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Progress in Opioid Research

Proceedings of the 1986 International Narcotics Research Conference

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service • Alcohol, Drug Abuse, and Mental Health Administration

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Editors:

John W. Holaday, Ph.D.
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NIDA Research Monograph 75

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Progress in Opioid Research

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This monograph is based upon papers from the 1986 International Narcotics Research Conference, held on July 6-11, 1986, in San Francisco, California. In the interest of rapid dissemination, it is published in the NIDA Research Monograph series as reviewed and submitted by the editors, whose names and affiliations follow.

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FOREWORD

The National Institute on Drug Abuse (NIDA) is pleased to publish Progress in Opioid Research, 1986, the proceedings of the 17th annual scientific meeting of the International Narcotic Research Conference (INRC). This meeting was held in San Francisco in July 1986.

The INRC is a unique international organization seeking to develop a fundamental understanding of the basic mechanisms of action of opioids, opioid peptides, and their receptors, research supported in large part by NIDA. This group and its meetings are different from others in the field because a definite effort is made to have young as well as senior investigators attend and present data and results of current, ongoing studies. For instance, in recent years, 75 per cent of the travel funds provided by the Division of Preclinical Research of NIDA for attendance at the meeting have been allocated to young investigators involved in laboratory research on opioids or related subjects. As is true for many other fields, excellence attracts more participants every year and this publication is the largest of any INRC Proceedings, with 155 papers and 113 abstracts included. The trio of editors worked swiftly and vigorously to review and assemble this compendium for publication by NIDA.

We are sure that this significant "state-of-the-art" volume of the latest research on the basic mechanisms of drug abuse will be of great value to members of the scientific community and other readers interested in the prevention and treatment of substance abuse.

Monique C. Braude, Ph.D.
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PREFACE

From our "bay view" in South San Francisco, we witnessed the recent developments in opioid research and reflected on the many notable scientific revelations from prior meetings of the INRC. Historically, the thrust of opioid research has progressed from classic in vivo and in vitro pharmacology toward a molecular description of events at a cellular and sub-cellular level. The 1966 Meeting of the INRC has provided us with an opportunity to view the cutting edge of research defining opioid ligands, receptors, and their functional consequences.

In many ways, the opiates have been at the forefront of scientific advances which were subsequently applied to other areas of biochemical, pharmacological, physiological and behavioral research. For example, the early definition of opiate receptors in 1973 has resulted in experimental strategies which have been directly applied to many other endogenous receptor systems. Clearly, the initial description of endogenous peptide ligands for opioid receptors in 1975 provided the major impetus for research in the neurobiology of many other opioid and non-opioid peptides. Likewise, the current emphasis on molecular biology as a means by which to define the biosynthesis and metabolism of endogenous opioids has obvious pertinence to other biologically active peptides. The complex biochemical procedures that have been developed to isolate and purify opiate receptors are already being applied to other receptor systems. Finally, the experimental strategies used to define the functional effects of endogenous opioids have resulted in a further understanding of the interactions between opioid and non-opioid systems.

Once again, this year's International Narcotics Research Conference provided a stimulating environment for the exchange of the most recent advances in opioid research. The program not only reflected many outstanding voluntary papers, but also included four invitational symposia and plenary lectures by international luminaries in pharmacology and the neurosciences. It was the intent of the program committee, under the leadership of Drs. Horace Loh and Nancy Lee, to utilize the plenary lectures as a means by which to acquaint opioid researchers with recent advances in related non-opioid fields. The first plenary lecturer, Dr. Daniel Koshland, discussed bacterial chemotaxis as a model sensory system. Dr. Julius Axelrod presented an update on the transduction of receptor mediated signals and the release of adrenocorticotropin. CTP binding proteins in signal transduction was the topic of the lecture by Dr. John Northrup, and Dr. 5. Numa provided the final plenary lecture on a molecular approach to the function of ionic channels. These outstanding presentations provided a refreshing break from the tachyphylactic effects of continued "opioid exposure", and also fostered new ideas and perspectives with pertinence to opioid research.

Unfortunately, despite the tireless efforts of many of our participants, the structures of isolated, purified opiate receptors were not available for presentation at this conference. Additionally, the fundamental problems of tolerance and physical dependence were only minimally addressed. Hopefully, future meetings of the INRC will provide a forum to reveal these advances as prior meetings have served to define the many advances described above.

Immediately following the INRC Meetings, the editorial board met for two days to review the papers and organize the monograph. In organizing this volume, it was our intention to provide a flowing framework, with related areas presented in logical groupings. We have attempted to arrange the papers into chapters which progress sequentially from biochemical studies of receptors and ligands through morphological descriptions of their localization to the final chapters defining the functional effects of opioid systems. Following the manuscripts, we have included the abstracts that were not submitted as papers.

As editors of this volume and participants in this year's meetings, we feel that the outstanding efforts of Dr. Horace Loh, Dr. Nancy Lee, Barbara Halperin and their colleagues in San Francisco, and Jean Paige in Washington, D.C., ensured both an outstanding scientific and social agenda for the meetings and the timely review and assemblage into this monograph of the many manuscripts based on those meetings. Their work was complemented by the tireless efforts of Dr. Eddie Way in his capacity as secretary to the IN RC. This meeting was generously supported by the National Institute on Drug Abuse, the U.S. Army Medical Research and Development Command, and several sponsors and contributors as listed in the program. Finally, Dr. Monique Braude, of the National Institute on Drug Abuse, provided guidance in our preparation of Progress in Opioid Research for publication in the NIDA Research Monograph series.

JOHN W. HOLADAY
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PURIFICATION OF KAPPA-OPIOID RECEPTOR SUBTYPE TO APPARENT HOMOGENEITY FROM FROG BRAIN

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M. Wollemann, K. Medzihradszky*

Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, P.O.Box 521, Hungary, and Central Research Institute for Chemistry of the Hungarian Academy of Sciences, II-1025, Budapest, P.O.:Box 17, Hungary.

ABSTRACT

Kappa-opioid receptor subtype was purified from digitonin solubilized preparation of frog brain membranes by hydrophobic and Sepharose 6B chromatography. 4800-fold purification over the membrane-bound receptors was achieved. The kappa receptor activity resides in a homo-oligomer of a single polypeptide of M_r 65,000.

INTRODUCTION

The opioid receptor system displays heterogeneity and consists of at least four major subtypes; i.e. mu, delta, kappa and sigma (Zukin & Zukin 1934). Recently, several laboratories have reported a partial purification of active opioid mu and delta binding sites (Cho et al. 1983, Gioannini et al. 1934 & 1985, Maneckjee et al. 1985, Fujioka Fujioka et al. 1935, Simonds et al. 1985, Demoliou-Mason & Barnard 1986). We have described earlier a succesful solubilization of opioid receptors from frog brain by the use of digitonin (Simon et al. 1984). This preparation was found to be a rich source of the kappa-subtype (72%). In solution this subclass was completely separated from the mu- and delta-sites by gel filtration on Sepharose-6B column (Simon et al. 1985) and it was purified on an affinity column (Simon et al. 1986). In the present work we purified the receptor by the use of hydrophobic chromatography.

MATERIALS AND METHODS

^{3}H-Ethylketocyclazocine (^{3}H-EKC: 0.74 TBq/mmol, 19.9 Ci/nmol) was purchased fron New England Nuclear. ^{3}H-Dansyl chloride (0.53 TBq/mmol, 14.2 Ci/mmol) was from Amersham.

Digitonin, bacitracin, phenylmethylsulfonyl fluoride (PMSF), dansyl chloride and polyethyleneimine (PEI) were obtained from Sigma Chemicals Co. Sepharose-6B beads, HMW calibration kit for gel filtration and LMW kit for gel electrophoresis were purchased by Pharmacia Fine Chemicals. All other chemicals were of analytical grade. Particulate membrane fraction of frog (Rana esculenta) brain was prepared in Tris buffer and solubilized with 1% digitonin as described earlier ($imon et al. 1984). Specific binding was measured with ^{3}H-EKC in the presence of blocking agents for mu and delta sites (Simon et al. 1934). Protein concentration was determined as described by Bradford (1976), or with ^{3}H-Dansyl chloride method (Schultz & Wassarman 1977). SDS polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) with slight modifications.

RESULTS AND DISCUSSION

Frog (Rana Esculenta) brain membranes were solubilized in 1% (ligitonin (Simon et al. 1984) and applied on Phenyl-Sepharose CL-B column with high salt concentration (0.5 M $(NH_4)_2SO_4$), after the removal the excess of the detergent. Proteins were eluted with decreasing salt concentration. ^{3}H-KC binding of the fractions was measured. The highest activity was found at low salt (0.06M-H_2O) concentration (fig. 1) indicating a hydrophobic character.

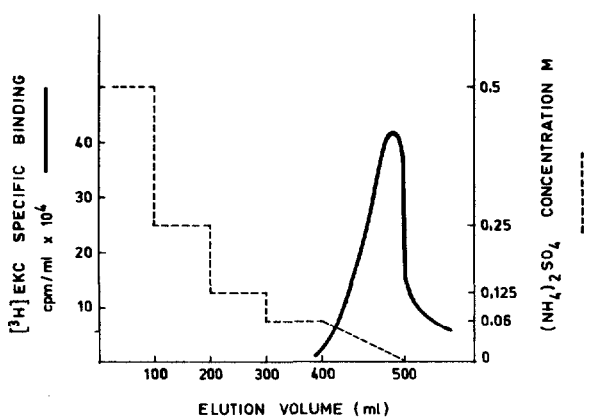

FIGURE 1. Phenyl-Sepharose CL-4B chromatography Solubilized opioid receptors were applied on Phenyl-Sepharose CL-4B column (32x0.8 cm) in 0.5M $(NH_4)_2SO_4$. Elution was carried out by decreasing salt concentration.EKC binding was measured.

The preparation exhibited high affinity for kappa-ligands as it is shown in table 1.

TABLE 1.

Relative potencies of opioid ligands in displacing ^{3}H-EKC binding

Unlabelled ligands	IC_{50}(nM)
Ethylketocyclazocine	30
Dynorphin(1-13)	10
U-50, 488 H	11

The pooled and concentrated fractions were applied on Sepharose 6B column, where a complete separation of the kappa subtype can be obtained (Simon et al. 1985). Fractions with high ^{3}H-EKC specific binding were collected and their protein contents were measured. Because of the very low mount, the ultrasensitive (5-10 ng per assay) ^{3}H-Dansyl chloride method was used .

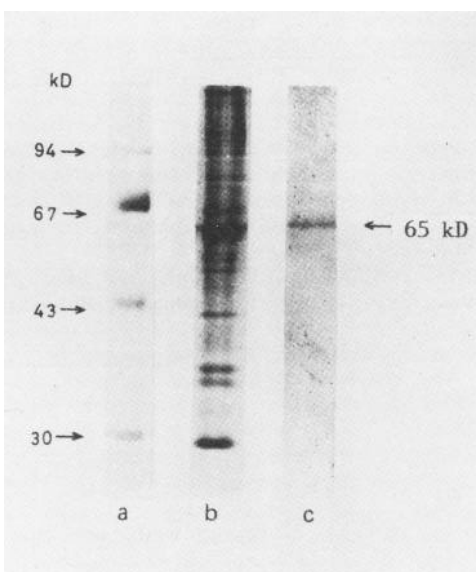

FIGURE 2. SDS polyacrylanide gelelectrophoresis
Lane 1.: molecular weight markers, lane 2.: digitonin extract, lane 3.: purified kappa fraction.

About 3360 pmol of ^{3}H-EKC per mg protein specific activity was obtained compared to 0.7 pmol/mg in the original membrane fraction. Approximately 4800-fold enrichment Of kappa-subtype over the menbrane-bound receptors was achieved. The kappa receptor activity resides in a homo-oligomer of a single polypeptide of M_r 65,000, as it is shown on fig. 2.
Results from other laboratories and our recent data suggest a general pattern of opioid receptors in which each subtype is due to a sinqle, specific type of subunit.

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CHARACTERIZATION, SOLUBILIZATION AND PURIFICATION OF AN OPIOID RECEPTOR FROM THE BRAIN OF THE FROG RANA RIDIBUNDA.

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ABSTRACT

The brain of the frog R. ridibunda contains a major opioid binding site which in vitro pharmacological profile is different from those of mammalian μ, δ- and κ-opioid sites. In digitonin extracts, this major opioid site exists as two molecular forms -10S and 12S- which are clearly resolved by sedimentation in sucrose gradients and which are thought to represent the opioid receptor alone (10S) or associated (12S) with a guanine nucleotide regulatory protein. Purification of the digitonin extracts by affinity chromatography on immobilized dynorphin, results in a single major protein component of apparent $M_r \sim 64,000$.

INTRODUCTION

Amphibian brain membranes display high levels of opioid binding (Ruegg et al. 1981, Puget et al. 1983, Simon et al. 1984). In addition, amphibian brain opioid receptors are readily solubilized under active form in reasonably high yields with digitonin alone (Ruegg et al. 1981, Simon et al. 1984). Therefore, the amphibian brain designates itself as a suitable source wherefrom to purify opioid receptors by affinity chromatography.

In this report, we have characterized the types of opioid sites present in frog brain membranes and we have analyzed the sedimentation behavior of the digitonin solubilized opioid receptors. Our results suggest that in the preparation, there is a major opioid receptor type different from mammalian μ, δ and κ types and which appears to exist independently of as well as in association with a guanine nucleotide regulatory protein.

MATERIALS AND METHOOS

Preparation and solubilization of the crude membrane fraction (0°C).

Freshly dissected whole brains from R. ridibunda frogs were homogeneized in 50 mM tris-HCl pH 7.4 ("buffer") and the homogenate was centrifuged in a Beckman rotor type 30 for 35 min at 28,000 rpm. The pellet was washed and resuspended in enough buffer to obtain a crude membrane fraction containing about 5 mg of protein per ml. The suspension was made 1 % (w/v) with digitonin (Fluka AG) and gently stirred for 30 min after which it was centrifuged in a Beckman rotor type 30 for 30 min at 35,000 rpm. The supernatant (2 mg of protein/ml) was taken as the soluble extract.

Equilibrium binding studies (25°C).

Equilibrium binding studies were carried out on 50 μl either of the crude membrane fraction (diluted 1 : 5) or on 30 μl aliquots of the soluble extract in a final volume of 0.5 ml using ^{3}H-etorphine (30-60 Ci/mmol, Amersham) or ^{3}H-diprenorphine (25-50 Ci/mmol, Amersham). All assays were in triplicate and incubation was for 1 hr at 25°C.

Nonspecific binding was determined in the presence of 10 µM diprenorphine. Free and bound radioligand were separated by rapid filtration on glass fiber disks (Whatman GF/B) which, in the case of the soluble extract, had been presoaked in PEI according to Bruns et al. (1983).

Sedimentation in sucrose gradients

0.4 ml aliquots of digitonin extract were layered on top of 10.6 ml linear 5 to 20 % (w:v) sucrose gradients in 50 mM tris-HCl pH 7.4 containing 0.01 % (w:v) digitonin. Centrifugation was at 40.000 rpm for 16 hrs in a Beckman rotor type SW 41 Ti after which fractions of 0.16 ml were collected. Each fraction was assayed for ^{3}H-etorphine (2 nM) or ^{3}H-diprenorphine (2 nM) in a final volume of about 0.2 ml by the rapid filtration method as modified by Bruns et al. (1983).

RESULTS AND DISCUSSION

The frog brain contains a major opioid receptor type that is neither µ, nor δ or κ.

Equilibrium saturation binding of the agonist ^{3}H-etorphine (^{3}H-ETO) and of the antagonist ^{3}H-diprenorphine (^{3}H-DIP) in frog brain membranes was clearly biphasic (figure 1). Sodium ions (120 mM NaCl) selectively reduced binding of ^{3}H-ETO at the high affinity site but not at the low affinity site suggesting the opioid nature of the former but not of the latter.
Examination of the potency of various unlabelled opioid ligands to compete with binding of 0.1 nM ^{3}H-ETO at the high affinity site revealed that the latter actually consisted of a mixture of several types including a major one (70 %) "pharmacological profile" in vitro was clearly different from those of mammalian µ-, δ- and κ-opioid sites. In particular, the major opioid site of the frog brain displayed moderate affinity toward DAGO (Ki = 60 nM) and DTLET (Ki = 120 nM) and low affinity toward U-50488 (Ki = 1,200 nM), i.e. toward selective ligands that normally bind µ, δ and κ sites respectively with nanomolar affinities.

Frog brain opioid receptors exist independently of and in association with a guanine nucleotide regulatory protein.

Because of the limited space allowed to the present report, only data from assays with ^{3}H-DIP in the presence cf 120 mM NaCl will be presented. Under these conditions, digitonin extracts from frog brain were found to contain in the range 1.2-1.5 pmol (/mg of protein) of a homogenous population of sites that bind ^{3}H-DIP with high affinity (Kd = 0.2 nM). Interestingly, the binding characteristics of ^{3}H-DIP were not modified in the least had the soluble extract been derived from NaCl (120 mM) or GppNHp (50 µM) pretreated membranes.
In contrast, the sedimentation profiles of opioid binding activity in the soluble extract were clearly heterogenous (figure 2). Opioid binding activity was found to be associated with a minor component at position 12S and with a major component at position 10S.
In extracts from membranes that had been incubated with 120 mM NaCl or with 50 µM GppNHp prior to solubilization, no activity was recovered at position 12S while, under these conditions, higher levels of ^{3}H-DIP binding were observed in association with the 10S component.
A simple way to explain these results is that the frog brain opioid receptor exists independently of (10S) as well as in association with (12S) a guanine nucleotide regulatory protein (N). The antagonist W-DIP does not discriminate the two forms in question hence linear

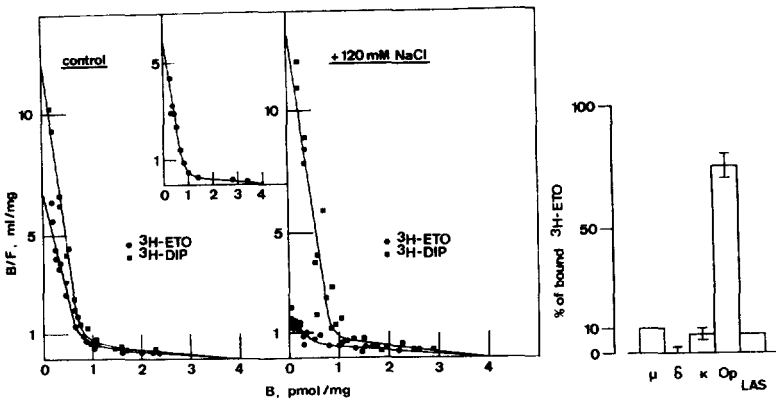

Figure 1: Scatchard transforms of the equilibrium adsorption isotherms of ^{3}H-etorphine (a) and of ^{3}H-diprenorphine (■) in the crude membrane fraction from the brain of R. ridibunda. (a) in 50 mM tris-HCl pH 7.4. The insert is the Scatchard transform of an isotopic dilution experiment using 0.1 nM ^{3}H-etorphine. The preparation contained 0.7-0.8 pmol/mg protein of a high affinity site (Kd = 0.1 nM) and 3.2-3.3 pmol/mg protein of a low affinity site (Kd = 10 nM). (b) in 50 mM tris-HCl pH 7.4 + 120 mM NaCl. The histogram at the right indicates the proportions of the various types of binding sites that were labelled in the presence of 0.1 nM ^{3}H-etorphine. Op represents the major opioid site and LAS the low affinity site.

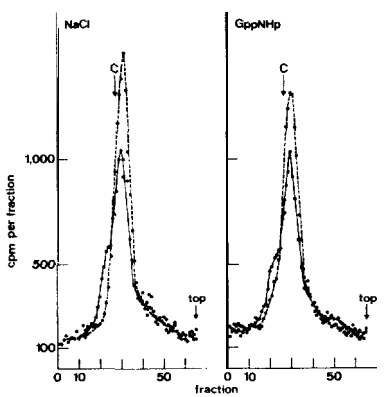

Figure 2: Sedimentation profiles of the opioid binding activity in digitonin extracts from the crude membrane fraction of the brain of R. ridibunda. Opioid binding activity was assayed for in each fraction of the gradients with ^{3}H-DIP (2 nM) in the presence of 120 mM NaCl. Solid line: the detergent extract was from untreated membranes. Dashed line: the detergent extract was from NaCl (120 mM) or from GppNHp (50 μM) preincubated membranes. Note that under these conditions no binding activity is recovered in association with the component that is sedimented slightly faster than catalase (C) used as velocity marker.

Scatchard transforms from equilibrium binding studies. 12S receptors appear to be converted into 10S receptors in the presence not only of sodium ions but also and most significantly of GppNHp, an effector of N proteins : this conversion manifests itself, in sedimentation studies, by a decreased binding of ^{3}H-DIP to the 12S component and by a concomittant increased binding of ^{3}H-DIP to the 10S component.

Purification

Opioid receptors in digitonin extracts from several thousands of frog brains are now being purified by affinity chromatography on immobilized dynorphin 1-13 and following essentially the protocol of Gioannini et al. (1985) for the purification of an active opioid-binding protein from bovine striatum. Our results are still very preliminary yet a purified preparation is routinely obtained that contains a major protein component of apparent M_r = 64.000. However, it has not been unambiguously shown yet that the purified protein actually carries the opioid binding site.

CONCLUSION

In addition to the frog brain opioid receptor, the μ-opioid receptor from rabbit cerebellum (Jauzac et al. 1986) and the κ-opioid receptor from guinea-pig cerebellum (Frances et al., unpublished) display the 10S and 12S forms which are thought to represent the receptor alone (10S) and the receptor associated (12S) with a N protein. It is therefore possible that all opioid receptor types share the property of interacting with a N-protein and it is tempting to speculate that opioid agonists in general modulate the permeability of the neuronal membrane (North 1986) through, primarily, the receptor-mediated activation of a N-protein. In this respect, opioid agonists in general would act, at the molecular level, in a way that is not any different from that of classical neuromodulators, serotonin, acetylcholine or noradrenaline (Siegelbaum and Tsien 1983).

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THE DIRECT DEMONSTRATION OF BINDING OF MU, DELTA AND KAPPA AGONISTS TO DIGITONIN-SOLUBILIZED OPIOID RECEPTORS FROM BOVINE STRIATUM

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ABSTRACT

Active opioid binding sites, that retain the ability to bind tritiated agonists have been obtained in good yield in digitonin /NaCl/Mg^{2+} extracts of morphine protected bovine striatal membranes. Ligand protection of binding sites and the presence of Mg ions were found to be absolute requirements for agonist binding in this solubilized opioid receptor preparation. Soluble preparations contained a ratio of mu: delta:kappa similar to that in the membranes.

INTRODUCTION

Solubilization, attempted purification and separation of the various opioid receptor types are important steps toward the elucidation of the molecular basis of receptor heterogeneity. Earlier studies from this laboratory demonstrated successful solubilization of active opioid binding sites from mammalian brain, using digitonin and high concentrations of NaCl (Howells et al. 1982). Binding was measured using antagonists because under these conditions little or no tritiated agonist binding could be detected. Recently Itzhak et al. (1984) were able to obtain opioid agonist binding after fractionation of the soluble binding sites on a sucrose density gradient. Opioid agonist binding activity has also been reported to Mg^{2+}/digitonin solubilized receptors from rat brain membranes (Demoliou-Mason and Barnard 1986) and to CHAPS-solubilized receptors (Simonds et al. 1980) In addition, some progress has been made in the identification of the ligand binding subunits of mu and delta receptors, using the technique of affinity labeling (Newman and Barnard 1984; Howard et al. 1985, 1986). In the present study, we describe a modification of our method for extracting from bovine striatal membranes active opioid binding sites that retain the ability to bind tritiated agonists as well as antagonists, with high affinity and in good yield.

MATERIALS AND METHODS

Particulate membrane fractions from fresh bovine striatum were prepared as previously described by this laboratory. The membrane preparations were stored at -70° in 0.32 M sucrose (1:6 w/v) until needed. For solubilization, crude

membrane preparations were thawed, diluted 1:1 with 50 mM Tris HCl containing 1 mM K_2 EDTA, and 10 mM $MgSO_4$ (buffer A) and incubated with 1 uM morphine sulfate, for 30 min at room temperature. The mixture was diluted with an equal volume of buffer A containing 1% digitonin/1 M NaCl and stirred on ice for 30 min followed by centrifugation at 100,000 x g at 4°C. The supernatant was removed and mixed 1:1 with 40% polyethylene glycol 6000 (PEG) in presence of 0.1% IgG. The suspension was then spun at 12,000 x g for 15 min at 4°C. The resulting pellet was rinsed twice with 7.5% PEG and resuspended in buffer A, 0.1 mM leupeptin ± NaCl. Duplicate 1 ml samples were incubated with tritiated ligands ± 1 uM unlabeled naloxone, for 60 min at 25°C. After incubation, samples were precipitated with 13% PEG in the presence of 0.15% IgG prior to filtration. Specific binding was assayed using membranes and solubilized preparations derived from equal amounts of tissue, at the same concentrations of radioligand and under identical conditions. Protein was determined by the method of Bradford (1972). ^{3}H-bremazocine (30 Ci/mmoll and ^{3}H[Tyr-D-Ser-Gly-Phe-Leu-Thr] enkephalin (DSTLE, 30 Ci/mmol) were purchased from NEN and ^{3}H-[D-Ala^2-Me-Phe^4-Gly-011 enkephalin (DAGO, 60 Ci/mmol) from Amersham. The following unlabeled drugs were used: Naloxone (Endo Laboratories), morphine sulfate (Merck). Other chemicals used were: digitonin (US Biochemical Corp.), polyethylene glycol 6000 (PEG) and bovine gamma-globulin (IgG) from Sigma, guanosine-5'triphosphate (GTP) from Boehringer.

RESULTS AND DISCUSSION

In an attempt to protect the opioid agonist binding from the detrimental effect of digitonin and NaCl present during the extraction procedure (Method I), bovine striatal membranes were incubated with morphine and $MgSO_4$ prior to solubilization (Method II). As shown in table 1, no specific binding of selective ^{3}H-labeled opioid peptides occurred under conditions of method I, while, as expected for ^{3}H-bremazocine and H-naltrexone the specific binding recovered represented 30-35%. In contrast, solubilization of membranes under conditions of method II, produced a good yield of soluble receptor activity for opioid peptides. This represented an 11-30% yield of ^{3}H-bremazocine binding activity. Saturation curves of binding yielded linear Scatchard plots for both soluble preparations with K_D = 1.9 nM, Bmax = 349 fmol/mg protein and K_D =1.5 nM, Bmax = 292 fmol/mg protein for methods I and II respectively (table II). The binding of selective opioid ligands to soluble binding sites (from method II) was saturable and of high affinity as indicated by the apparent K_D and Bmax values summarized in table II. This demonstrates that distinct subtype specificities, characteristic of membrane-bound receptors, were still retained in the soluble preparation. In addition, the ratio of mu:delta:kappa was similar in the soluble

fraction as in the membranes.

Table I
Yields* of agonist and antagonist binding in soluble receptors extracted from bovine striatal membranes using methods I and II

Extract	PEG pptation	Protein %	Recovery of specific binding (%) Naltr. (3nM)	Bremaz. (3nM)	DAGO (6nM)	DSTLE (6nM)
Method I	- **	50	30	35	0	0
	+	30	18	21	0	0
Method II	+	25	21	19	20	11

*Percent yields were calculated as follows: (Specific binding to soluble fraction/specific binding to membrane fraction x 100)
**Binding in extract not precipitated by PEG was carried out after 1:5 dilution to reduce digitonin concentration to 0.1%.

Table II
Comparison of affinity constants and maximal binding values or solubilized bovine striatal membranes, processed by methods I and II

Ligand	Method I K_d (nM)	Method I Bmax (fmol/mg protein)	Method II K_d (nM)	Method II Bmax (fmol/mg protein)
^{3}H-Bremazocine	1.9±0.2	349±32	1.5±0.13	292±21
^{3}H-Bremazocine (+100 mMDAGO + 100mM DPDPE)	N.D.*	N.D.	1.5	135
^{3}H-DAGO	N.D.	N.D.	2.5±0.2	65±12
^{3}H-DSTLE	N.D.	N.D.	6.2	45

*N.D. - not detectable
Values represent mean of at least 3 determinations ± S.E.
When no S.E. is given only 2 determinations were done.

As shown in table III, specific mu opioid binding to the solubilized receptors was still sensitive to the inhibitory effect of GTP. In fact at 100 uM GTP it seemed to be more sensitive than to membrane-bound receptors. In the membrane Mg^{2+} was able to reverse the GTP-induced reduction in ^{3}H-DAGO binding. A similar

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reversal of GTP-induced decrease in ^{3}H-dihydromorphine binding by manganese was reported by Childers and Snyder (1980).

Table III

Effect of GTP and Mg^{2+} on mu type opioid binding to membranes* and solubilized fraction of bovine striatum

Specific binding of ^{3}H-DAGO (fmol/mg protein)

	Conc NaCl	Additions			
	(mM)	None	GTP(100uM)	Mg^{2+}(10mM)	Mg^{2+}+GTP
Membranes	0	64	45	60	74
	10	50	30	56	85
	25	33	20	58	75
	100	13	7.5	42	47
Soluble (Method II)	0	0	0	43	0
	100	0	0	42	0

Results are the average of duplicate experiments.
*Specific binding was determined following incubation of 1 ml striatal membrane (1 mg/protein/ml) with ^{3}H-DAGO (1 nM) in the absence and presence of 1 uM unlabeled naloxone for 60 min, 25°C.
**Specific binding was determined following incubation of 1 ml solubilized striatal membranes "Method II" (0.6 mg/protein/ml) with ^{3}H-DAGO (6 nM) in the absence and presence of 1 uM unlabeled naloxone for 60 min, 25°C.

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IDENTIFICATION OF RAT BRAIN OPIOID (ENKEPHALIN) RECEPTOR BY PHOTOAFFINITY LABELING

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ABSTRACT

A photoreactive, radioactive enkephalin derivative was prepared and purified by high performance liquid chromatography. Rat brain and spinal cord plasma membranes were incubated with this radioiodinated photoprobe and were subsequently photolysed. Autoradiography of the sodium dodecyl sulfate gel electrophoresis of the solubilized and reduced mernbranes showed that a protein having an apparent molecular weight of 46,000 daltons was specifically labeled, suggesting that this protein may be the opioid (enkephalin) receptor.

INTRODUCTION

Extensive studies have been made on the endogenous opioid peptides and their interactions (Smith and Loh 1981; Barnard and Demolion-Mason 1983; Zukin 1984). In order to identify the opioid (enkephalin) receptor in situ, the technique of photoaffinity labeling was employed. The usse of photogenerated reagents for the labeling of biological receptor sites has been reviewed (Bayley and Knowles 1977; Eberle and de Graan 1985). A number of photoreactive enkephalin derivatives designed to investigate the opioid receptor have been reoorted (Hazum et al. 1979: Lee et al. 1979; Smolarskv and Koshland 1980; Garbay-Jaurequiberry et al. 1984; Zioudron et al.' 1983; Fujioka et al. 1984; Yeung 1985). In this communication, we report the preparation and purification of a radioiodinated, para-azido-L-phenylalanyl derivative of enkephalin. This photosensitive and radioactive enkephalin tracer was used to identify the opioid receptor in the plasma membranes from rat neural tissues. A plasma membrane protein with an apparent molecular weight of 46,000 daltons was specifically labeled. It is likely that this protein is a protein of the opioid (enkephalin) receptor.

MATERIALS AND METHODS

Preparation and Purification of ^{125}I(D-Ala^2, p-N_3-Phe^4-Met^5)Enkepha-

Purified (D-Ala^2, p-N_3-Phe^4-Met^5)enkephalin (AP-Enk) (Yeung 1985) was radioiodinated with carrier free ^{125}I (Amersham Radiochemicals) and chloramine-T as described by Hunter and Greenwood (1962). Separation of the iodinated mixture on a Sep-Pak C_{18} reverse phase cartridge was carried out as described by Yeung (1984). The radioactive photoprobe was further purified by high performance liquid chromatography (HPLC) on a Lichrosorb RP-8 (5 micron) column (0.4 x 25 cm) using a linear gradient of 0.05% trifluoroacetic acid in water and acetonitrile, pH 3.0. Fractions collected from the column were analyzed by thin-layer chromatography (tlc) on a cellulose Chromagram sheet (Eastman type 13255), developed in I-butanol:ethanol:2 N NH_4OH (v/v, 5:1:2), followed by autoradiography on Kodak AR5-X-OMat film. The extent of radioiodination of AP-Enk was determined by alkaline hydrolysis, followed by analyzing the hydrolysate for (^{125}I) monoiodotyrosine and, (^{125}I)diiodotyrosine on tlc.

Binding and Photoaffinity Labeling

Plasma membranes from the rat brain (minus cerebellum), spinal cord and cerebellum were prepared as the microsomal fractions from 250 g male Wister rats according to the method of Posner et al. (1974). These membrane preparations (3-4 mg/ml) were incubated in the dark in a polypropylene microfuge tube containing 275 µl of 0.05 M Tris-HCl, pH 7.5, 0.05 mM each of benzamidine hydrochloride and phenylmethyl-sulfonyl fluoride and approximately 0.5 µCi/ml of the purified radio-active photoreactive enkephalin at 24°C for 30 minutes. (D-Ala^2 Met^5)Enkephalin (4.3 nmol) was added where appropriate to determine the non-specific binding. The reaction mixture was photolysed for 30 seconds using a 100-W high pressure mercury lamp, and was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on a gradient slab of 10-15% (Yip et al. 1982). The membrane was reduced bv boiling for 15 minutes in U.l M dithiothreitol and 3% SDS. solubilized material was run on the gel. After staining, destaining and drying, the gel was exposed to Kodak AR5-X-OMat film, using an intensifying screen (Swanstrom and Shank 1978).

RESULTS AND DISCUSSION

The crude radioactive photoreactive enkephalin analogue recovered from Sep-Pak minicolumn (SP-2) was further ourified bv HPLC. Four radioactive fractions were eluted from the Lichrosorb column (Figure 1a). The purity of the fractions were assessed by tlc. Figure lb shows the relative mobilities of the fractions. The R_f values of HPLC-1 and HPLC-2 were similar to that of radioactive iodine stan-dard. HPLC-3 and HPLC-4 (R_f values 0.72 and 0.76 respectively) showed single major component with a trace amount of free iodine. Alkaline hydrolysis of HPLC-3 and HPLC-4 followed by tlc showed that both of these fractions were monoiodinated. Although HPLC-1 was a major radioactive component, both HPLC-1 and HPLC-2 did not show any specific binding by rat brain plasma membranes. In contrast, the specific binding for HPLC-3 and HPLC-4 were estimated to be 90% and

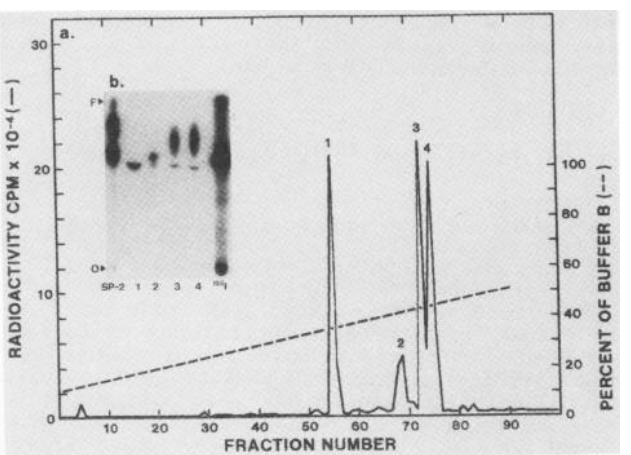

FIGURE 1. (a) Llution profile of ^{125}I-AP-Enk recovered from Sep-Pak minicolumn (SP-2). Radioactive fractions 1-4 were pooled for further characterization. (b) Autoradiogram of thin-layer chromatography of pooled fractions. carrier free Na^{125}I standard in water; 0 and F indicate origin and solvent front respectively.

72% respectively of the total binding. Since HPLC-3 was found to be more active in radioreceptor binding assay, while HPLC-4 retained some binding activity, HPLC-3 was used for the photoaffinity labeling experiment.

Binding of HPLC-3 to rat brain (minus cerebellum) plasma membrane, followed by photolysis, led to covalent labeling of membrane protein as shown by autoradiography (figure 2). A radioactive band was specifically labeled (lane 1) since the labeling of the band was abolished in the presence of an excess (2.5 µg) of (D-Ala^2, Met^5)-enkephalin (lane 20). Such labeling was most likely the result of net cross-linking of ^{125}I(AP-Enk) HPLC-3 to the protein through the para-azido-L-phenylalanine. The apparent molecular weight of the radioactive band was estimated to be 46 kDa (ranqe 44 to 48 kDa). Considering the anomalous behavior of glycoprotein in SDS-gel electrophoresis, the molecular weight of the labeled band may be less than 46 kDa. The labeling of this membrane protein was totally dependent on photolysis. since a control not exoosed to liaht showed no such labeling (lane 3). Prephotolysing the radioactive tracer for 5 minutes before use resulted in no specific labeling of the 46-kDa protein (lanes 6-8). Exposure of the membrane to the light source for 1 minute before use in incubation and subsequent experiments did not seem to affect the ability of the photoprobe to label the specific 46-kDa protein. A similar 46 kDa radioactive band was also found to be covalently cross-linked and identified in the rat spinal cord plasma membranes. However, no such protein or any other protein band were specifically labeled when rat cerebellum plasma membrane was used. These observations strongly suggest that the 46-kDa protein may be the opioid receptor. It is apparent that a wide range of

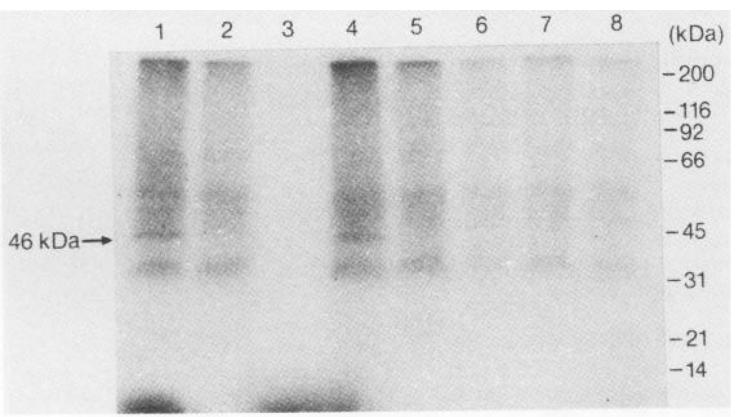

FIGURE 2. Autoradiogram of an SDS gradient gel of rat: brain plasma membranes labeled with ^{125}I-Ap-Enk HPLC-3, enatured and reduced. Excess (D-Ala^2, Met^5)enkephalin was used in lanes 2, 5 & 7; while lines 3 and 8 served as dark control. The band (M_r 46 kDa) specifically photolabeled is indicated by an arrow. The molecular weight standards (Biorad) used were myosin (200,000), β-galactosidase 116,250), phosphorylase B (92, 500), bovine serum albumin (66,200), ovalbumin (45, 000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

molecular weights for the opioid receptor complex and its subunit has been obtained from a number of direct and indirect approaches. In

this study using the direct photoaffinity labeling approach, we estimate the molecular weight of the opioid (enkephalin) receptor protein to be 46 kDa. This molecular weight estimate is within the range of the reported values of 25-65 kDa for the subunit.

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PHOTOAFFINITY LABELING OF OPIATE RECEPTORS WITH ^{3}H-ETORPHINE: POSSIBLE SPECIES DIFFERENCES IN GLYCOSYLATION

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ABSTRACT

Opiate receptors from whole rat brain (minus cerebellum) and cow striatum were labeled irreversibly using the intrinsic photolability of ^{3}H-etorphine. After incubation with 2 nM ^{3}H-etorphine and centrifugal washing, membranes were irradiated with light of 254 nm. Non-specific binding was determined by carrying out incubations in presence and absence of 10 uM levallorphan. Specific binding in photolabeled membranes was 75-80%, with a photo-incorporation yield of approximately 50%. Photolabeled membranes were extracted with CHAPS/Lubrol and unbound ^{3}H-etorphine was removed by dialysis and passage over Sephadex G-25. Solubilized proteins were then subjected to chromatography on wheat germ agglutinin, and retained proteins were eluted with N-acetyl D-glucosamine (NAG). Protein profiles from rat brain and cow striatum were identical, with 89% of the total protein flowing through unretained and 11% eluted by NAG. However, the profile of radioactivity was markedly different in the two species. With rat, the specific activity (cpm/A_{280}) was the same for flow-through and NAG-eluate. With cow, the specific activity of the NAG-eluate was 17 times greater than the flow-through. These results indicate that cow striatum and rat whole brain contain populations of opiate receptors which are glycosylated differently.

INTRODUCTION

Gioannini et al. (1982) have shown that opiate receptors from several species are retained by wheat germ agglutinin and can be eluted with N-acetyl D-glucosamine. This indicates that opiate receptors are glycoproteins. These workers also found that varying proportions of the total binding activity appeared in flow-through and NAG-eluate in different species, indicating a possible heterogeneity in receptor glycosylation across species. Howevever, results are difficult to interpret in this regard since the ^{3}H-ligand could dissociate from receptor and appear in the flow-through. We have developed a method to irreversibly label receptors by utilizing the intrinsic photolability of various ^{3}H-opiates (Bowen et al. 1985, Kooper et al. 1985, submitted). Using this method, opiate receptors can be irreversibly labeled with high specificity and yield. Here we report that rat whole brain and cow striatal receptors photolabeled with ^{3}H-etorphine exhibit differences in their ability to be retained on wheat germ agglutinin. Therefore, opiate receptors may be glycosylated differently across species or across brain regions.

METHODS

Lysed P_2 membrane fraction was prepared from the brains (minus cerebellum) of male Sprague-Dawley rats (150-200 gm) and freshly' obtained cow striatum. Membranes (1 mg protein/ml) were incubated with 2 nM ^{3}H-etorphine in 10 mM TRIS-HCl pH 7.4 at 25°C for 90 min. Non-specific binding was determined by incubating in presence of 10 uM levallorphan. Labeled membranes were washed twice by repeated centrifugation and resuspension in ice-cold incubation buffer. Resuspended membranes were either irradiated on ice for 10 min with ultraviolet light of 254 nm (Mineralight R52G, Ultra-Violet Products, San Gabriel, CA.) at a distance of 2 cm or allowed to stand in room light (DARK-treated) for 10 min. Denaturation was carried out by heating for 10 min at 90°C. To determine results of these treatments, 500 ul aliquots were filtered over glass fiber filters and washed with ice-cold TRIS-HCl pH 7.4. Filters were counted after overnight extraction in scintillation cocktail.

Photolabeled membranes were solubilized by homogenization in 13 mM CHAPS/1% Lubrol. The homogenate was incubated for 15-30 min at 25°. The 105,000 x g supernatant was then dialyzed overnight at 4°C against 10 mM TRIS-HCl pH 7.4 or gel filtered on Sephadex G-25 (eluted with buffer containing 10 mM CHAPS) to remove free ^{3}H-etorphine.

Chromatography on wheat germ Lectin-Sepharose 6MB was carried out as follows. Extract was applied to the column in 1 ml aliquots and allowed to incubate in the bed for 15 min at 25°C. The column was washed with 10 mM TRIS-HCl pH 7.4 containing 10 mM CHAPS and 1 mM EDTA. Retained proteins were eluted with the same buffer containing .5 M N-acetyl D-glucosamine. Protein was estimated by absorbance at 280 nm and a 50 ul aliquot of each 1 ml fraction was counted in a scintillation spectrometer to detect tritium. Specific activities of labeled proteins in the flow-through and NAG-eluate were estimated by determining the areas under the cpm and A_{280} peaks and taking their ratios.

RESULTS AND DISCUSSION

Table 1 shows results of photolabeling of rat whole brain and cow striatal receptors with ^{3}H-etorphine. After incubation with ^{3}H-etorphine and washing to remove unbound ligand, membranes were irradiated at 254 nm or allowed to stand in room light. Irreversible binding was assessed by heat denaturation. Denaturation after dark treatment caused a loss of 97% and 95% of specific binding in rat and cow membranes, respectively. However, irradiation before denaturation markedly reduced the loss of specific binding, For rat, 60% of specific binding in irradiated membranes survived denaturation. For cow, 39% remained. Importantly, the percent specific binding in irradiated/denatured samples was 75% and 79% for rat and cow, respectively. Thus this method produced covalent labeling of opiate receptors in rat and cow. The receptors were labeled in high yield and with high signal-to-noise ratio.

TABLE 1. Photolabeling of Rat and Cow Membranes with ^{3}H-Etorphine

	RAT				COW			
	BK	TOT	SB	% S B	B K	TOT	SB	% S B
Dark	405	2363	1958	83	279	4007	3728	93
Dark/Denat.	376	433	57	13	192	365	173	47
UV	312	1455	1143	79	268	2465	2197	89
UV/Denat.	231	971	686	75	225	1076	851	79
			Yield	60%			Yield	39%

Membranes photolabeled with ^{3}H-etorphine were solubilized with 13 mM CHAPS/1% Lubrol and the soluble proteins were subjected to chromatography on wheat germ Lectin-Sepharose 6MB. The elution profile is shown in Figure 1. For both rat and cow, approximately 89% of the total protein flowed through the column unretained, while 11% was retained and eluted with NAG. However, the profile of radioactivity was markedly different for the two preparations. Table 2 shows an analysis of the specific activity in the flow-through and NAG eluate from the two preparations, estimated by determining the areas under the cpm and A_{280} peaks. For rat, the specific activity of the flow-through and NAG eluate were the same. However, for cow striatal receptors, the specific activity of the NAG eluate was 17 times higher than that in the flow-through.

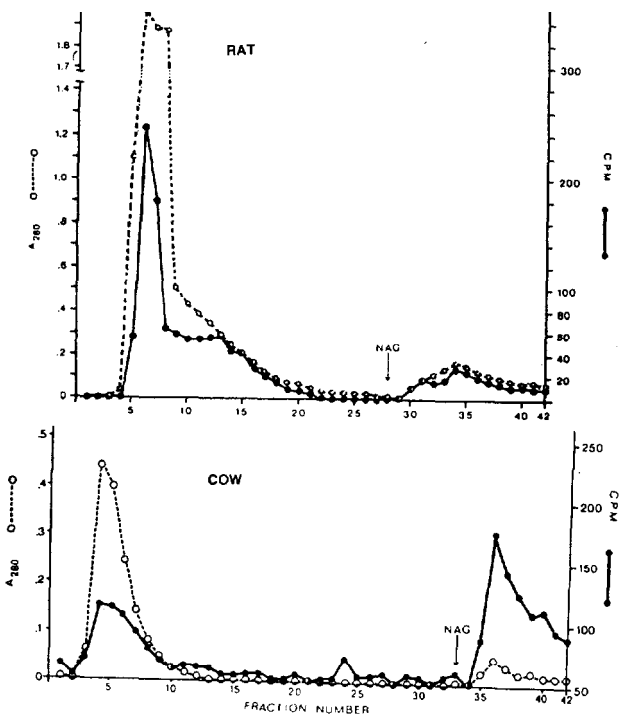

FIGURE 1. Wheat germ agglutinin chromatography of CHAPS/Lubrol extracts from membranes photolabeled with ^{3}H-etorphine.

TABLE 2. Comparison of Specific Activities of Peaks from WGA-Chromatography

	RAT			cow		
	area (cm^2)			area (cm^2)		
	cpm	A_{280}	cpm/A_{280}	cpm	A_{280}	cpm/A_{280}
FLOW-THRU	26.73	47.38	0.56	7.26	14.85	0.49
NAG-ELUATE	3.99	5.95	0.67	14.57	1.75	8.33
SP. ACT. RATIO NAG-ELUATE : FLOW-THRU		1.2 : 1			17.0 : 1	

These results are consistent with th se of Gioannini et al. (1982) who observed that of total soluble ^{3}H-diprenorphine binding in rat, 23% was recovered in the flow-through and 31% was recovered in NAG-eluate. For cow striatum, these values were 11% in flow-through and 47% in NAG-eluate. In the study described here, we utilized covalently bound H-etorphine, so that dissociation of ligand is not a factor. The results reported here have several interpretations. Opiate receptors in different species may be glycosylated differently. In this case, rat brain would contain a larger population of receptors which lack affinity for wheat germ agglutinin. Alternatively, different brain regions may contain receptors which are glycosylated differently. The cow preparation is from striatum while the rat preparation is from whole brain (minus cerebellum). Striatal receptors from the two species would have to be compared to resolve this issue. Finally, ^{3}H-etorphine will label mu, delta, and kappa receptors. The differences in the species and/or regions may reflect a difference in glycosylation among receptor types. Further purification of receptors will be needed in order to determine glycosylation state of receptor types. The method of photolabeling utilized here may facilitate achieving this goal.

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BINDING TO MU AND DELTA OPIOID SITES BUT NOT KAPPA SITES IS INHIBITED BY FAB FRAGMENTS FROM A MONOCLONAL ANTIBODY DIRECTED AGAINST THE OPIOID RECEPTOR

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ABSTRACT

Fab fragments from a monoclonal antibody, OR-689.2.4, directed against the opioid receptor selectively inhibited opioid binding to rat and guinea pig neural membranes. In a titrable manner, the Fab fragments noncompetitively inhibited the binding of the μ selective peptide [^{3}H][D-Ala^2,MePhe^4,Gly-ol^6] enkephalin (DAGO) and the δ selective peptide, [^{3}H][D-Pen^2,D-Pen^5] enkephalin (DPDPE), to neural membranes. The binding of [^{125}I-Tyr^{27}] endorphin was also blocked by the Fab fragments. In contrast, κ opioid binding, as measured by the binding of [^{3}H]bremazocine to rat neural membranes and guinea pig cerebellum in the presence of μ and δ blockers was not significantly altered by the Fab fragments. When μ sites were blocked with DAGO, the Fab fragments suppressed the binding of [^{3}H]DPDPE to the same degree as when the μ binding sites was not blocked. The Fab fragments also inhibited binding to the μ site regardless of whether or not the δ site was blocked. This monoclonal antibody is directed against a 35,000 dalton protein. Since the antibody is able to inhibit μ and δ binding but not κ opioid binding, it appears that the 35,000 dalton protein is an integral component of μ and δ opioid receptors but not κ receptors.

INTRODUCTION

As a tool to probe the molecular basis of the multiple opioid receptors, a monoclonal antibody, OR-689.2.4, capable of partially inhibiting opioid binding to rat neural membranes has been produced (Bidlack and Denton 1984). This IgM was able to block and displace opioid ligands from rat neural membranes. The specificity of this immunoglobulin has been demonstrated by its inability to inhibit the binding of 5 different nonopioid ligands to neural membranes and the ineffectiveness of other mouse immunoglobulins to block the binding opioids to neural membranes (Bidlack and Denton 1985). The antibody, directed against a 35,000 dalton protien, immunoprecipitated opioid binding sites from a solubilized preparation (Bidlack and Denton 1985). Because it is an IgM with a molecular weight of 980,000, the ability of the antibody to penetrate tissue is hampered. As a consequence, *a* procedure was developed for obtaining Fab fragments, with a molecular weight of 48,000, from this IgM (Bidlack and Mabie in press). This study describes the selective inhibition of opioid ligands by OR-689.2.4 Fab fragments.

MATERIALS AND METHODS

Generating the OR-689.2.4 monoclonal IgM and Fab fragments

The IgM, OR-689.2.4, was produced by immunizing a BALB/c mouse with a partially purified opioid receptor complex consisting of proteins with molecular weights of 43,000, 35,000 and 23,000 (Bidlack et al. 1981; Harwell et al. 1984). The monoclonal IgM was detected by radioimmunoassay using the ^{125}I-labeled antigen and by its ability to inhibit opioid binding to rat neural membranes (Bidlack and Denton 1984). The Fab fragments from the IgM were generated by digesting the IgM with trypsin in the presence of 10 mM cysteine and were purified by gel filtration (Bidlack and Mabie in press).

Opioid binding to rat and guinea pig neural membranes in the presence of OR-689.2.4 Fab fragments

Neural membranes from male Sprague-Dawley and Hartley guinea pigs were prepared, excluding cerebellar tissue, as previously described by Pasternak et al. (1975). Guinea pig cerebellar membranes were prepared in a similar manner, In a final volume of 1 ml, 0.25 mg of neural membranes protein was incubated wth OR-689.2.4 Fab fragments for 60 min at 25 C. Radiolabeled ligand was then added and the incubation continued for 60 min. Binding of [^{125}I-Tyr^{27}] β_{1h}-endorphin was measured in the presence of 0.2% bovine serum albumin and 100 µg/ml bacitracin. The glass fiber filters were soaked in 0.2% polyethylenimine prior to use. Bound radioactivity was determined by filtering samples through Whatman GF/B glass fiber filters. All results are reported as specific binding- the difference between binding in the presence and absence of 10 µM unlabeled ligand. Binding of 0.1 nM [^{3}H]bremazocine to rat neural membranes and guinea pig cerebellum in the presence of 100 nM DAGO and either 100 nM DADLE or 1 µM CPDPE was regarded as binding to κ sites.

Measuring the ability of the OR-689.2.4 Fab fragments to inhibit binding to membranes when binding sites are blocked

To determine if the Fab fragments could inhibit the binding to µ sites when δ sites were blocked, the following protocol was followed. Membranes were incubated with 100 nM DPDPE for 30 min to block δ sites. OR-689.2.4 Fab fragments at a concentration of 250 nM were added. Following a 60 min incubation, 0.5 nM [^{3}H]DAGO was added. After an additional 60 min incubation, the samples were filtered.

RESULTS AND DISCUSSION

As shown in Fig. 1, OR-689.34 Fab fragment% inhibited binding to the binding of [^{125}I]endorphin, [^{3}H]DAGO, and [^{3}H]DPDPE to rat neural membranes in a titrable manner. The IC_{50} value for the inhibition of [^{125}I]β-endorphin binding was 400 nM, while the IC_{50} value for the inhibition of binding of the µ and δ ligands was about 3-fold greater. As previously shown, the Fab fragments are noncompetitive inhibitors of µ and δ binding to rat neural membranes (Bidlack and O'Malley in press).

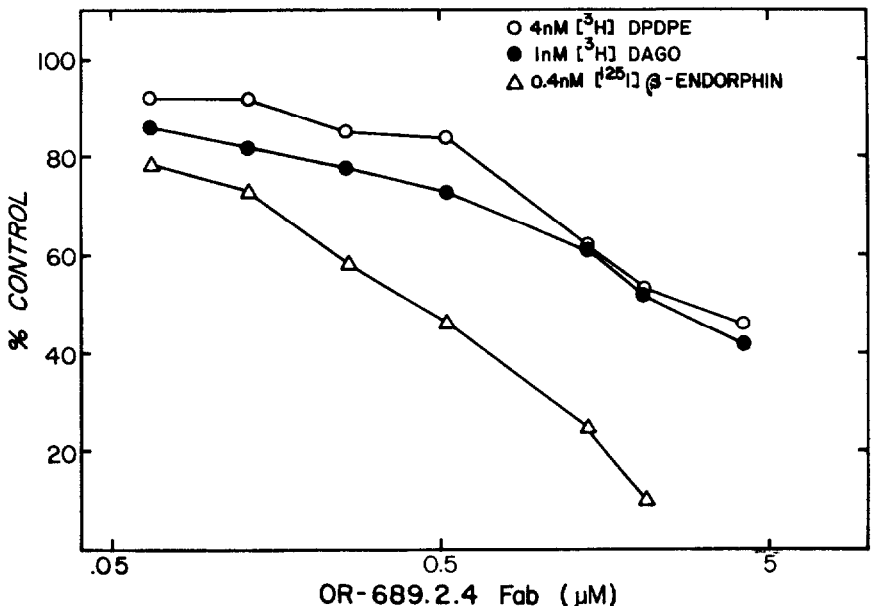

FIGURE 1. Inhibition of opioid binding to rat neural membranes by OR-689.2.4 Fab fragments. Binding was measured as described in "Materials and Methods."

Kappa binding to rat neural membranes and guinea pig cerebellum was determined by the binding of 0.1 nM [^{3}H]bremazocine in the presence of μ and δ blockers. In the absence of blockers, 500 nM OR-689.2.4 Fab fragments inhibited the binding of 0.1 nM [^{3}H]bremazocine to rat neural membranes by 27% (Bidlack and O'Malley in press). However, in the presence of 100 nM DAGO and 100 nM DADLE, the Fab fragments did not have a significant effect on the binding of 0.1 nM [^{3}H]bremazocine. The Fab fragments did not alter the binding of 0.1 nM [^{3}H]bremazocine to guinea pig cerebellum regardless of whether μ and δ blockers were present (Bidlack and O'Malley in press). The antibody inhibited the binding of [^{3}H]DAGO and [^{3}H]DPDPE to guinea pig neural membranes.

Since the antibody is directed against a 3-dimensional epitope on the 35,000 dalton protein (Bidlack and Denton 1985), it may be possible to use the antibody to probe conformational changes of the receptor. In an attempt to address the question of interconversion between μ and δ binding sites, the ability of the OR-689.2.4 Fab fragments to inhibit the binding of 0.5 nM [^{3}H]DAGO to neural membranes was determined after suppression of δ sites by 100 nM DPDPE. As detailed in Bidlack and O'Malley (in press), the Fab fragments inhibited the binding of 0.5 nM [^{3}H]DAGO to the same degree regardless of whether δ sites were or were not blocked by DPDPE. The same results were obtained when μ sites were block with DAGO and the ability of the Fab fragments to inhibit the binding of [^{3}H]DPDPE was measured. Under the experimental conditions used, there is not an interconversion of μ and δ sites that results in the inability of the antibody to inhibit binding to the other site

when one site is blocked.

The monoclonal antibody is directed against a 35,000 dalton protein (Bidlack and Denton 1985). Evidence supporting the role of the 35,000 dalton protein as a component of the opioid receptor includes crosslinking studies using enkephalin-like peptides (Zukin and Kream 1979; Zukin et al. 1980), human [^{125}I-Tyr^{27}] β-endorphin (Howard et al. 1985; Howard et al. 1986) and [^{125}I-Tyr^{27},Leu^{5}], β-endorphin (Helmeste et al. in press). Affinity labeling studies using [^{125}I]14-bromoacetamidomorphine also resulted in the specific labeling of the 35,000 dalton protein (Bidlack et al. 1982). Purification studies using an opioid agonist affinity column (Bidlack et al. 1981; Maneckjee et al. 1985) and an opioid antagonist affinity column (Bidlack et al. 1982) have resulted in the specific elution of a 35,000 dalton protein. The monoclonal antibody directed against the 35,000 dalton protein immunoprecipitated opioid binding sites from a solubilized preparation of rat neural membranes (Bidlack and Denton 1985). These studies strongly suggest that the 35,000 dalton protein is a component of the opioid receptor, probably of the μ and δ receptors but not the κ receptor.

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MONOCLONAL ANTIIDIOTYPIC ANTIBODIES WHICH RECOGNIZED THE BINDING SITE OF DELTA RECEPTOR : FINE SPECIFICITY OF THE ANTIIDIOTYPIC ANTIBODIES

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ABSTRACT

Monoclonal antiidiotypic antibodies (Mab2), specific for the binding unit of the opiate δ receptor, were generated in rats immunized with rabbit anti-DADLE antibodies (ab1). The fine specificity of the seven clones screened in the fusion was studied using a solid phase test where the abl antibodies were coated to the plate. In this respect, 4 different set of monoclonal antibodies can be distinguished. The antibody affinities were comparable (Kd 1.10^{-8} M), but the specificity patterns were completely different.

INTRODUCTION

A powerful approach to the production of antireceptor antibodies is now fully exploited for a number of different cell receptors. This approach employs the concept that antibodies directed against pharmacologically active ligands have a three dimensional binding site which is somewhat analogous to the natural receptor. Consequently, when antiidiotypic antibodies (ab2) are produced against these anti-ligand antibodies (ab1), some of the antiidiotypic antibodies, which mimic the original ligand, are able to bind to cell surface receptors. Anti-idiotypic antibodies, which have specificity of a variety of hormone and neuro-transmetter receptors have been produced following immunization against specific anti-ligand antibodies (Cleveland et al. 1983, Farid and Lo 1985, David and Isom 1985). Using the antiidiotypic approach, we developed monoclonal anti-idiotypic antibodies, specific for the binding site of the δ receptor. These (ab2) antibodies were generated in rats immunized with rabbit anti-DADLE antibodies. These anti-idiotypic antibodies bind the cell surface of the NG108-15 cells and precipitated a surface exposed 53Kd protein on the same cells (Cupo et al. data to be published). The present study reports a detailed analysis of the specificity of the seven monoclonal antiidiotypic antibodies screened in the fusion and allows us to distinguish four different antiidiotypic antibodies sets according to their structural requirements.

MATERIALS AND METHODS

Purification of monoclonal antibodies from ascitic fluids

3.10^6 hybridoma cells of each different clone were intraperitoneally injected in nude mouse: (Swiss strain) in order to produce ascitic fluids. After collection, 5 ml of each ascitic fluid were precipitated by 50% ammonium sulfate and purified by DEAE-A52 chromatography according to the Bazin's procedure for the IgG2a isotype (Bazin et al. 1974).

Anti-idiotypic test

DADLE was cross-linked by carbodiimide with bovine serum albumin (as irrelevant carrier). Flexible PVC microtiter plates (Limbro plate SMRC 96) were coated with 50 µl of conjugate solution (1 nmole of DADLE) for 4 h at room temperature. The solution was then removed and the wells were filled for 4 hours with PBS-0.5% BSA to saturate the remaining binding site of the wells. The plates were washed 3 times with PBS, then dried and stored at 4°C for weeks until used. A cascade of monoclonal antibody dilutions and a solution of rabbit anti-DADLE immunserum (diluted 1/2000 in PBS-0.1% BSA) were preincubated at 37°C for 2 h 30 in plastic tube. Then 50 µl of this mixture was transfered into conjugate-coated wells at 4°C for 2 h. The wells were washed 3 times with PBS, then incubated with 50 µl of iodinated protein A (1.10^5 cpm) for 30 min at room temperature then washed again and counted. Polyclonal rat immunserum (anti-anti-DADLE, diluted 1/100) and irrelevant rat monoclonal antibodies (anti-AMPc) served as controls.

Affinity and cross-reactivity measurements

The rabbit anti-DADLE antibodies (ab1) were purified onto a protein A-Sepharose column. The total IgG solution (1.10 M) was coated on PVC plates (50 µl/well) following the same procedure than in the antiidiotypic test. The seven monoclonal antiidiotypic antibodies were ^{125}I-iodinated, diluted in PBS-0.1% BSA and stored at 4°C. The inhibition of binding of iodinated antiidiotypic antibodies Mab2, by ab2 was assessed by simultaneous incubation in abl (anti-DADLE antibodies) coated wells of 25 µl of iodinated Mab2 (100,000 cpm) and 25 µl of ab2 at concentrations ranging from 10^{-5} M to 10^{-11} M. The incubation was performed for 24 h at 4°C, the wells were then washed 3 times in PBS and counted.

RESULTS AND DISCUSSION

Antiidiotypic activity

The antiidiotypic activity of each purified monoclonal antibody (Mab2) was characterized by its ability to inhibit the interaction ab1-DADLE. The 11 and 141 clones were able to inhibit 100% of the interaction ab1-DADLE, The plateau was reached with an Mab2 concentration around 1.5×10^{-7}M. For the 5 and 106 clones, the ab2 concentration necessary to reach a plateau was identical, but these antibodies were able to inhibit 85-90% of the abl-DADLE interaction. The 51 and 70 clones and 16 clone inhibited only 50% and 30% of the interaction ab1-DADLE, respectively. To obtain this inhibition, the Mab2 concentration was around $1.5.10^{-6}$ M. We can conclude, that the 11 and 141 antibodies recognized all the abl population in a same manner or more probably the same epitope exhibited by all the polyclonal anti-ligand antibodies while the other clones 5-106, 51-70 and 16 recognized 85-90%, 50% and 30% of the abl population, respectively.

The results were shown in Figure 1.

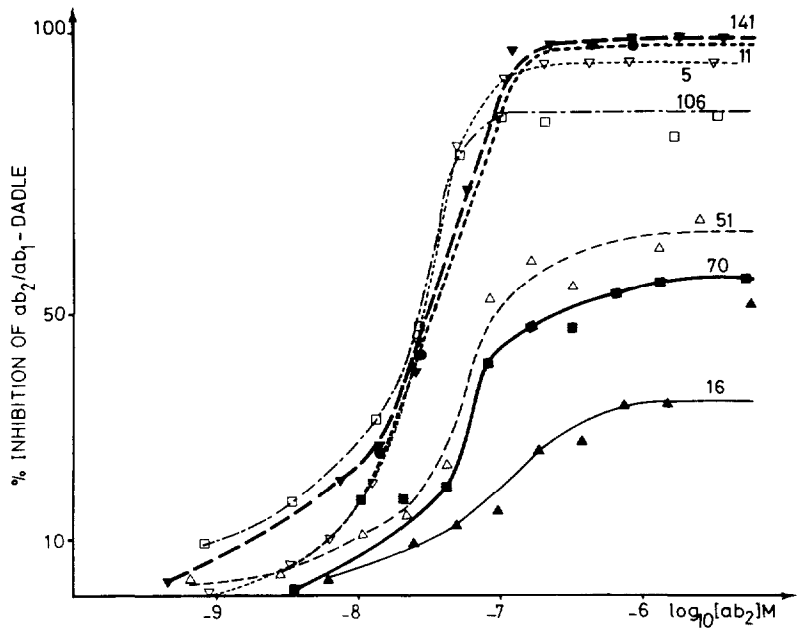

FIGURE 1. Competition curve for the purified monoclonal antibodies.

Fine specificity of the anti-idiotypic antibodies (Mab2)

The affinity and the specificity of each purified Mab2 was realized by competition studies using ^{125}I-labelled purified Mab2. The table exhibited the results. The affinity (IC50) was expressed in term of concentration (M) able to inhibit 50% of the ^{125}I-MabZ-ab1 interaction. The specificity pattern allows us to distinguish 4 different Mab2 sets. Appeared to be identical 5 and 106 clones, 51 and 70 clones and 11 and 141 clones. Their affinities are similar and close to 1.10^{-8}M but their specificities are different. The 5, 11 and 16 Mab2 did not recognized the epitope recognized by the 51 Mab2; moreover the fixation of these Mab2 on their respective epitope did not interfere with the specific binding of the 5 Mab2. For sake of clarity, we will named the epitope recognized by the monoclonal antibody according to the number of the antibody. The 11 and 51 Mab2 did not recognized the 16 epitope but the 5 Mab2 might be, either, exhibited a weak cross-reaction with the 16 epitope (f around 930) or its fixation on its specific epitope weakly disturbed the specific binding of the 16 Mab2. We can propose the same explanation for the specificity study of the 11 Mab2 ; the 16 and 51 Mab2 did not recognize the 11 epitope and the 5 Mab2 cross-reacted weakly with the 11 epitope directly or not. The specificity pattern of the 5 cloned showed that 16 and 51 clones did not recognize the 5 epitope but the 11 Mab2 strongly inhibited the 5 specific binding.

TABLE. FINE SPECIFICITY OF THE ANTI-IDIOTYPIC ANTIBODIES (versus ab1)

	Iodinated M,ab						
	5	106	51	70	11	141	16
5	9.10^{-9}M	7.10^{-9}M	nI	nI	1.25	1.12	nI
106	7.10^{-9}M	$7.1.10^{-9}$M	nI	nI	2.5	2.5	nI
51	nI	nI	$1.2.10^{-8}$M	$1.2.10^{-8}$M	nI	nI	nI
70	nI	nI	$1.2.10^{-8}$M	$1.2.10^{-8}$M	nI	nI	nI
11	240	240	nI	nI	1.10^{-8}M	1.10^{-8}M	nI
141	150	75	nI	nI	1.10^{-8}M	$9.7.10^{-8}$M	nI
16	930	320	nI	nI	nI	nI	$3,10^{-9}$M

nI= no inhibition was observed using high concentration of Mab2 (C > 10^{-6}M). The number expressed the cross-reactive factor f=IC50 analogue versus IC50 homologue.

We can imagine either that the 11 and 5 epitopes are structurally related epitopes or that the binding of the 11 Mab2 induced dramatically structural change of the 5 epitope, but clearly the two epitopes were carried by the same abl antibodies.

In conclusion, using the antiidiotypic approach, we have obtained 7 monoclonal antibodies (Mab2) which recognized the binding unit of the δ receptor. These 7 Mab2 exhibit similar affinity but the antiidiotypic activity and the specificity pattern allows us to distinguish four different monoclonal antibody sets.

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ANTI-IDIOTYPIC ANTIBODIES RAISED AGAINST ANTI-METENKEPHALIN ANTIBODIES IN RABBITS

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ABSTRACT

Anti-idiotypic antibodies were raised in rabbits immunized against immunoglobulins purified from anti-metenkephalin antibody. The antibodies competed dose-dependently with metenkephalin in binding to anti-metenkephalin antibody on a solid phase metenkephalin enzyme-linked immunosorbant assay (ELISA). The titers of the anti-idiotypic activity appeared characteristically in transient peaks and troughs in alternation with anti-metenkephalin activities and were retained after purification on various affinity columns.

INTRODUCTION

Anti-idiotypes (anti-idiotypic antibody) and antibodies have been proved to be useful tools in the study of receptor structure and function (Venter et al. 1984). The mechanism of interaction between a ligand and its antibody might be quite different from its binding to a receptor. However, when an idiotypic antibody, raised from an anti-ligand antibody, binds to the receptor, it is always directed at the active binding site of the receptor. This implies that, under circumstances when it is difficult to prepare anti-receptor antibodies from the purified receptor directly, it might be feasible to use the anti-idiotypic approach. In this way one obtains an anti-receptor antibody without purifying the receptor (fig. 1).

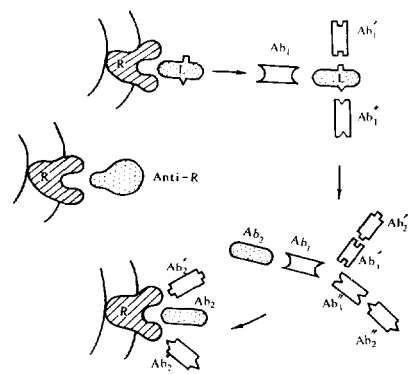

FIGURE 1. Anti-idiotypic approach of producing anti-receptor antibodies. L: ligand, R: receptor, Anti-R: anti-receptor antibody.

While working on opiate receptors, we have been trying to produce polyclonal and monoclonal anti-idiotypic antibodies as an immunological reagent and pharmacological tool to study the structure and function of opiate receptors. We have succeeded in raising anti-metenkephalin antibody (Ab_1) in rabbits and use the purified anti-metenk immunoglobulin as immunogen to produce polyclonal anti-idiotypic antibody (Ab_2) against it.

METHODS

1. Immunization of rabbits:

Rabbits were immunized at multiple spots intradermally over the back with metenkephalin (metenk) conjugated to thyroglobulin at a dosage of 200 μg of free metenk emulsified in complete Freund's adjuvant for primary immunization, and 100 μg for monthly boostings thereafter. The animals were bled to death when a reasonable high titer of anti-metenk antibody was detected in the serum. The antiserum was then purified down to its IgG (IgG,) with a Protein A-Sepharose 4B column. This purified Ab_1 was used as immunogen at 2 mg in complete Freund's adjuvant for primary immunization and 1 mg thereafter to raise Ab^2 in rabbits. The best protocol we followed finally was to immunize

the rabbits once a week within the first month and biweekly thereafter or whenever necessary.

2. ELISA for the characterization of anti-metenk antibody and Ab_2 against it:

Multicell polystyrene plate coated with metenk-BSA conjugate 0.1 μg/well was incubated with the rabbit anti-metenk antibody to be characterized, followed by goat-antirabbit IgG horseradish peroxidase conjugate and then OPD added with H_2O_2 as its substrate, to develop a color reaction readable at 492 nm with a spectrometer. For anti-idiotypic antibody against anti-metenk antibody, the anti-serum to be characterized was first incubated with a standard Ab_1, before they were added to the well and proceed as mentioned above.

3. Purification of the anti-idiotypic antiserum:

Normal rabbit IgG or metenk was coupled to CNBr-activated Sepharose 4B to form normal IgG-Spherose 4B and metenk-Sepharose 4B affinity columns. These are used to separate anti-metenk and non-specific anti-IgG activities included in the anti-metenk anti-idiotypic antiserum.

RESULTS

1. The specificity and sensitivity of the ELISA (Liu et a1. 1985), developed for the characterization of anti-metenk antibody and anti-idiotypic antibody against it:

The readings of O.D. at 492 nm on ELISA were linear with serial dilutions of anti-metenk antibody or its IgG; it was even more sensitive than radio-immunoassay in monitoring the titer of anti-metenk antibody (fig. 2). The binding of Ab_1 or its IgG to the solid phase metenk was displaceable by free metenk in the solution and is sensitive up to less than 0.5 μg or 1 nmol of free metenk (fig. 3). So it could also be utilized for the characterization of the anti-idiotypic activities developed against Ab_2.

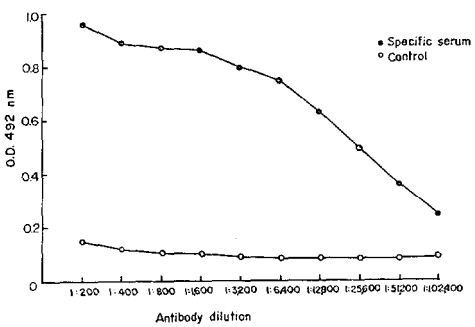

FIGURE 2. Rabbit anti-metenk antiserum dilution curve.

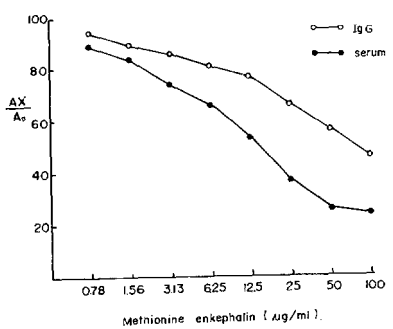

FIGURE 3. Inhibitory effect of free metenk on the binding of antiserum or its IgG to metenk-BSA conjugate coated on the polystyrene plate. 50 μl of either antiserum or its IgG were preincubated with 50 μ1 of free metenk in twofold serial dilutions at 37°, 1 hr, before ELISA.

2. The development of anti-iditiotypic antibody against Ab, in rabbits:

Rabbits immunized against anti-metenk IgG, were bled at different intervals after immunization and monitored for their titer of anti-idiotypic activities in their serum. As shown in fig. 4A and 4B, in three out of four rabbits immunized with IgG_1, their anti-idiotypic activities went up and down in peaks and troughs, and did not follow closely the protocol of immunization.

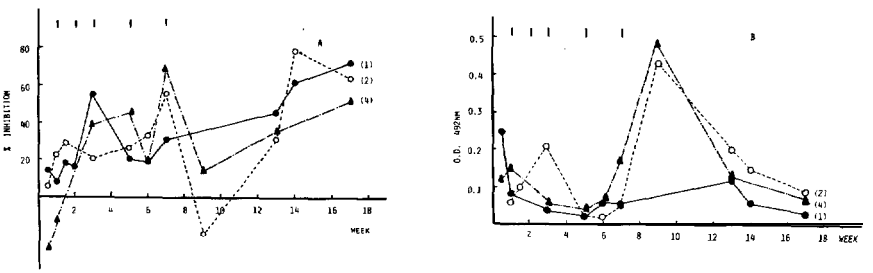

FIGURE 4. (A) The inhibitory effect of anti-metenk anti-idiotypic antiserum on the binding of anti-metenk antiserum and solid phase metenk. (B) The binding of anti-metenk anti-idiotypic antiserum on solid phase metenk. The anti-metenk anti-idiotype antiserum was drawn after immunization with anti-metenk IgG at dilferent time intervals as indicated on the abscissa. Number in parenthesis indicates the number of rabbits.

At the same time, there were corresponding fluctuations of anti-metenk activities in the serum, interlocking with anti-idiotypic activities, as demonstrated clearly in fig. 5 for individual rabbits.

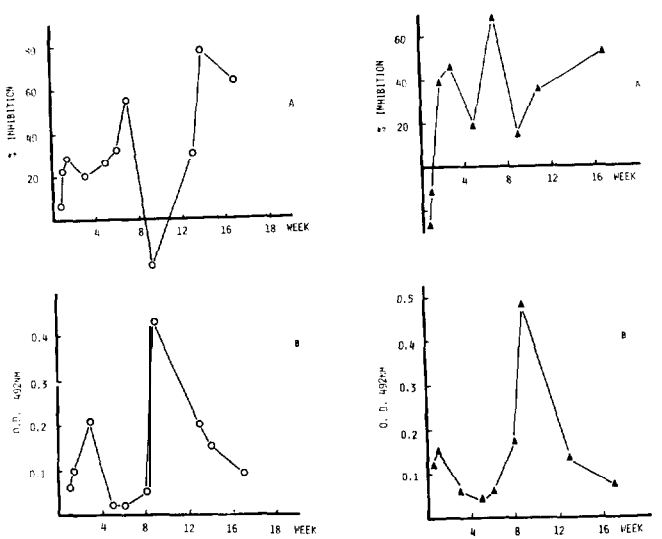

FIGURE 5. The generation of anti-idiotypic antibodies following immunization with anti-metenk IgG in Rabbit #2 (left) and #4 (right). (A) The inhibitory effect of anti-metenk anti-idiotypic antiserum on the binding of anti-metenk antiserum and solid phase metenk. (B) The binding of the antiserum to solid phase metenk.

3. The effect of purification of anti-idiotypic activities on normal rabbit IgG- and metenk- Sepharose 4B columns:

The anti-idiotypic activities were retained in effluents when the anti-idiotypic antiserum was added in batches to normal IgG- or metenk- Sepharose 4B affinity gels. At the same time the eluates or the filtrates, obtained after the gels were treated with 0.1 M acetic acid (pH 2.4) and neutralized immediately by saturated Tris buffer, showed no significant anti-idiotypic activities (fig. 6).

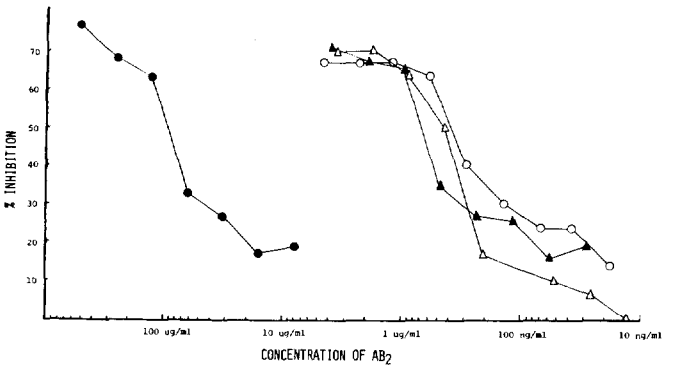

FIGURE 6. The inhibition of purified anti-metenk anti-idiotypic antibody on the binding of anti-metenk with solid phase metenk. ●——● serum, ▲——▲ IgG, ○——○ effluent from normal IgG-Sepharose 4B, Δ——Δ effluent from metenk-Sepharose 4B.

DISCUSSION

Our results indicate that anti-idiotypic antibody could be generated against anti-metenk antibody in rabbits immunized against anti-metenk IgG. Their appearance is in line with the "immune network theory" proposed by Jerne (1974). The characteristic peaks and troughs, in alternation with anti-metenk activities, were probably the result of a self-regulatory mechanism within the immune system of the body. Analogous findings have been reported in the literature with rabbits immunized against anti-alprenolol IgG by Strosberg's group (Couraud et al. 1983). Our results also indicate a transient nature of the anti-idiotypic activity. No definite relation could be found between its appearance in the serum and the protocol of immunization so that optimal timing of its presence in the serum was only arbitrary. The amount of blood that could be drawn from the animal was also limited. Further characterization of the anti-idiotypic antiserum we obtained are now still underway, especially with regard to its direct binding to opiate receptors. At the same time efforts are being made to repeat the same procedure in BALB/c mice in an attempt to obtain an almost unlimited supply of monoclonal anti-idiotypic antibody.

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DEVELOPMENT OF A BIOTIN-AVIDIN PROBE FOR DETECTING OPIOID RECEPTORS

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ABSTRACT

Biotinylated derivatives of β$_h$-endorphin (β$_h$-EP) with C_6 spacer arm, inserted between biotin and β$_h$-EP, were synthesized and isolated by HPLC. Liquid secondary ion mass spectrometry (LSIMS) indicated the presence of 1 to 4 biotin substituents per β$_h$-EP molecule, and in combination with the analysis of tryptic peptide fragments, specified the location of the biotinylated lysine residue. Affinities to μ receptors decreased with increasing biotinylation number. Association of the biotinylated ligands with avidin retained or even enhanced IC_{50} values at the μ site, thus, matching the relative binding affinity of underivatized β$_h$-EP with the monobiotinylated derivatives. Hence, monobiotinylated β$_h$-EP represents a versatile opioid receptor probe.

INTRODUCTION

The avidin-biotin sandwich system represents a versatile tool in biochemistry and molecular biology (Korpela 1984). Bifunctional biotinylated receptor ligands have been employed for purification (Finn et al. 1984) or histochemical detection of hormone and neurotransmitter receptors with high resolution (Childs et al. 1983, Atlas et al. 1978). Previously reported biotinylated leucine-enkephalin derivatives failed to crosslink the opioid receptor and avidin with sufficiently high affinity (Koman and Terenius 1980). Here we report the synthesis of several biotinylated β$_h$-EP analogs with a C_6 spacer arm and varying positon and number of biotinylation. Among these, avidin-bound monobiotinylated derivatives, modulated near the COOH terminal end were found to bind to the μ receptor with a similar affinity to that of underivatized β$_h$-EP and hence, represent a promising probe for the opioid receptor.

MATERIALS AND METHODS

β$_h$-EP was kindly provided by Dr. Chao Hao Li (University of California, San Francisco). The following compounds were obtained from the indicated sources: dimethylsulfoxide (DMSO), bacitracin, trifluoroacetic acid (TFA), bovine serum albumin (BSA), 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) from Sigma, egg white avidin (binding capacity: 12.8 μg biotin/mg), biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (biotin-XNHS), D(+)-biotin from Calbiochem, [^{3}H]DAGO (50 Ci/mmol) from Amersham and diprenorphine-HCl from NIDA.

Synthesis and identification

β$_h$-EP (100 μg in 100 μl of 0.1 M $NaHCO_3$, pH:8.3) was mixed with 300 μl of biotin-XNHS (20 μg in DMSO). After 30 min, the reaction was stopped by addition of 100 μl 1M acetic acid. Separation of mono- and bis-biotinylated products was performed by reversed phase HPLC (μ Bondpack C18 column, Waters) using a 40 min linear gradient

(initial solvent:acetonitrile/0.1% TFA in H_2O (28/72, v:v); final solvent: acetonitrile/0.1% TFA in H_2O (34/66, v:v> with a flow rate of 1 ml/min. UV detection was performed at 210 nM. Peaks were collected and concentrated under a stream of nitrogen. Reaction products were identified by LSIMS, as described by Gibson et al. (1984), with or without prior tryptic digestion and subsequent HPLC separation.

Binding studies

Ability of biotinylated analogs to bind to avidin was tested spectrometrically, by displacing HABA from avidin (Green, 1970). Competitive receptor binding studies were performed in washed rat brain membranes (brain without cerbellum, Sprague-Dawley, male, 120-140 g) with [^{3}H]DAGO tracer (1 nM) at 20°C, as described by Yu et al. (1986) with the exception of the incubation buffer (50mM Tris-HCl, 0.1% BSA, 0.01% bacitracin, pH:7.4). Ligands were used after rechromatography of isolated peaks, shown in figure 1. Binding in the presence of avidin was tested with preformed ligand-avidin complexes at a fixed avidin concentration of 0.5 µM. Bound and unbound tracers were separated by filtration through Whatman GF/B filters. Relative receptor affinities were calculated from IC_{50} values according to Yu et al. (1986).

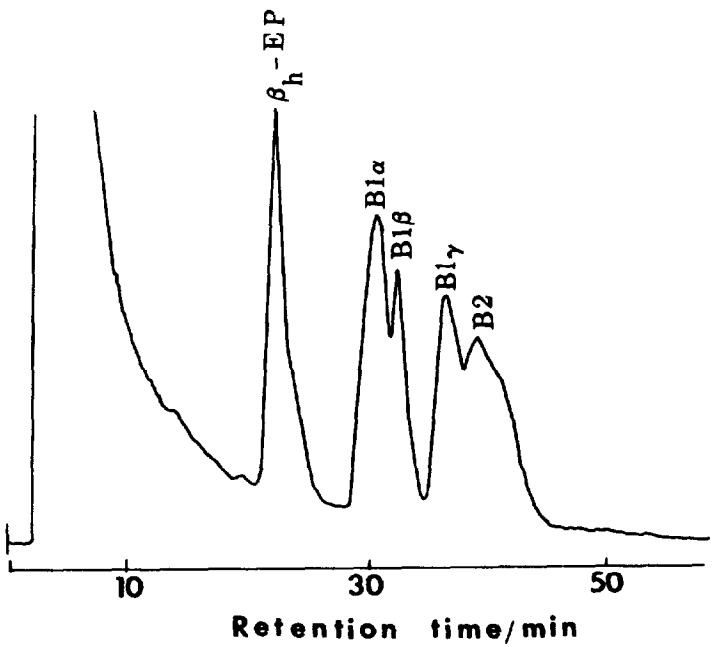

FIGURE 1. HPLC separation of monobiotinylated analogs. For separation conditions see materials and methods (B1α, B1β, B1γ: monobiotinylated isomers; B2: mixture of bis-biotínylated products).

RESULTS AND DISCUSSION

We used β_h-EP as parent compound for developing bifunctional ligands, since it binds to the opioid receptor with high affinity (Ho et al. 1983) and slow dissociation kinetics (Howard et al. 1985). Further, β_h-EP can be covalently crosslinked to the receptor sites (Howard et al. 1985), which could serve to establish avidin-affinity chromatography for receptor purification. β_h-EP can be readily biotinylated via free amino groups of lysine that are mainly located in the a helical part, near the COOH terminal end, where modulations may have little affect on receptor binding (Taylor et al. 1983). Biotin was introduced via a C_6 spacer arm, since systemic studies showed the necessity of a spacer of at least 8Å length to fully retain avidin binding to macromolecule biotin-conjugates (Green et al. 1971). Reaction of β_h-EP with biotin-XNHS resulted in a mixture of mono- to tetra-biotinylated analogs, that were separated by reversed phase HPLC according to their biotinylation number (data not shown). Mono-biotinylated derivatives were further resolved by two-step chromatography into several isomers (figure 1). Identification was performed by LSIMS, with or without prior tryptic digestion. Biotinylation of a lysine eliminates a potential tryptic cleavage site; thus, in place of two peptides generated from trypsin cleavage of native β_h-EP only one new peptide containing biotin will be generated. LSIMS of peptide fragments, after HPLC separation readily specified number and position of the biotin residue (table 1).

TABLE 1. Relative IC_{50} values for μ receptor binding of biotinylated β_h-EP derivatives. Relative IC_{50} values are calculated as described in materials and methods. IC_{50} values of β_h-EP for μ sites were 1.6 ± 0.7 (n=7) and were identical in the presence of absence of avidin. B1 and B2 represent derivatives, separated according to the biotinylation number (mixture of isomers).

Ligand	Rel IC_{50}		Position of biotinylation
	without avidin	with avidin	
β_h-EP	1	1	
B1	4		monobiotinylated
B1α	4.3(4.1-4.5)	1.1(0.7-1.4)	Lys 28, 29 or 24
B1β	5.5(3.5-7.8)	3.0(2.0-4.0)	Lys 9
B1γ	8.5(6.5-10.5)	2.9(1.7-4.2)	Lys 19
B2	18 (16-19)	10	bisbiotinylated

Mono- and bis-biotinylated compounds displaced HABA from avidin in a stoichiometric manner directly related to the biotinylation number, which suggested that each biotin residue was accessible to avidin. Affinities to μ sites were determined in rat brain homogenate, using underivatized β_h-EP (figure 1) as internal standard (IC_{50}: 1.6 ± 0.7 nM, n=7). There was a decrease of binding affinity as a function of biotinylation number (table 1). Monobiotinylated derivatives exhibited binding affinities approximately 5 times less than that of the parent compound, with no significant effect of the biotin position on the binding potency. The hydrophilic region between

residues 6-12 is thought to be responsible for the potency of β_h-EP binding to the μ sites (Taylor et al. 1982), however, biotinylation at Lys 9 did not affect the binding potency relative to biotinylation near the COOH terminal.

In order to test the ability of the bifunctional ligands to crosslink receptor sites with avidin, binding studies were performed in the presence of excess of avidin (0.5 μM). This insured the presence of biotinylated β_h-EP-avidin complexes at a 1:1 ratio over the whole spectrum of ligand concentrations. Neither tracer binding nor the IC_{50} value of β_h-EP were affected by this concentration of avidin. IC_{50} values of mono- and bis-biotinylated analogs shifted to higher affinities by a factor of 2-4. A decrease of IC_{50} values in the presence of avidin has not been previously reported for biotinylated receptor ligands. However from these data alone, a higher receptor affinity of the complex cannot be readily deduced. High nonspecific binding to glass and plastic presents problems in receptor assays with β_h-EP. Assuming that nonspecific binding is reduced for avidin associated ligand, a shift of the competition curve to lower IC_{50} values will be the result, without any change in intrinsic receptor affinity of the complex over that of the free ligand. Because of the high affinity of the monobiotinylated β_h-endorphin Bla (with the substituent near the COOH terminal), which is retained or even enhanced after tight association with avidin, this derivative represents the most promising probe of the opioid receptor.

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CONSIDERATIONS ON THE NATURE OF μ, κ and σ RECEPTORS

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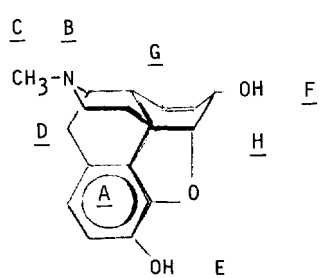
ABSTRACT

It is assumed that opioid receptors are flexible, distorted by agonists but not by antagonists. Combined with Martin's model of the receptors, this assumption permits reasonable interpretation of experimental facts.

This is a progress report on an attempt to reinterpret Martin's picture of opioid receptors (1983) in light of the hypothesis that receptors are flexible, inactive in their native conformation but activated when drugs distort them (Gero 1973). Drugs attaching without distortion have no effect and thus function as competitive antagonists and may furnish information on the native receptor. Agonists tell about the nature of receptor distortion.

Space limitation precludes reporting all results so far obtained. Only some of the more important findings and the principal items of evidence will be presented.

Martin distinguishes two "nuclear" sites in opioid receptors, an aromatic ring binding site A and an anionic site B, and six major (plus several minor) "satellite" sites which either reinforce drug binding to the nuclear sites or (keeping the drug away from the nuclear sites) hinder it. The location of the binding sites around the morphine molecule is shown in the following sketch:



This writer also postulated two nuclear sites (Gero 1978), assuming further that the two sites are so located that they can bind a drug molecule strainlessly if its cationic group is about 6½ Å from the center of the benzene ring. The cation-binding nuclear site in μ receptors is C, not B, and it binds not the N

atom but the methyl goup attached to it which, for instance in morphine, the quintessential μ agonist, carries the bulk of the cationic charge (Kaufman et al. 1974). In morphine and its derivatives, including morphinans and benzomorphans, the N-methyl is only 4½ Å from the center of the benzene ring, and the rigidity of the polycyclic ring system permits no alteration of this distance. Binding can therefore occur only by forcing the receptor to narrow the A-C distance, hence such drugs are generally μ agonists. If, however, allyl replaces the N-methyl, resonance shifts the cationic charge to the terminal C atom of the allyl group. This C atom is 6½ Å from the center of the benzene ring, hence nalorphine, levallorphan, etc. do not distort the receptor and are μ antagonists. A similar charge shift occurs in N-cyclopropylmethyl and N-cyclobutylmethyl.

The first step in the attachment of the drug to the receptor is probably the attraction of the cationic head of the drug to the anionic receptor site. Then the other sites come into play and add their binding energy to the formation of the drug-receptor complex. It is possible that not all other sites come close enough to the drug molecule to contribute to binding. The final conformational adjustment of the drug-receptor interaction will be that which provides the greatest free-energy decrease and therefore the most stable - i.e. strongest - attachment. In this the benzene ring of the drug plays a special role: if in the aggregate more free energy is lost by binding it to site A even at the cost of receptor distortion, the drug will be an agonist, otherwise an antagonist. The significance of the nuclear sites IS that their distance from each other in the drug-receptor complex determines whether or not a drug is an agonist. It is also apparent that there must be a great variety of possibilities for antagonistic binding: a drug needs to occupy only part of the binding sites in the receptor - of which site A may or may not be one - in order to function as an antagonist.

Among the satellite sites, site F seems to be particularly important. We think that it is an electrophilic site with particular affinity for a carbonyl oxygen at about 8 Å from the N-methyl (or 7 ½Å from the N), therefore it favors the attachment of hydromorphone, naloxone, etc. In some cases sites C and F may hold a drug molecule quite strongly, and if no steric factors keep the drug molecule from pivoting around the axis formed by these two sites and away from site A, we have an antagonist. On the other hand, in a drug like meperidine sites C and F can also bind the N-methyl and the carbonyl O, but then site A-binds the benzene ring with its center 4½ Å from the N-methyl. Hence meperidine is an agonist. In fentanyl this distance is 7½ Å,, so here sites A and C are forced apart rather than together. That is still a distortion, and fentanyl is also an agonist.

Site G appears to be a pocket that can accomodate both the polar 14-OH of oxymorphone, naloxone, etc. and the nonpolar hydrocarbon group of etorphine and other oripavines at the same place.

The κ receptor follows Martin's model: the nuclear sites are A and B, and C is a satellite site that can bind large alkyl -

groups. The electrophilic site F is important also: the antagonists naloxone, naltrexone and WIN 44:441-3 all have a carbonyl group 7½ Å from the N atom. The case of WIN 44,441-3 is particularly instructive: it is a benzomorphan with the center of the benzene ring 4½ Å from the N-methyl (and 4 Å from the N) and should therefore be an agonist, but since it has only a methyl group attached to the N, binding to site C would be weak. On the other hand, just below the N-methyl (when drawn like morphine in the illustration above) there is a cyclopentyl ring which can bind strongly to site C. The molecule can therefore attach its N to site B, then turn clockwise so that the cyclopentyl group faces site C and the carbonyl O site F, but then the benzene ring is too far from site A to attract it and to deform the receptor.

Where the δ receptor fits in remains to be seen.

As to σ receptors, a number of benzomorphans, morphinans and morphines have been listed as weak to moderate agonists or antagonists, but the really potent σ agonists are phencyclidine and ketamine, which have very little resemblance to opioid agonists, and really potent σ antagonists are butyrophenones such as haloperidol and spiperone, which have just as little obvious resemblance to opioid antagonists. However, if we write the structure of butyrophenones as N-H··· O=C chelates (which is certainly proper) we see a benzene ring and an alkylated N in similar mutual position as in μ antagonists. The σ ı receptor therefore may contain sites A, B and C, also site E attracted to the fluorine atom, the strongly negative end of a dipole, and perhaps site F as welI (see next paragraph).

Among morphine derivatives nalorphine is an agonist, naloxone an antagonist, morphine a weak antagonist. Thus here, too, the allyl is just a large alkyl group attaching to site C after site B has bound the N. In nalorphine this places the benzene ring close enough to site A to bind it, necessarily with distortion. If nalorphine distorts the receptor but naloxone does not, we must conclude that, as with κ receptors, the 6-carbonyl binds strongly to site F and allows the molecule to pivot away from site A. Morphine-has no 6-carbonyl and only methyl on the N which does not bind site C strongly enough to bring the benzene ring within binding distance of site A. Hence morphine is attached only to site B and functions simply as a weak antagonist.

Phencyclidine and ketamine can attach strongly to sites A, B and C, but only at the cost of very great distortion: here the N is closer to the benzene ring and sites A and B are forced together to fit an N-to-phenyl distance-of only 3 Å, in contrast to 4 Å in μ and κ agonists.

It is an intriguing question why μ, κ, and σ receptors have so much in common that some of the same drugs can act on them. This writer would answer that the basic features of these receptors - that is, sites A, B and C - must occur rather commonly in proteins, and when they are-present where appropriate

drugs can cause protein distortion with physiological consequences, we are dealing with a receptor. The individual characteristics that differentiate the several receptors will most probably lie in the satellite sites. It will be an interesting challenge to molecular pharmacology to unravel both the similarities and the differences between the classes of opioid receptors and to find what features they correlate with.

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A NOVEL COMPREHENSIVE OPIATE-RECEPTOR MODEL

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ABSTRACT

A new, comprehensive opiate-receptor model based on calculations of possible events at the molecular level is proposed. Agonist versus antagonist action, ultra long-lasting activity and N-dealkylation accompanying receptor binding are included.

MATERIALS AND METHODS

The syntheses and pharmacological characterization of the ultra-long lasting compounds in this study are described by Kolb et al. (1985) and Koman et al. (1986). Theoretical calculations were carried out as described by Snyder et al. (1985, 1986).

RESULTS AND DISCUSSION

Recently we proposed a mu-opiate receptor model (Snyder et al. 1985) predicated on the concept that analgesia is the end-result of proton transfer from nitrogen of piperidine ring-containing opiates to the receptor (Dimaio et al. 1979). In this communication we extend the outlines of the model to include the phenomenon of long-lasting antagonist activity and N-demethylation attending receptor action.

According to the present model the agonists bear equatorial N-CH_3 and axial N-H functions, while the receptor features two negative centers anchoring the positively charged ammonium head group by means of intermolecular electrostatic bridges. Coulombic attraction is dominated by the role of polar C-H bonds on the rigid opiate (Sites 1 and 2, Fig. 1).

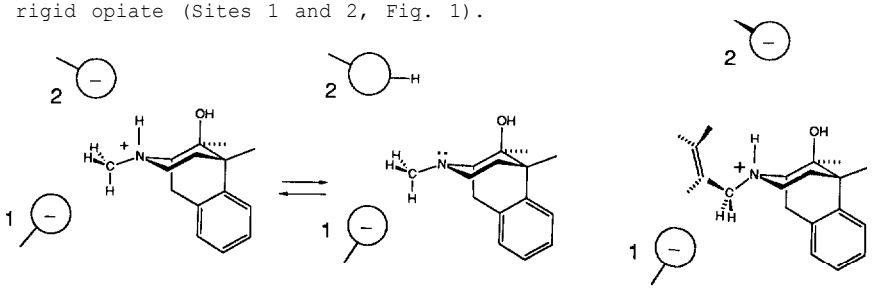

FIGURE 1. Antagonist and agonist cations bind to the receptor through polar bonds. The antagonist N-R group hinders proton transfer; the agonist N-H is readily deprotonated.

Biological action is initiated by transfer of the N-H proton from the drug to the receptor. In response to the proton shift, the receptor protein undergoes a conformational interconversion inducing the amplification required for the ultimate effect. Simultaneously, the agonist salt is converted to a neutral free base. Positive charge at the N-head group is eliminated, electrostatic binding is seriously weakened and the agonist is released from the receptor binding pocket.

The positive, protonated head group of an antagonist, typically carrying a somewhat bulky N-allyl or N-cyclopropylmethyl substituent, binds in a similar fashion. However, by virtue of the binding at Site 1 (fig. 1), the equatorial N-alkyl assumes a rotational state which hinders proton transfer and displaces site 2 (Snyder et al. 1985). Head group charge is retained, receptor conformation is conserved and the antagonists remain bound longer than the agonists. The latter is supported by the experimentally observed slower off-rates for antagonists (Kurowski et al. 1982). The former is substantiated by pharmacological evidence that the mu-opiate receptor exists in two distinct molecular forms assigned as agonist and antagonist (Snyder 1975).

Recently, we have discovered a series of novel ultra-long lasting opiate antagonists (Kolb et al. 1985; Koman et al. 1986). These compounds combine two chemically discrete and spatially demanding molecular fragments. The one is an opiate antagonist, e.g. naloxone. The second is a bulky moiety such as a fluorescein or a steroid bound to the C-6 position of the rigid antagonist via a thiosemicarbazone or an azine spacer, respectively. The azino-compounds are prolonged blockers in the brain opiate-receptor preparation, but not in the guinea pig ileum. By contrast, the thiosemicarbazones are long lasting in both preparations. Displacement studies with [^{3}H] DHM indicate that all compounds in question access the same overall receptor binding site.

Selected conformations of examples in the mono-axine, double-azine and thiosemicarbazone classes were geometrically relaxed by molecular mechanics. The naloxone moieties of each were subsequently superimposed by a least squares fit. Examination of the extended non-opiate fragments by computer graphics portrays a qualitative structural differentiation among the classes. In particular, the preliminary analysis suggests distinct subsidiary binding sites for the various bulky groups.

Superposition of the double azines, e.g. the hybrid azine between naloxone and androstene dione (NAL-AD-NAL), and the naloxone-estrone mono-azine (NAL-E) intimates the presence of two common binding sites. One is a hydrophobic patch which can accept the nonpolar steroidal moieties. The other is a hydrogen-bond-accepting site which can access the -OH group of the phenol ring in either the estrone unit of NAL-E or the second naloxone component of NAL-AD-NAL (fig. 2). In this scheme the two classes -- mono and double azines -- are able to stimulate similar receptor responses.

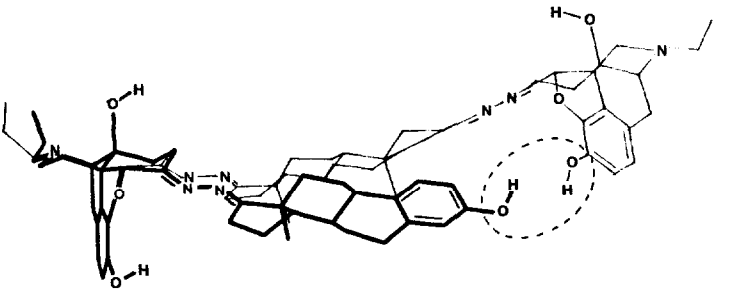

FIGURE 2. NAL-E (dark) and NAL-AD-NAL superimposed at the naloxone fragment. The circled OH's may access a common receptor site.

The thiosemicarbazones, e.g. 6-fluoresceinyl- and 6-rhodaminyl naloxone thiosemicarbazones (NAL-F and NAL-R respectively), are characterized by a bulky 2,5-dibensopyran end group. The latter is calculated to be nearly perpendicular to the rest of the planar C-6 attachment (fig. 3).

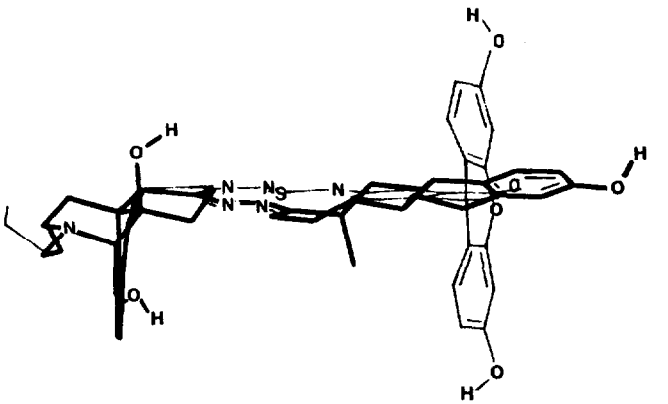

FIGURE 3. NAL-E and NAL-F superimposed at the naloxone fragment. The dibenxopyran group (light) is perpendicular to the average molecular plane.

No meaningful superposition was possible between the end groups of the hybrid opiate-steroid mono-axines and the opiate-fluorescein thiosemicarbazones because of profound structural differences. The thiosemicarbaxone spacer is highly polar and thus would appear incompatible with the above described hydrophobic patch, although it occupies the same molecular space (fig. 4). Furthermore, the planar dibenzopyran moieties are complemented by highly polar substituents that conceivably associate with a polar peptide region of the receptor or possibly the lipid head-groups of an adjacent membrane. In conclusion, the thiosemicarbazones are proposed to achieve their long-lasting effects by binding to either of the latter polar regions. This suggests in addition that both the brain preparation and the gpi contain similar environments for accommodating the fluorescein

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derivatives in the antagonist receptor conformation. The action of long-lasting agonists (Koman et al., 1986) can likewise be incorporated in this scheme as will be discussed in a forthcoming paper.

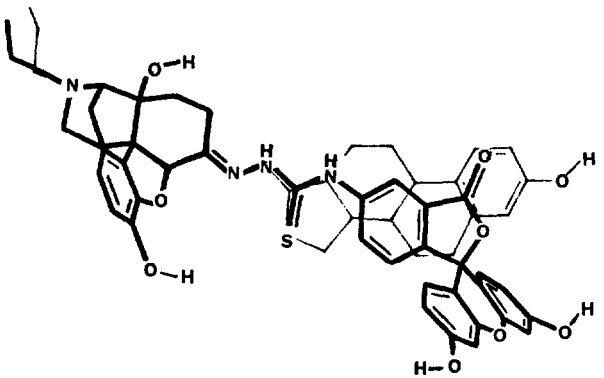

FIGURE 4. NAL-F (dark) and NAL-E superimposed at the naloxone fragment. The polar thiosemicarbazone of the former spatially matches the nonpolar steroid of the latter.

Finally, transformation of the receptor protein to the agonist conformation may activate N-dealkylases which convert a fraction of the freed agonists to their nor-congeners. Evidence that this reaction is initiated by oxidative electron transfer from nitrogen has been summarized by Kolb (Kolb 1984). The process was previously thought to be the genesis of the analgesic response, though calculations on the energetics of $N^{+\bullet}$ radical cation formation render this speculation unlikely (Snyder et al. 1986). Presently, we consider oxidative N-dealkylation to be a secondary event possibly associated with side effects such as euphoria and addiction.

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STEREOCHEMISTRY OF NOVEL DELTA SELECTIVE OPIATE ANTAGONISTS

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ABSTRACT

We have discovered novel delta selective opiate antagonists of a non-peptide nature. Our lead compound at the present time is the mixed azine between estrone and naloxone, the compound named EH-NAL. In this paper we describe the stereochemistry of EH-NAL and an EH-NAL analog, the mixed azine between pregnenolone and naltrexone, the compound named PH-NX. The stereochemical assignments are based on the high-resolution ^{13}C-NMR studies and on the X-ray structural determinations.

INTRODUCTION

Koman (1985) and Koman et al. (1986) studied the interaction of the mixed azines between steroids and naloxone with various opioid receptor types in vitro. We summarize here their findings for the EH-NAL, the mixed azine between estrone and naloxone. The potency of EH-NAL as an antagonist of opioid effects on the electrically evoked contractions of the mouse vas deferens (M.v.d.) and guinea-pig ileum myenteric plexus-longitudinal muscle (G.p.i) preparations was compared to that of naloxone. EH-NAL was 9-fold more potent than naloxone in antagonizing the effects of D-Ala^2-Leu^5-enkephalin in the M.v.d., and 22-fold less potent in antagonizing normorphine in the G.p.i. The two compounds were also compared for their capacity to displace the binding of [^{3}H]-dihydromorphine, [^{3}H]-D-Ala^2-Leu^5-enkephalin and [^{3}H]-ethylketocyclazocine to rat brain membranes. The relative affinities for delta-, mu- and kappa-sites were 0.70, 0.16, 0.14 for EH-NAL and 0.05, 0.87, 0.08 for naloxone, respectively. Thus, in contrast to naloxone which is mu selective, EH-NAL is a delta-selective antagonist.

In order to explain the unexpected delta selectivity of EH-NAL, we undertook a detailed stereochemical analysis of this molecule. Also, we designed another probe for delta receptor, the mixed azine between pregnenolone and naltrexone, the compound named PH-NX. In PH-NX the steroidal unit is separated from the opiate unit by an

extra carbon as compared to EH-NAL. PH-NX has an OH group at the same steroidal carbon as EH-NAL (C-3 of the ring A), but lacks the aromatic pi cloud of the phenol ring of EH-NAL (Fig. 1). A comparison of the delta selectivities of EH-NAL and PH-NX thus may help elucidate the biological roles of the phenolic pi cloud and the absolute length of the steroidal unit in imparting the delta selectivity.

MATERIALS AND METHODS

The ^{13}C-NMR spectra were taken on a 400 MHz IBM-Bruker WM-400 instrument. The X-ray measurements were performed on a Nonius CAD4- and a Syntex P_3 diffractometers.

The mixed azines between estrone and naloxone (EH-NAL) and pregnenolone and naltrexone (PH-NX) were prepared by reacting the free base of the opioid ketone naloxone or naltrexone with the steroidal hydrazone estrone hydrazone (EH) or pregnenolone hydrazone (PH), respectively. The experimental procedure of Kolb and Hua (1984) was followed. EH was prepared as described by Dandliker et al. (1978). PH was synthesized by the same method. Stereochemical determinations by ^{13}C-NMR described below were done in a manner analogous to that described by Kolb and Gober (1983) and Kolb and Hua (1984).

RESULTS AND DISCUSSION

Stereochemistry of EH, a precursor of EH-NAL: Based on the ^{13}C-NMR, EH was a single hydrazone; only one C-17 (hydrazone carbon) was observed (at 157.76 ppm), and only one azine with acetone-d, was found (C-17 of the azine at 173.56 ppm) (solvent $CHCl_3$: acetone-d,). C-l 124.98; C-2 111.66; C-3 153.73; C-4 114.01; C-5 136.32; C-6 29.08: C-7 25.79: C-8 37.25: C-9 42.98: C-10 129.84; C-11 26.01; C-12 33.03; C-13 43.31; C-14 51.20; C-15 25.14; C-16 21.72; C-17 157.76 (the hydrazone carbon); C-18 15.41. The stereochemistry of the hydrazone was assigned to be anti since the syn isomer is sterically very crowded. Namely, the distance between the syn NH, and 12ßH is only 1.2 Å (based on Dreiding models).

Stereochemistry of PH, a precursor of PH-NX: In the ^{13}C-NMR only one peak per carbon was observed, indicating the presence of only one isomer. The inspection of the Dreiding model of PH suggests the anti isomer as a more favorable one, since the syn isomer is sterically crowded due to a clashing between the NH, group of the hydrazone and the angular C-18 methyl group and also the 16ßH and 12ßH. The X-ray structural determination confirms the anti orientation in PH. Some characteristic ^{13}C-NMR shifts (dmso-d_6): C-1 38.43; C-3 70.06; C-4 42.22; C-5 141.31; C-6 120.42; C-9 49.83: C-10 36.16: C-11 22.73; C-13 43.13; C-14 55.75; C-16 36.99; C-17 58.35; C-18 13.04; C-19 19.20; C-20 147.28 (the hydrazone carbon): C-21 15.81.

Stereochemistry of EH-NAL: Based on the ^{13}C-NMR, EH was a single azine; only one peak per carbon was observed.

The stereochemistry of the azine bond was assigned to be anti on the steroidal C-17, because of the anti orientation of EH, and anti on the opiate C-6 due to the preference for the formation of the anti azine of naloxone (Kolb and Hua 1984). Some characteristic ^{13}C-NMR shifts for the opiate and steroidal units of EH-NAL are given (in $CDCl_3$): The opiate unit: C-1 119.76; C-2 118.01: C-3 139.59; C-4 143.70; C-5 87.80; C-6 162.30 (the azine carbon); C-9 63.38: C-11 124.01; C-12 129.61; C-13 49.96: C-14 70.31; C-16 44.75: C-17 57.64: C-18 137.70; C-19 118.34. The steroidal unit: C-1 126.29; C-2 113.83; C-3 153.52; C-4 115.90; C-5 135.20; C-8 37.95: C-9 43.68: C-10 131.51; C-13 44.25: C-14 52.12; C-17 175.79 (the azine carbon): C-18 16.60.

Stereochemistry of PH-NX: The crude PH-NX was subjected to a ^{13}C-NMR analysis, which revealed the presence of two azine isomers: steroid C-20 anti-opiate C-6 anti (major) and steroid C-20 anti-opiate C-6 syn (minor). The recrystallization of the crude PH-NX from EtOH-CHCl, provided a single azine isomer (by ^{13}C-NMR). The structure of the latter isomer was determined by X-ray and found to be anti (steroid)-anti (opiate) gauche azine (Fig. 2). The C=N-N=C torsion angle was -123°, indicating gauche geometry of the azine bond. Some characteristic ^{13}C-NMR shifts (in dmso-d_6) of the latter anti-anti azine: The opiate unit: C-1 118.57; C-2 117.00; C-3 139.12; C-4 143.68; C-5 87.56: C-6 159.12 (the azine carbon): C-9 61.47; C-11 123.85; C-12 130.63; C-13 47.89; C-14 69.51; C-16 43.36; C-18 9.17; C-19 3.70; C-20 3.47 (the latter two shifts could be interchanged). The steroidal unit: C-1 38.23: C-3 69.96: C-4 42.16; C-5 141.25; C-6 120.27: C-9 49.63; C-14 55.85: C-18 13.16; C-19 19.09; C-20 157.24 (the azine carbon): C-21 18.51. Some characteristic ^{13}C-NMR shifts for the opiate unit of the minor isomer: C-6 160.17 (the azine carbon): C-1 118.93; C-2 117.18; C-5 89.34: C-14 69.67: C-9 61.39. The steroidal azine carbon: C-20 156.92.

Both EH-NAL and PH-NX are configurationally much more rigid than typical delta substrates which are peptides. Namely, both EH-NAL and PH-NX have as a major point of flexibility in the molecule just one bond -- the N-N bond of the azine linkage. Thus, these azines may be useful as semi-rigid probes for mapping the delta receptor. EH-NAL contains an aromatic ring (steroidal A ring) which can be overlapped with Ph^4-residue of enkephalins (Koman 1985; Koman et al. 1986; Pilipauskas et al. 1986). PH-NX was found to be an active opiate antagonist (more active than EH-NAL) on G.p.i. (Koman private communication). The biological tests for the delta selectivity of PH-NX are underway.

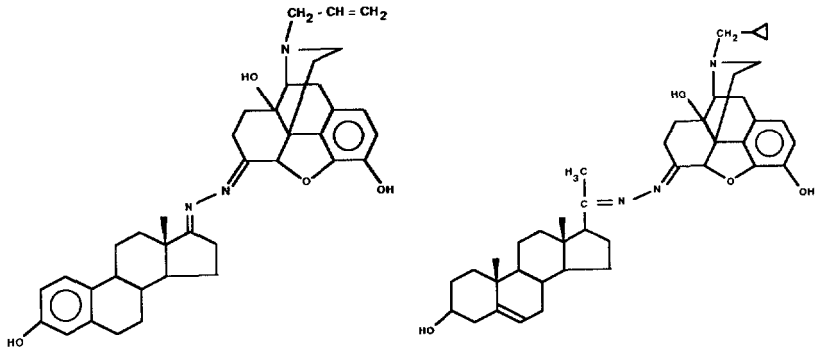

FIGURE 1. Structures of EH-NAL and PH-NX.

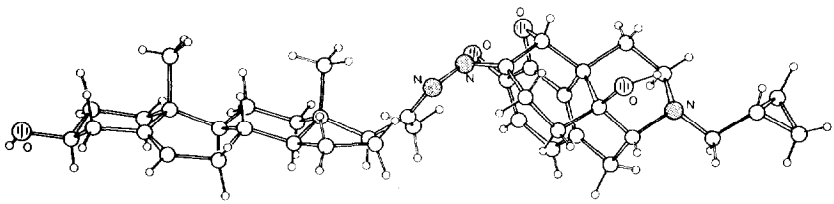

FIGURE 2. The X-ray structure of PH-NX.

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STRUCTURE ACTIVITY STUDIES OF A SERIES OF 4-(m-OH PHENYL) PIPERIDINES

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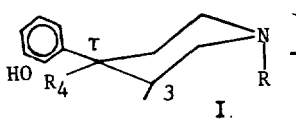
ABSTRACT

A series of 4-(m-OH phenyl)-piperidine analogs with R_4=methyl or t-butyl and NR=methyl, allyl and phenethyl have been synthesized; and their receptor affinities, in vivo analgetic agonism and antagonism, and energy-conformational profiles determined. These analogs bind selectively and with moderate to high affinity to opioid μ-receptors. Binding in their preferred phenyl axial conformation appears to lead to meperidine-like agonism. In addition, for some R_4=methyl but not t-butyl compounds, binding of an energy-accessible phenyl equatorial conformation produces antagonism.

INTRODUCTION

The 4-ϕ-piperidine class of opioids, I, are thought to bind and act at the same type of μ-receptors as morphine. Unlike morphine in which the phenyl ring is fused in a fixed axial position with respect to the piperidine ring, these fragments are more flexible and can assume both phenyl axial and phenyl equatorial conformations. Consequently, in the 4-ϕ- piperidines, antagonism was found to be modulated primarily by the R_4 substituent and the presence of a j-methyl and a m-OH phenolic group, rather than by N-substituents as in morphine. In particular, potent pure antagonists have been prepared in this family (Zimmerman et al. 1978), for a number of R_4 substituents with a 3-methyl group trans (β) to them.



I.

R4 / R	methyl	t-butyl
methyl	1a	2a
allyl	1b	2b
phenethyl	1c.	2c

Previous theoretical studies (Burt and Loew 1980; Loew et al. 1981; Froimowitz and Kollman 1984), strongly indicate that the observed antagonism is initiated by binding of an energy-preferred, phenyl-equatorial conformer of these analogs in an orientation relative to morphine with overlapping m-OH phenyl groups, as shown in figure la. However, both phenyl equatorial and phenyl axial conformers have been implicated in agonist activity.

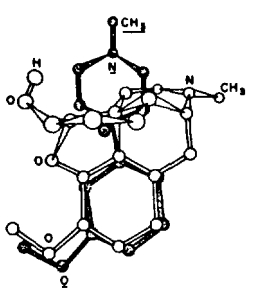


Fig. 1a: Proposed antagonist conformer and receptorsite orientation relative to morphine for 4-ϕ-piperidines.

In order to further investigate the role of relative stabilities of phenyl axial versus phenyl equatorial conformers in modulating relative agonist/antagonist activity, a number of 4-(m-OH ϕ) piperidine derivatives in which the R_4 group alone could affect conformational preferences were selected for synthesis and

analyses. In particular, the effects of replacing a compact methyl group by a bulky tertiary butyl group at the R_4 position and of N-R variations (analogs 1-2a-c) on conformation, receptor affinity, and in vivo analgetic agonism and antagonism were investigated.

METHODS AND PROCEDURES

A. CHEMICAL SYNTHESIS: All analogs, 1-2a-c were produced by modification of a known procedure (McElvain and Clemens 1958).

B. OPIOID RECEPTOR BINDING: Competitive inhibition by analogs 1-2a-c in rat brain homogenate of four tritium labled ligandc (naloxone, D-Ala, D-Leu enkephalin, ethylketocyclazocine and dihydromorphine) was determined by procedures and data analysis described previously (Cheng et al. 1986). Data obtained were analysed by a modified version of the program LIGAND (Munson and Rodbard 1980) which predicts a set of self consistent receptor binding affinities and capacities assuming different receptor site models.

C. IN VIVO PHARMACOLOGY: Analgesic agonist activity and antagonism to morphine analgesia of compounds 1-2a-c and of morphine, meperidine and nalorphine as standards were evaluated in the mouse tail flick test by procedures which we have described previously. (Cheng et al. 1986).

D. THEORETICAL STUDIES: All calculations were performed using a molecular mechanics method called MOLMEC (Oie et al. 1981). The initial geometries were optimized with respect to all variables. To obtain energy profiles for phenyl ring rotation, constrained optimizations were performed keeping the dihedral angle $\tau(C_8C_7C_4C_m$ fig. 1b) fixed at a set of values with intervals of 30 degrees.

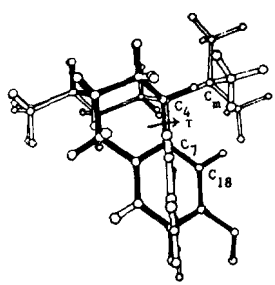


Fig. 1b: Overlap of the optimized phenyl axial conformer of 4-t-butyl-4-(m-OH)-phenyl piperidine with the benromorphan ring of morphine.

RESULTS AND DISCUSSION

The results of a 5-receptor site model obtained when all the data was analyzed simulaneously are shown in table 1. The five site model identifies sites we have called μ_1, μ_2, delta and kappa. A fifth site, not shown, with very high capacity and low affinities for all ligands is most likely unrelated to opioid activity. The six 4-phenyl piperidines studied (1-2a-c) are all μ-selective having high to moderate affinities at μ1 an μ2, with values at delta and kappa being one to two orders of magnitude lower. The results of in vivo studies of analgetic agonist and antagonist potencies are shown in table 2.

TABLE 1. Receptor Affinities(Kd n=) Maximum Binding Capacities(pmol/gm)

	u1	u2	δ	κ
Nal.	0.13	3.6	19.8	4.1
DADL	0.71	21.0	2.1	3700
EKC	0.55	3.1	15.7	12.1
DHM	0.13	10.9	98.0	6800
4-(m-OH-Phenyl)piperidines				
1a	27.4	17.2	1500	4400
1b	179.0	77.5	4400	560
1c	2.5	3.7	420	830
2a	0.2	1.9	93	1560
2b	2.3	14.3	430	3360
2c	14.0	6.2	200	780
Bmax	2.1	23.3	7.3	9.3

The 4-t-butyl series are pure agonists with potencies about 1/10th that of morphine, similar to meperidine, while two of the 4-methyl analogs, 1b, 1c, show measurable antagonist activity, about 1/20 that of nalorphine.

TABLE 2. Analgesic Agonist and Antagonist Potencies (μmol/kg.s.c.) in House Tall-of flick Procedure

Compound.	ED50	AD50[a]
1a	25	>280
1b	>300	150
1c	70	44
2a	27	>244
2b	23	>226
2c	48	>191
meperidine	25	N.A.
morphine	3	N.A.
nalorphine	828	2

[a]Antagonism of tail flick inhibition induced by 21 umole/kg of morphine sulfate, s.c.

The results of energy conformation studies of four 4-(m-OH-phenyl) piperidines with R_4=t-butyl (2a), methyl (1a) and the latter with an a-or β-3-methyl substituent (3α, 3β) are summarized in table 3. As seen in this table, a piperidine chair conformer is much more stable than a twist boat conformer. The analogs are ordered in decreasing stability of the optimized phenyl axial piperidine chair conformer. The phenyl ring torsion angle (τ'), for the lowest energy form is given in parenthesis. The energy (ΔE min/morph) required to rotate the optimum axial phenyl ring conformer to a morphine-like value (τ'=32') is also shown.

TABLE 3. Optimized Conformational Energies[a] for 4-(m-OH phenyl) Piperdines

ANALOG	ΔE(boat)	ΔE(equat)	ΔE(min/morph)	Activity
2a	6.8	5.1(89°)	15.9(32°)	pure ag.
1a	6.0	0.9(80°)	4.4(32°)	ag/ant
3α	-	-0.9(50°)	-	ag/ant[b]
3β	-	-2.6(50°)	-	pure ant[b]

a) Energies in kcal/mol relative to optimized phen. axial/piperidine chairconformer.
b) Data taken from (Zimmerman et al 1978)

Previous theoretical studies (Loew et al. 1980, Burt and Loew 1981) have led to the hypothesis that in meperidine- and prodine-like compounds, both agonist and antagonist activity appear to be elicited by lowest energy piperidine-chair, phenyl equatorial conformers binding to the μ-receptor in a different orientations. For the 4-alkyl-4-(m-OH phenyl)-piperidines, however, a different relationship between relative agonist/antagonist potency and phenyl equatorial and axial conformations is emerging. As summarized in table 3, our results reinforce the previous association of phenyl equatorial conformers with antagonist activity but demonstrate for the first time a clear association of phenyl axial conformations with the initiation of agonist activity. The 4-t-butyl compounds are the first simple 4-alkyl-4-arylpiperidines predicted to have a definitive phenyl axial conformation. These compounds are pure agonists, providing strong evidence that a phenyl axial conformation leads to agonist activity. As shown in table 3, the 4-methyl compound, la, also has a preferred phenyl axial conformation but with a much more accessible phenyl equatorial conformation. While the N-methyl analog is in fact a pure agonist, the N-phenethyl analog lc has significant antagonist activity. Because both phenyl axial and phenyl equatorial conformers have energies within 1 Kcal/male, the N-substituent appears to affect the relative extent to which each conformer binds to the receptor. Thus lc can bind in both phenyl axial and phenyl equatorial conformations and exhibits agonist and antagonist activity.

Further evidence for association of phenyl equatorial conformers with antagonist activity and phenyl axial conformers with ago-

nist activity comes from our energy conformation study of the effect of adding a 3-methyl group cis(3α) and trans(3β) to the 4-methyl group. As shown in table 3, the pure antagonism of 2 appears to be due to the preferential stabilization of the phenyl equatorial conformer by 3.5 kcal/mole and verifies the role of phenyl equatorial conformers in initiating antagonist activity. By contrast, in the cis isomer, 3α , the phenyl axial and phenyl equatorial conformed are within 1kcal/mole, consistent with its observed mixed agonist/antagonist activity.

Our studies not only provide further evidence for phenyl equatorial conformers initiating antagonist activity and phenyl axial conformers agonist activity, they also provide insight into why N-substituent variations do not modulate agonist/antagonist activity as they do in fused ring opioids. Previously, we have postulated that important overlaps of both the amine nitrogen and phenolic OH must be preserved in opioids for N-substituent modulation to correspond to that in fused ring analogs. Thus, since the phenyl ring is axial in these rigid opioids, it is possible that, at least in the 4-t butyl series in which the axial conformer is the most definitive low energy form, N-substituent modification could lead to an agonist/antagonist profile similar to morphine. However, as shown in table 3 and in figure lb, for both 1a and 2a in the lowest energy phenyl axial conformer, the phenylring is nearly perpendicular to the piperidine ring, a very different relative orientation than in morphine. Thus, in neither series does the optimum torsion angle of the phenyl group resemble that of morphine and considerable energy is required (table 3) to rotate it to that angle. As a results, no N-substituent variation effects similar to those in fused ring opioids are observed in these 4-alkyl piperidines.

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THEORETICAL STRUCTURE-ACTIVITY STUDIES OF METHYL-SUBSTITUTED 4-(m-OH PHENYL) PIPERIDINES

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ABSTRACT

The theoretically determined molecular structures of N-protonated 1,3,4,6 methyl-substituted 4-(m-OH phenyl) piperidines are correlated to their experimentally derived analgesic activities. It is concluded that the orientation of the 3-methyl group plays a crucial role in determining agonism and antagonism.

INTRODUCTION

The 4-ϕ-piperidines are known since 1939 to be capable -of exhibiting strong analgesic agonism. In 1977 the first example of this class of compounds was found (Zimmerman and Nickander 1977) which is a pure narcotic antagonist. Unlike the fused-ring molecules, antagonism was not obtained by varying the N-methyl substituent, but rather by introducing two methyl substituents in the 3,4-trans position of the piperidine ring. Subsequently, several analogs with methyl groups mainly in the 1,3,4,6 positions have been synthesized and tested (Zimmerman et al. 1980, Huegi et al. 1984), and it has been suggested (Zimmerman et al. 1980) that antagonism is introduced by a phenyl equatorial conformer. Molecular mechanics calculations of the various isomers were performed in order to better understand conformational features which modulate their agonist and antagonist activity.

METHODS AND PROCEDURES

All calculations were carried out using the molecular mechanics program MOLMEC (Oie et al. 1981). Unless otherwise noted, all geometries and energies are the result of a complete optimization with respect to bond lengths, bond angles, and torsion angles. In all studies the piperidine ring was calculated with a chair conformation and protonated nitrogen atom. In cases where several rotamers exist with different values of the torsion angle t (t = C8C7C4X, fig. 1) we report only the energetically lowest-lying conformer.

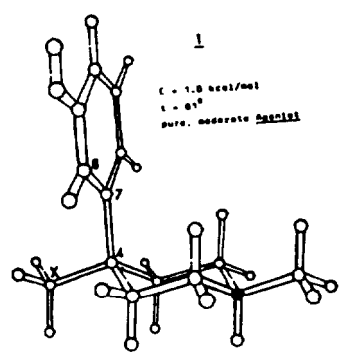

FIGURE 1

RESULTS AND DISCUSSION

The theoretically obtained structures of the various isomers are shown in figures 1 and 2. For each isomer only the global minimum is shown, which represents one of the diastereomers corresponding to the (-) 3-methyl and (+) 6-methyl group, respectively. The value of E indicates the relative energy of the higher energy conformer due to piperidine-ring inversion (equatorial-axial) with the N-methyl group kept in an equatorial

position. The figures also exhibit the value for t calculated for each global minimum. In some cases, the energy E to rotate the phenyl group to a constrained value for t is given in parenthesis. The experimentally derived analgesic activities were taken from Loew et al. (this volume) for 1, from Zimmerman et al. (1980) for compounds 2 - 7, and from Huegi et al. (1984) for compound 8. We assume that the published results relate to racemates, since the separation of the optical isomers was not reported.

Structure 1 has a methyl substituent only in the 4-position. The phenyl-axial conformer is favored by 1.0 kcal/mol relative to the phenyl-equatorial form, and this compound is reported as a pure, moderate agonist (fig. 1). If an additional methyl group is added in the 3-position trans to the 4-methyl (2, fig. 2), the phenyl-equatorial conformer is now favored by 2.3 kcal. 2 is a pure, potent antagonist. If still another methyl group is added to 2 in the equatorial 6-position, compound 3 results. The shapes of 2 and 3 are nearly identical except for- the additional methyl group in 3. Like 2, 3 is a pure, potent antagonist. The conclusion is that the methyl group in the 6-position of 3 is unimportant for analgesic antagonism.

If the 3,4 methyl groups in 3 are not trans, but cis to each other, compound 4 is formed. As in 3, the phenyl equatorial conformer of 4 is clearly favored over the phenyl axial form. However, 4 is a pure, potent agonist! Two structural differences may be related to this reversal of activity: The 3-methyl group is equatorial in 4, but axial in 3, and the torsion angle t is very different for both compounds. The energy to rotate the phenyl group of 3 and 4 from their respective equilibrium values to the constraint value of the other isomer is quite high (fig. 2). Thus, at this point it can not be decided which structural difference between 3 and 4 is responsible for the change in activity.

Compound 5 has an all-equatorial arrangement of the 3,4,6-trimethyl, groups. The phenyl-axial isomer is favored by 2.4 kcal/mol (fig. 2), and 5 is a pure, potent agonist. Comparing 5 with 4 shows that the-4-methyl and 4-phenyl groups are reversed, while the rest of the structure is the same. The interesting conclusion is that both phenyl axial and phenyl equatorial conformers can lead to agonist activity.

In compound 6 the two methyl groups in the 3,4 position are cis to each other. The phenyl equatorial form is favored for 6, which is a mixed, moderate analgesic agonist/antagonist (fig. 2). The structure of 6 is nearly identical to 4, except that the methyl group in the 6 position is missing. What introduces antagonism in 6? For 3, the 6-methyl group was found to be unimportant for antagonism. Unless the 6-methyl group has a different effect on antagonism in 4 compared to 6, the only remaining explanation for the occurence of antagonism in 6 is the reduced energy difference to the less stable conformer, which is only 1.8 kcal/mol in 6, but 4.2 kcal/mol in 4. Thus, antagonism would be introduced by the presence of the energetically higher, phenyl axial form, 6a. This prediction can be tested by additional studies of compound 7 (fig. 2). In 7, the additional methyl group in the 6 position stabilizes the structure of the phenyl axial minor isomer 6a sufficiently to become clearly the most stable form. Unfortunately, 7 was only tested for analgesic agonism, not for

antagonism. If 7 were an antagonist, it would clearly implicate phenyl axial conformers for the first time in such activities.

Analog 8, which has two methyl groups in the 3 position (fig. 2) does not bind to opiate receptor sites, and shows no analgesic agonism or antagonism. The phenyl equatorial form is favored, and a comparison with 2, a pure antagonist, and with 6 in which the 3-methyl equatorial form is most likely the agonist -(compare 4), shows that the value of t in 8 is intermediate between 2 and 6. However, the different t value of 8 should not cause the lack of activity since rotation from the-equilibrium value of t = 36° to the value for 2 (t = 68°) and 6 (t = 0°) is within the feasible energy range (fig. 2). The only remaining explanation for the inactivity of 8 as an opiate is the presence of two methyl groups in the 3 position, each of which requires different orientations of the compound at the receptor binding site to be accomodated.

The combination of all conclusions reached so far lead to a picture which is different, but compatible with previous suggestions. We conclude that for 1,3,4,6 methyl substituted 4-ø-piperidines, a dominant factor in the structure activity correlation of opiate activity is the orientation of the methyl group in the 3 position of the piperidine ring. If this methyl group is axial, the compound will be an antagonist, if it is equatorial, it should be an agonist. If both orientations are occupied by dimethyl groups, the compound is inactive. This points to a mechanism where the 3-methyl group occupies a crucial steric position in the molecule. It may be compared with a key which fits in the "agonist key-hole" when the 3-methyl group is equatorial, and it fits in the "antagonist key-hole" when the 3-methyl group is axial. Both keys cannot fit into these key holes simultaneously if methyl groups are found in both orientations in the same molecule.

This conclusion may now be used to predict activities of the unknown compounds 9 and 10. In 9, the phenyl equatorial conformer with the methyl group equatoria1 is clearly favored (fig. 2) and thus, is expected to exhibit analgesic agonism. For 10, with the methyl group in the axial position, the phenyl equatorial conformer is the more stable form, but only by 1.5 kcal/mol. Consequently, 10 is expected to be an antagonist with the possibility to show mixed activity since the less stable isomer with the methyl group in the equatorial position might also be active.

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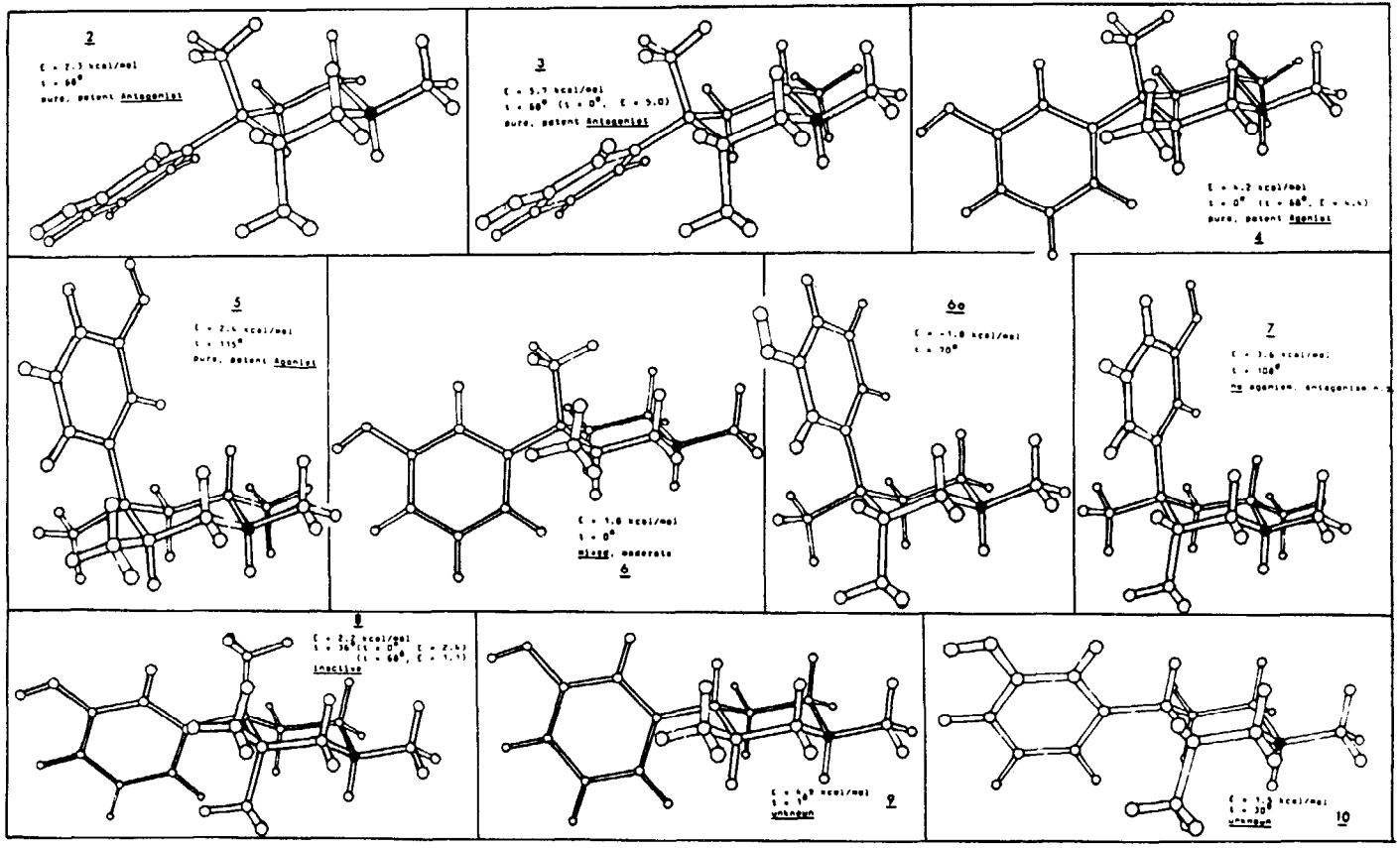
2
pure, potent Antagonist
3
pure, potent Antagonist
4
pure, potent Agonist
5
pure, potent Agonist
6
6a
7
8
inactive
9
unknown
10
unknown

FIGURE 2

CONFORMATIONAL STUDIES AND RECEPTOR BINDING OF DELTA SELECTIVE OPIOID PEPTIDES

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ABSTRACT

Detailed energy-conformation studies of a linear (DTLET) and four cyclic (DPDPE, DPLPE, DPLCE, DCLPE), δ-selective opioid peptides were combined with computer assisted detailed receptor binding studies. The results of these studies have allowed the identification of a low energy conformer common to all of these analogs which could be responsible for their high affinity δ-receptor binding. This conformer contains multiple intramolecular H-bonds and is very different from the beta-II type structure previously postulated to lead to high affinity μ-receptor binding. This μ-binding conformer was found either to have higher energies or be greatly distorted in these δ selective analogs.

INTRODUCTION

Receptor binding studies (Chang and Cuatrecasas 1979) of enkephalin analogs in rat brain homogenates have established that these peptides bind to at least two distinct classes of opioid receptor sites called μ and δ. Further studies of the biochemistry and pharmacology of δ-receptors will be aided by the recent discovery of two linear, δ-selective, hexapeptides, Tyr-D-Ser(D-Thr)-Gly-Phe-Leu-Thr(DSLET, DTLET) (Zajac et al. 1983) as well as a series of δ-selective cyclic enkephalin pentapeptides (Mosberg et al. 1983): (D-Pen2, D-Pen5 enkephalin, (DPDPE), its isomer (DPLPE), and two analogs with a cysteine replacing one penicillaminone residue (DCLPE, DPLCE). To more clearly delineate affinities to μ- and δ-receptors, detailed computer assisted binding has been done for the six δ selective peptides shown in table 1.

TABLE 1. Receptor Affinities[a] and Calculated Relative Energies[b] and Overlap[c] of Candidate High-Delta Affinity Conformers for Linear and Cyclic Opioid Peptides.

ANALOG	u1	u2	delta	E	rms
DPLCE	14	79	0.8	0	0.40
DTLET	25	5	1.0	1.9	0.00
DSLET	7	45	1.2	-	-
DPLPE	102	520	1.3	0.0	0.45
DCLPE	5	8	1.9	2.3	0.50
DPDPE	338	2700	13.0	1.2	1.47

a. Receptor affinities, (Kd nm), for a five-site model
b. Energy, (kcal/mol), of each analog relative to its lowest energy conformer
c. The rms value is the minimum root mean square distance between matched atoms in DTLET and each cyclic analog

While the successful design of δ-selective analogs has been accomplished, the conformational requirements for high affinity binding to δ-receptors and how these are different from those favorable for μ-receptor binding are not yet understood. Nor have commonalities between linear and cyclic δ-selective analogs been explored. In previous studies of highly μ-selective morphiceptin analogs (Loew et al. 1986) and enkephalins (Loew et al. 1982), we have identified and characterized common candidate BII[1]-Type peptide conformers leading to high affinity binding to μ-receptors. In a complementary fashion, the energy conformational studies reported here of one linear (DTLET) and the four cyclic δ-selective peptides

shown in table 1, were designed to identify and characterize peptide conformations leading to high affinity binding to δ-1receptors.

METHODS AND PROCEDURES

OPIOID RECEPTOR BINDING: Competitive inhibition of all peptide analogs in rat brain homogenate of five labeled ligands [^{3}H] Naloxone, DADL, DSLET, EKC AND DHM were determined by procedures and data analysis described previously (Cheng et al. 1986). Data obtained were analyzed by a modified version of the program LIGAND (Munson and Rodbard 1980) which predicts a set of self consistent receptor binding affinities and capacities assuming different receptor site models.

Conformational energies were calculated using a modified version of the potential energy terms in the Empirical Conformational Energy Program, ECEPP, (Momany et al. 1975), and optimized using a new energy minimization program (PEP) developed in our laboratory. Initial conformations to be optimized were constructed using effficient interactive structure generating programs coupled to graphics displays contained in the programs called MOLECULE (Egan et al. 1982) Coordinates and atomic parameters of the unusual L-and D-penicillamines were developed systematically from a related known X-Ray structure (Rosenfield and Parthasarathy 1975). The extent of overlap between any two conformers was determined by using a program called MOBLS, which determines the minimum root mean square (rms) distance between user-selected, matched atoms in two rigid molecules.

Different search strategies were used to span the conformational space for the linear and cyclic peptides studies. For the linear haxapeptide, DTLET, an "aufbau" procedure was used, starting with optimized conformers for each single amino acids, and linking these to form initial hexapeptide conformers. This procedure led to 30 different optimized structures with a relative energy range of $\Delta E \leq$ 7.5 kcal/mol. For the cyclic peptide DPLPE, the main search strategy used involved a systematic exploration to determine sets of 14 ring torsion angle values that allowed cyclic structures to be formed, using a program developed in our laboratory based on an existing ring closing algorithm (Go and Scheraga 1970). A total of 248,000 conformations were subjected to cyclic ring closure searches. These conformations were screened using distance, energy and similarity criteria. Screening of all of the successfully cyclized structures, resulted in 31 unique conformers with optimized energies within 7.5 Kcal/mol of the lowest energy structures. These 31 unique DPLPE conformers were used to construct initial conformations for the three remaining cyclic peptides DPDPE, DPLCE and DCLPE and additional conformers for the linear peptide DTLET. Conversely, the 30 lowest energy DTLET conformers were used as templates to construct additional initial conformer for each of the four cyclic peptides. In this way a set of 62 energy optimized conformers were obtained for DTLET and the four cyclic peptides DPDPE, DPLPE, DPDCE and DCDPE.

RESULTS AND DISCUSSION

The receptor binding data was analyzed using a five-receptor site model which gave the best fit to all of the experimental data. called μ1, μ2, δ and κ and a fifth site with very high capacity and low affinities for all ligands, most likely unrelated to opioid activity. Table 1 shows the affinities of the six analogs studies

for sites labeled μ1,μ2 and δ, verifying their high affinity at δ and indicating that DPDPE and DPLPE have the most pronounced δ selectivity with respect to both sites 31 cyclic structures. After optimization of the three other cyclic analogs, using low energy DPLPE conformers as templates, a single common low energy conformer for all four cyclic analogs resulted. Moreover, when these 31 low energy conformers were used as templates to construct initial conformers for the linear peptide DTLET, all resulting optimized conformers had energies more than 7.5 kcal/mole higher than the lowest energy conformer found independently for DTLET by the aufbau procedure.

The search for common low energy conformers was much more fruitful when the reverse strategy was tried, using the low energy DTLET structures found from the aufbau procedure as templates for the cyclic analogs. After optimization, 16 conformers of DPLPE had energies less than 7.5 kcal/mol above their lowest energy conformers. These DPLPE conformers were then used as templates for the remaining cyclic peptides, and the optimized conformers were all examined for similarities to their corresponding DTLET conformer using the rms procedure. One low energy DTLET structure, with three strong intramolecular H-Bonds (fig. 1) and a relative energy of 1.6 kcal/mol, was the template for the lowest energy conformer of DPLPE found by any of the search strategies used. Furthermore, this DPLPE conformer yielded low energy optimized structures for the three other cyclic analogs with great similarity to DTLET. No other conformer was found which was more similar, or had lower energies for all 5 analogs. This conformer is then an excellent candidate for high affinity binding to the δ-receptor. Figure 1 shows the superposition of this candidate conformer for DTLET and DPLPE and illustrates their similarity and internal H-bonding.

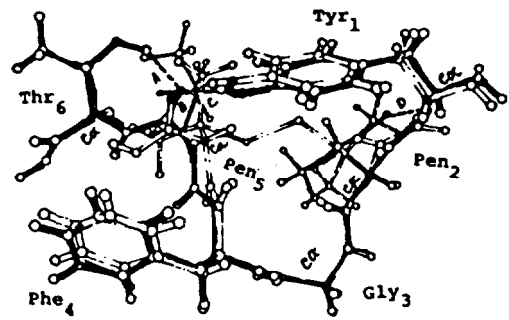

Figure 1:
DPLPE and DTLET Overlapped in the Candidate Delta-binding Conformer.

H Bonds		Energy
A. DTLET	Thr6 OH--O=C Leu 5	-5.75
B. DPLPE	Pen5 C=O--HO Tyr1	-5.78
C. DTLET	Leu5 C=O--HO Tyr1	-5.88
D. DTLET	Thr2 OH--O=C Tyr1	-5.78

DTLET
DPLPE

Given in table 1, is the correlation of δ-receptor affinities, with calculated relative energies and overlap of the candidate conformer of each cyclic analog with that of DTLET. Consistent with its lower δ-affinity, only DPDPE seems appreciably different from the others in its extent of similarity with DTLET. These results indicate that a conformational effect, and perhaps the position of the terminal COOH group and its involvment in two internal H bonds, one to the TyrOH and the other to the DPEN-2 carbonyl, rather than an energy difference, could account for its lower delta affinity. The candidate low energy conformers chosen

for DPDPE and DPLPE are also consistent with preliminary conformational results for these analogs using NMR methods (Mosberg **1986)** and should aid in their further interpretation.

The candidate peptide conformer leading to high affinity δ-receptor binding is very different from the $\beta\Pi^1$ form previously proposed for favorable μ-receptor binding of D-Ala2-met-enkephalin amide. Preliminary studies indicate that such conformers favorable to μ-receptor binding are either higher energy forms or are greatly distorted in these δ-selective analogs.

The conformer identified in this study as responsible for high affinity δ-selective binding, clearly illustrates the key role for the two threonine residues in DTLET. They are involved in two very strong intramolecular H bonds: Thr2 with the Tyr-OH and Thr6 with the Leu C=O. This latter moiety is also involved in H-Bonding with the Tyr-OH. The multiple intramolecular H-bonds formed by these residues apparently ensures a compact conformation very suitable to δ-selective binding. Cyclization appears to accomplish this same purpose, cyclic analogs favoring a conformation very similar to the multiply H-bonded linear peptide DTLET. This conformation retains one strong H-bond between the TyrOH and the terminal carbonyl group. The unique H-bonded conformations of these analogs must be well suited for interaction with a δ-receptor site. High affinity could be the result of an excellent steric fit at this site, assured by the internal H-bonds or the cyclization. Additionally, once this fit is assured, the intramolecular H-bonds can be replaced by more favorable interactions with appropriate receptor residues to which the opioid peptide has been guided by its unique conformat ion.

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SITE-DEPENDENT EFFECTS OF IONS ON μ-, δ- AND κ-OPIOID BINDING IN SUSPENSIONS OF GUINEA-PIG BRAIN MEMBRANES

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ABSTRACT

NaCl, LiCl, NH_4Cl and KC1 inhibit the equilibrium binding of peptide agonists at each of the μ-, δ- and κ- sites; the orders of potencies and the slopes of the dose-response curves are site-dependent. In contrast, the monovalent salt choline chloride inhibits μ- and κ-binding but has little effect on δ-binding. The profiles of activity of $MnCl_2$, $CaCl_2$ and $MgCl_2$ are also site-dependent but differ markedly from those of the monovalent salts.

INTRODUCTION

In many of the early investigations of the effects of monovalent and divalent salts on opioid binding, the opioids used were interacting with more than one of the μ-, δ- or κ- sites. In more recent papers, in which more selective labelling techniques have been used, site-dependent differences in the effects of salts on opioid binding in rat brain have been indicated by several groups. The aim of this investigation was to compare systematically the effects of the chloride salts of monovalent and divalent ions on equilibrium binding at μ-, δ- and K-binding sites of guinea-pig brain, using selective labelling conditions. A detailed report of the results will be published elsewhere (Paterson et al. 1986).

MATERIALS AND METHODS

The effects of monovalent and divalent salts on equilibrium binding at μ-, δ- and κ-opioid sites were investigated in membrane suspensions (10 mg tissue/ml 50 mM Tris-HCl, pH 7.4 at 25°C) of guinea-pig brain. In the brain minus cerebellum the μ-sites were labelled with [^{3}HI-[D-Ala^2,MePhe^4,Gly-o1^5]enkephalin (1 nM; 1221-2442 GBq/mmol, Amersham International) and the δ-sites with [^{3}H]-[D-Pen^2,D-Pen^5] enkephalin (1.5 nM; 1665 GBq/mmol, Amersham International). For investigations at the κ-site, binding of [^{3}H]-dynorphin A (1-9) (0.1 nM; 1258 GBq/mmol, Amersham International) was determined in the cerebellum, in which 84% of the opioid binding sites are of the κ-type; bestatin (30 μM) and captopril (300 μM) were added to reduce the degradation of the labelled peptide. Specific binding was the difference between the binding in the absence and presence of diprenorphine (1.2 μM; Reckitt and Colman). For a detailed account of the experimental design see Paterson et al. (1986).

RESULTS

NaCl, LiCl, NH_4Cl and KCl caused a dose-dependent inhibition of the binding of the peptide agonists at each of the μ-, δ- and κ-sites. On the other hand, choline chloride, which produced dose-dependent

inhibition of μ- and κ- binding had, at concentrations up to 100 mM, no inhibitory effects on δ- binding. The orders of potencies of the salts were different at each site, as seen from the values for threshold of inhibition (fig. 1). Furthermore, the slopes of the dose-

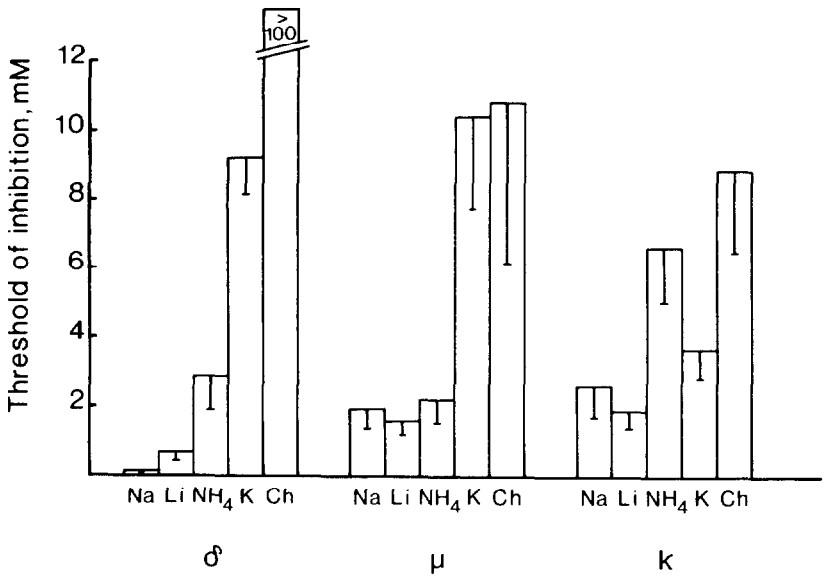

FIGURE 1. Thresholds of inhibition for effects of the chloride salts of monovalent ions on binding at δ-, μ- and κ- opioid sites. Values are the means ± s.e.m. of 3-10 observations and were obtained by extrapolation of the linear regression of log mM salt against change (%) in binding. Ch: choline.

response curves also differed at each site. They were highest at the κ- site (mean = 51% change in binding/log mM salt), intermediate at the μ-site (mean = 38% change in binding/log mM salt) and lowest at the δ- site (mean = 28% change in binding/log mM salt).

The effects of divalent salts differed from those of monovalent salts and were also site-dependent. At the δ-site, $MnCl_2$, $MgCl_2$ and $CaCl_2$ markedly potentiated binding by up to 50%; no inhibition of binding was observed at the highest concentration used (16 mM). In contrast, at the μ-site low concentrations of $MnCl_2$ and $MgCl_2$, but not $CaCl_2$, slightly potentiated binding and at higher concentrations all three salts inhibited binding. In further contrast, κ-binding was inhibited by $MnCl_2$, $MgCl_2$ and $CaCl_2$ but no potentiation was observed.

DISCUSSION

Since throughout this investigation chloride salts have been used the

differences in their effects can be attributed to the cations. Since the orders of potencies and the slopes of the dose-response curves are site-dependent, the mechanisms by which the monovalent cations inhibit binding appear to differ at each site. Furthermore, the actions of divalent salts differ from those of monovalent salts. For inhibition of μ- and δ-binding by monovalent salts the slopes of the dose-response curves are shallower than would be expected for competitive interactions, a finding in contrast to observations for NaCl in rat brain (Kouakou et al. 1982; Zajac and Roques 1985). These discrepancies may be due to differences in the species or in the assay conditions.

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BINDING CHARACTFRISTICS OF [D-Ala^2-MePhe^4-Glyol^5] ENKEPHALIN AND [D-Pen^2,D-Pen^5] ENKEPHALIN IN RAT BRAIN: INHIBITION STUDIES WITH FENTANYL-LIKE DRUGS

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ABSTRACT

We have investigated the binding characteristics of two tritiated enkephalin analogues [D-Ala^2-MePhe^4-Glyol^5]enkephalin (DAGO) and [D-Pen^2, D-Pen^5]enkephalin (DPDPE) which are highly selective for μ and δ opioid sites respectively, in homogenates of whole rat brain and of the pons/medulla region. In addition, we have explored the binding profile of four 4-anilinopiperidine drugs (fentanyls) by means of inhibition studies. Our studies show that the δ ¡ involvement in pons/medulla of rat is small compared with that in whole brain and with μ binding in both whole brain and pons/medulla. In addition the rank order of affinities of the fentanyl series is the same at the μ and δ site, but the affinities are between 1 and 600-fold greater at the former site.

INTRODUCTION

Foremost of the adverse effects observed with the use of opioid drugs as analgesics or anaesthetic/analgesics is respiratory depression. Although this has been known for a considerable time, little progress has been made in determining the mechanism of action of opioids in this regard. Investigations at the receptor level have until recently been impeded by the lack of highly specific, high affinity ligands for μ- and δ··opioid sites. The advent at DAGO and DPDPE has overcome this problem and these enkephalin analogues are now available at usefully high specific activities.

We have begun <u>in vitro</u> experiments into the binding characteristics of these ligands and of an homologous series of potent synthetic opioids, the fentanyls, in rat brain homogenates, in particular from areas known to be involved- in respiratory control.

METHODS AND MATERIALS

Adult male Wistar albino rats, University of Surrey strain (250g) were killed by decapitation, the brains rapidly removed and quickly dissected according to the procedure of Glowinski and Iversen (1966). The procedure for preparation of membranes for use in binding studies was identical to that of Gillan and Kosterlitz (1982)

Saturation assay mixtures comprised a 2ml final volume of the membrane preparation, containing 19mg original wet weight of tissue. Non-specific and total binding of the ^{3}H-DAGO and ^{3}H-DPDPE was determined in the presence and absence, respectively, of 100-fold excess of unlabelled diprenorphine or naloxone. The assay tubes were incubated in a shaking water bath for 40 mins (^{3}H-DAGO) or 50 mins (^{3}H-DPDPE) at 25°C, and incubation was terminated by rapid filtration over glass fibre filters (Whatman GF/B), then immediately washed three times with 5ml ice-cold buffer. The filters were transferred to scintillation vials and extracted overnight in 0.5ml Triton X100: Toluene (20:80) before addition of 3.5ml Unisolve 1 scintillant followed by liquid scintillation counting. A ^{3}H-Hexadecane internal standard was included for quench correction, and all assays were performed in triplicate.

For inhibition studies, the protocol above was followed, but the concentration of ^3H ligand was held constant whilst that of the cold displacing drug was increased. Non-specific binding was assessed both in the presence and absence of the displacing drug.

Data from saturation experiments were analysed by Scatchard plots and those from inhibition assays by Hill plots. Slopes and intercepts were calculated from the values of at least four independent experiments, derived by linear regression of the data.

^3H-DAGO (57.5 Ci/mmole) and ^3H-DPDPE (28.0 Ci/mmole) were obtained from Amersham International, U.K. Alfentanil, carfentanil, fentanyl and sufentanil were gifts fron Janssen Pharmaceuticals, Belgium. Naloxone was a gift from Endo Laboratories, U.S.A., and Diprenorphine, a gift from Reckitt and Colman, U.K. The labelled peptides were highly purified by HPLC.

RESULTS AND DISCUSSION

Binding of tritiated enkephalin analogues:

The specific binding of both ^3H-DAGO and ^3H-DPDPE to whole brain and pons/meduIla membranes demonstrated a rectangular hyperbolic relationship to free ligand concentration, and Scatchard plots were always linear. However, it was not possible to obtain acceptable correlation coefficients for Scatchard plots from single experiments with ^3H-DPDPE in pons/meduIla homogenates. Accordingly, binding constants were determined by simultaneous analysis of the data from five separate experiments, with r = 0.95. There was no significant difference between the B_{max} or the K values for the μ specific ligand in the two tissues (table 1). Whilst this was also true for the K_D value for ^3H-DPDPE in pons/medulla homogenate, the maximum number of binding sites for ^3H-DPDPE in pons/medulla homogenates was less than one third that of the whole brain. Moreover, the specific binding of ^3H-DAGO in whole brain at 1nM represented 91% of the total binding, but the equivalent value for H-DPDPE at K_D in pons/medulla was only 38%. There was a positive correlation between the non-specific binding of both ligands in both tissues and the time taken at 25°C for half-maximal specific binding of the ligand to be reached (r = 0.98), and also between the non-specific binding and the time taken for a constant ratio of specific/non-specific binding to be reached (r = 0.99).

TABLE 1. Comparative binding characteristics of ^3H-DAGO and ^3H-DPDPE in whole brain and pons/medulla homogenates of rat.

	^3H-DAGO		^3H-DPDPE	
	Whole brain	pans/medulla	Whole brain	*pons/medulla
B_{max} (fmol mg^{-1} protein)	167 ± 11	145 ± 6	175 ± 23	54.9
K_D (nM)	1.1 ± 0.2	1.5 ± 0.1	9.4 ± 1.0	10.2
Non-specific binding (% of total binding)	8.7	13.6	30.6	61.7
Time to half-maximal specific binding (mins)	8.8	9.4	13.1	27.5
Time to constant ratio of specific to non-specific binding (mins)	15	25	50	<120

Each value is the mean ± s.e.m. of at least four independent determinations.
*n = 5, but determined by simultaneous analysis of all the data.

Several studies have concluded that the depression of respiration produced by opioids is mediated via μ or δ but not κ receptors (Santiago and Edelman 1985). However, the low selectivity of some of the ligands used has presented difficulties in interpretation. Our quantitative studies show that the δ involvement in rat pons/meduila is small compared with that in whole brain, and with μ binding in both regions. This militates against δ mediation of respiratory depression, but does not exclude it.

Inhibition studies:
The majority of inhibition studies produced Hill plots with near-integral slopes and correlation coefficients, indicating interaction of the tracer ligands with one receptor population (table 2). We have thus far found no evidence for the existence of μ-isoreceptors (Pasternak 3986) in rat brain.

The rank order of affinities of the fentanyl series is the same at the μ and δ site, (and also the same as the rank order of potency in production of respiratory depression by these drugs in rats (Janssen 1982), but the affinities are between 1 and 600-fold greater at the former site. This appears to implicate a μ receptor involvement in the depression of respiration caused by the fentanyls. However, the considerable differences in the relative affinities of the drug series at the μ and δ sites provides a potential tool for testing this hypothesis.

TABLE 2. Binding profile of a series of 4-anilinopiperidine drugs at the μ and opioid receptors of rat brain

	Carfentanil	Sufentanil	Fentanyl	Alfentanil
*K_I at δ site (nM)	0.44 ± 0.02	21.1 ± 0.8	259 ± 4.3	22100 ± 3700
Hill plot slope	1.00 ± 0.03	1.1 ± 0.06	0.91 ± 0.13	0.83 ± 0.07
**K_I at μ site (nM)	0.42 ± 0.04	0.77 ± 0.10	3.3 ± 0.45	38.9 ± 3.7
Hill plot slope	0.96 ± 0.01	0.97 ± 0.01	0.98 ± 0.01	0.97 ± 0.01
K_I δ/K_I μ	1.1 ± 0.05	28 ± 1.0	78 ± 13	570 ± 90
Relative μ affinity	7.9	4.3	1.000	0.085
Relative δ affinity (fentanyl = 1.000)	47.8	12.3	1.000	0.012

Each value is the mean ± s.e.m. of at least four independent determinations.

*The marker ligand was ^{3}H DPDPE at 0.5 K_D.

**The marker ligand was ^{3}H DAGO at K_D.

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DEVELOPMENT OF δ-OPIOID RECEPTORS IN RAT BRAIN CHARACTERISED BY [^{3}H]-[DPen^2,D-Pen^3] ENKEPHALIN BINDING

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ABSTRACT

The development of rat brain δ-opioid receptors has been studied in the posnatal period using the highly selective δ-site ligand [^{3}H]-[D-Pen^2,D-Pen^3] enkephalin (DPDPE) in binding studies. Between day 10 and day 25 there was a three-fold increase in the number of binding sites (expressed per mg protein) and the K_D was constant throughout the postnatal period. Specific binding could not be detected earlier than day 10.

INTRODUCTION

There is good evidence that there is differential development of μ, δ and κ opioid binding sites (Spain et al. 1985). The understanding of this heterogeneity has been hampered, however, because of the lack of selective ligands for the receptor subtypes.

With the recent availabilty of ^{3}H-DPDPE which has been shown to be a highly selective binding ligand for the δ-opioid receptor (Cotton et al. 1985) an accurate determination of δ-site ontogeny has become possible. We report here ^{3}H-DPDPE binding in the postnatal period and reveal inconsistencies with previous ontogenetic studies using less selective ligands.

METHODS

Wistar albino rats of mixed sexes were used in all experiments and receptor binding assays (in Tris HCl, pH 7.4 at 25°C for 40 mins) were carried out as described by Cotton et al. (1985) in whole brain homogenates. ^{3}H-DPDPE (25-27Ci/mmol, Amersham) was purified by HPLC prior to use. Scatchard analysis of saturation curves was used to determine K_D and B_{max}.

RESULTS AND DISCUSSION

Table 1 shows the maximal binding capacities and affinities for ^{3}H-DPDPE during postnatal development. Specific binding was absent in animals less than 10 days old and this contrasts with data for [^{3}H]-[D-Ala^2,D-Leu^5] enkephalin (DADL) where binding was reported during the first postnatal week (Spain et al. 1985). In addition the three-fold increase in number of binding sites between the second and fourth postnatal week is of a greater magnitude than reported for ^{3}H-DADL. In common with all ontogenetic binding studies using opioid ligands the affinity of ^{3}H-DPDPE remains constant during postnatal development.

In conclusion this study is the first report of A-receptor ontogeny using a ligand highly selective for this receptor and confirm that the major development of δ-receptors occurs between the second and fourth postnatal weeks. It should be pointed out however that difficulties were encountered in obtaining acceptable Scatchard plots in young animals due to the low specific binding of ^{3}H-DPDPE and this poses problems in the use of this ligand for ontogenetic studies.

TABLE 1. Binding characteristics of [^{3}H] - [D-Pen^2, D-Pen^{5}I enkephalin in the developing rat brain

Age (days)	Dissociation constant (K_D, nM)	Binding capacity (B_{max}, fmol/mg protein)
10	10.5 ± 1.9	34.5 ± 6.2
15	10.2 ± 1.7	49.3 ± 4.6
21	9.7 ± 1.1	78.6 ± 7.5
25	12.7 ± 2.7	102.8 ± 13.6
30	9.7 ± 0.9	107.6 ± 6.1
35	9.7 ± 1.4	111.8 ± 15.2
40	8.5 ± 0.5	103.3 ± 11.5
45	11.1 ± 1.8	119.4 ± 11.2
50	7.2 ± 0.4	99.9 ± 12.0

Values are the mean ± s.e.m. of 4 Scatchard plots.

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COMPARISON OF THE EFFECTS OF CALCIUM CONCENTRATION ON MU AND KAPPA AGONIST ACTIONS IN THE GUINEA PIG ILEUM

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ABSTRACT

The effect of calcium on responses to selective mu (fentanyl and DAGO) and selective kappa agonists was investigated on guinea pig ileum. The dose-response curves were shifted to the right in a non-parallel fashion with increasing calcium concentration (0.9-3.6 mM). However U50488 was shifted more than either fentanyl or DAGO. Both fentanyl and U50488 could not produce 100% inhibition of twitch in 3.6mM calcium. It is concluded that the efficacy of the agonists changes with calcium concentration and that the kappa agonist U50488 is affected more than the mu agonists because kappa receptor activation directly inhibits calcium entry whereas mu receptors inhibit calcium by increasing potassium conductance.

INTRODUCTION

It has been shown that kappa receptor activation probably inhibits calcium entry in neurons of the myenteric plexus (Cherubini & North 1985). This is in contrast to mu agonists which appear to indirectly reduce calcium-entry by increasing potassium conductance. Although there have been previous reports of the effect of varying calcium concentration on morphine actions in the guinea pig ileum (Opmeer & van Ree 1979) there is little information available on the effect of such manipulation on kappa agonists. We have compared the effect of a range of calcium concentrations on the actions of the kappa selective agonist U50488 and the mu selective compounds fentanyl and [D-Ala^2,MePhe^4,Gly(ol)^5]-enkephalin (DAGO) on the guinea pig isolated ileum.

MATERIALS AND METHODS

Male Dunkin-Hartley guinea pigs (250-400g) were killed by cervical dislocation and the ileum removed and placed in Tyrode's solution of the following composition: (mM) NaCl 136.9, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, KCl 2.7, $MgCl_2$ 1.05, D-glucose 5.55 and $CaCl_2$ 1.8. The pieces of ileum were then mounted on coaxial electrodes and electrically

stimulated with 0.5ms pulses at 0.1Hz at supramaximal voltage. The 10ml organ baths were maintained at 37°C and the solutions bubbled with $95\%O_2/5\%CO_2$. Washing was by overflow. The ability of the agonist to inhibit the electrically evoked twitch was measured in Tyrode's with 0.9, 1.8 and 3.6mM calcium. The calcium concentration of the solutions was altered by adding calcium chloride to the reservoir. The osmolarity of the solution varied between 282 and 287 milliosmoles.

RESULTS

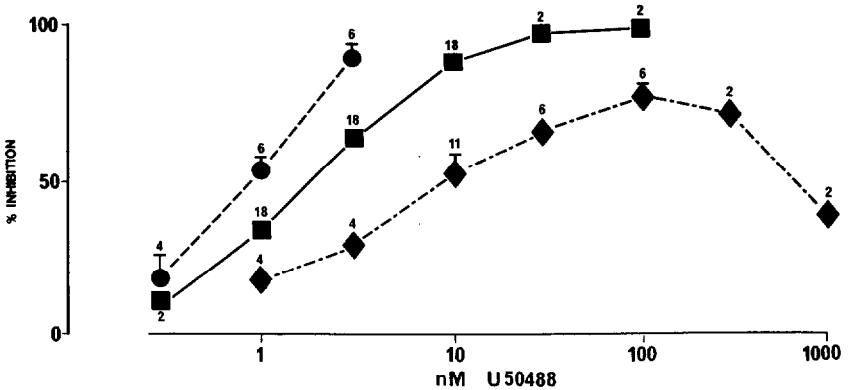

FIGURE 1 Dose-response curves for the kappa selective compound U50488. The curves were shifted to the right in a non-parallel fashion with increasing calcium concentration. (● =0.9mM, ■ =1.8mM and ◆ =3.6mM calcium). In 3.6mM calcium 100% inhibition of the twitch was not seen.

The mu and kappa ligands tested in this study reduced the electrically evoked twitch of guinea pig isolated ileum. This effect was found to be dependent upon external calcium concentration.
As is shown in table 1, the dose ratio produced by the change from 1.8 to 3.6mM calcium for U50488 is significantly greater than that for either fentanyl or DAGO. The dose-response curves were shifted to the right in a non-parallel fashion with increasing concentration of calcium. At the highest calcium concentration (3.6mM) it was not possible to obtain 100% suppression of twitch with either fentanyl or U50488 (figures 1,2).

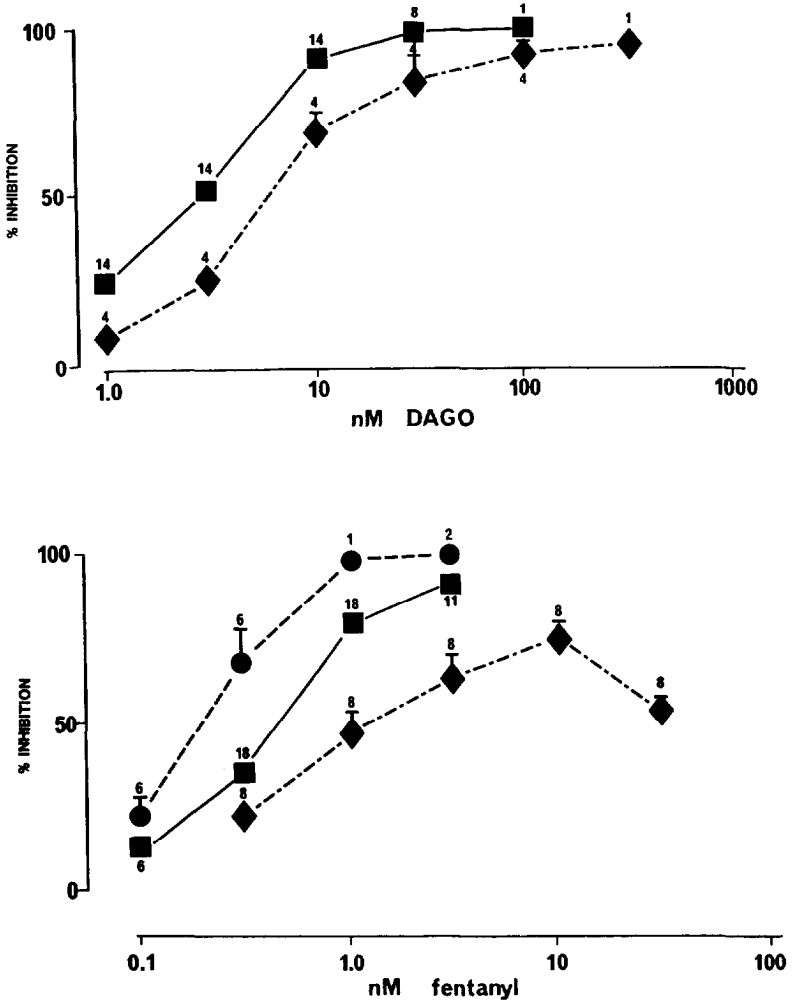

FIGURE 2 Dose-response curves for the mu selective ligands fentanyl and DAGO in increasing calcium concentration. The curves were shifted to the right. Allthough the DAGO curves appeared to be parallel the fentanyl curves were not and did not reach maximum inhibition.(● =0.9mM, ■ =1.8mM and ◆ =3.6mM calcium)

DISCUSSION

The data presented suggest that mu and kappa agonists may inhibit calcium entry by differing mechanisms. This supports the finding that kappa agonists reduce

[Ca++]	Fentanyl	dose ratio	U50488	dose ratio	DAGO	dose ratio
(mM)	IC50±sem(n) (nM)	mean±sem(n)	IC50±sem(n) (nM)	mean±sem(n)	IC50±sem(n) (nM)	mean±sem(n)
0.9	0.24±0.08(5)	0.54±0.24(3)	0.92±0.14(6)	0.53±0.07(6)		
1.8	0.55±0.11(8)		1.98±0.17(10)		3.3±0.16(4)	
3.6	2.20±0.65(8)	3.77±1.09(6)	13.3±2.75(4)	7.55±1.62(8)	6.5±1.00(4)	2.0±0.4(4)

TABLE 1 The effect of calcium concentration on the effectiveness of U5C488, fentanyl and DAGO, in reducing the electrically evoked twitch of guinea pig ileum. The potencies of all three agonists are reduced by increasing the calcium concentration. However the dose-ratio for U50488 in 1.8 and 3.6 mM calcium is significantly larger (*p<0.1, Student's unpaired t-test) than for either fentanyl or DAGO.

transmitter release directly by inhibiting calcium entry (Cherubini & North 1985) whereas mu agonists act indirectly by increasing potassium conductance to hyperpolarise the nerve terminal and subsequently reduce calcium entry. Hayes and Sheehan (1986) have shown that compounds which appear to be partial agonists and antagonists on the rat vas deferens, a tissue with a low receptor reserve, increase their efficacy in lowered calcium concentrations suggesting an increase in the receptor reserve. Therefore it is possible that in higher calcium concentrations the receptor reserve for both mu and kappa receptors is reduced in guinea pig ileum and so the efficacy of what appear to be full agonists is reduced to that of partial agonists. This could explain the reduced maximum responses seen with fentanyl and U50488. The ability of DAGO to produce a full inhibition of the twitch in the presence of 3.6mM calcium suggests a high efficacy, in comparison with fentanyl. The possibility that the guinea pig ileum opioid receptor reserve is calcium dependent could only be adequately investigated, however, by using an irreversible receptor antagonist such as **ß**-CNA (Takemori et al. 1986).

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KAPPA OPIOID BINDING SITES IN RAT SPINAL CORD

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ABSTRACT

The spinal cord of the rat is shown to contain only a low level of kappa-binding sites, comparable to rat brain. [^{3}H]Bremazocine appears to label an additional class of sites which do not appear to be of mu, delta, or kappa specificity.

INTRODUCTION

Intrathecally administered opioids are believed to act directly at the spinal level to prevent the passage of nociceptive information to higher centres (Yaksh 1981). Pharmacological studies of opioids after intrathecal administration show that ligands with mu, delta, and kappa selectivity are active in various test systems used'to evaluate analgesia (Tung and Yaksh 1982, Schmauss and Yaksh 1984). Characterisation of opioid receptors in the rat cord by ligand binding assays have demonstrated the presence of a large number of putative kappa sites (Gouarderes et al. 1981, Traynor and Rance 1984). Such sites have properties inconsistent with a single site (Traynor and Rance 1985) and the existence of sub-types has been suggested (Attali et al. 1982). In this paper we present a partial characterization of these sites.

MATERIALS AND METHODS

The following labelled ligands were used: [^{3}H]Bremazocine (30 Ci.mmol^{-1}) (New England Nuclear) and [^{3}H]Dynorphin(1-9) (26 Ci.mmol^{-1}) (Amersham International). Unlabelled ligands used were Tyr-D-Ala-Gly-MePhe-$NHCH_2$-CH_2OH (GLYOL), D-Ala_2-D-Leu_5-Enkephalin (DADLE) (Sigma U.K.) and Trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene acetamide (U-50488H) (Upjohn). Peptidase inhibitors used were Bestatin (Cambridge Research Biochemicals) and Captopril (Merck).

Binding to rat lumbo-sacral spinal cord membranes was performed in Tris buffer, pH 7.4, at 37°C or 25°C (for [^{3}H]Dynorphin(1-9)) as previously described (Traynor and Rance 1985). In the case of [^{3}H]Dynorphin(1-9) the peptidase inhibitors bestatin (30µM) and captopril (300µM) were included to prevent degradation of the labelled ligand. Binding to homogenates of guinea-pig cerebellum (Robson et al 1984) was performed in 20mM Hepes buffer or Hepes-buffered Krebs solution containing 10µM GppNHp for 40 min at 25°C. Non-specific binding was defined using 1µM Diprenorphine.

RESULTS AND DISCUSSION

[^{3}H]Bremazocine exhibited high affinity binding to rat lumbo-sacral cord membranes. However, after suppression of binding to mu and delta sites, with 100nM each of GLYOL and DADLE per 0.5nM [^{3}H]ligand, displacement of the remaining bound [^{3}H]Bremazocine was not consistent with a single kappa site. Thus ligands for mu (GLYOL), delta (DADLE) and kappa (U-50488H) sites displaced the remaining specifically bound [^{3}H]Bremazocine with low Hill coefficients (table 1). Indeed for DADLE it was possible to discern two definite components with affinities of 321nM and 25890nM respectively. Displacement by naloxone suggested all of the specifically bound [^{3}H]Bremazocine was opioid in nature.

TABLE 1. Inhibitory effects of opioids on the binding of [^{3}H]Bremazocine to rat lumbo-sacral membranes in the presence of GLYOL (100nM) and DADLE (100nM).

Competing Ligand	Ki(nM)	Hill Coefficient
GLYOL	1338 ± 309	0.83 ± 0.04 (3)
DADLE	8945 ± 4825	0.61 ± 0.07 (3)
U-50488H	57.3 ± 7.8	0.52 ± 0.01(4)
Naloxone*	11.0	

* Naloxone displaced a mean of 99.1% of the specifically bound [3H]Bremazocine in the two experiments.

In order to gain further insight into the heterogeneous site we have studied the binding of [^{3}H]Bremazocine in the presence of a high concentration of DADLE (5-10μM) to suppress the higher affinity of the two kappa components. The remaining binding is characteristic of a classical kappa site (table 2).

TABLE 2. Inhibitory effects of opioids on the binding of [^{3}H]Bremazocine to rat lumbo-sacral membranes in the presence of DADLE (5μM).

Competing Ligand	Ki(nM)	Hill Coefficient
GLYOL	6306 ± 1333	1.12 ± 0.13 (3)
DADLE	2573 ± 638	0.84 ± 0.10 (3)
U-50488H	15.63 ± 2.84	0.77 ± 0.08 (3)

Furthermore. in the presence of this high DAOLE concentration the number of [^{3}H]Bremazocine binding sites was markedly reduced bringing it into line with the number of sites labelled by the kappa-selective peptide [^{3}H]Dynorphin(1-9) (table 3). These findings suggest that the true kappa site accounts for only 13% of the total [^{3}H]Bremazocine binding in the cord, rather than the much higher percentage previously supposed (Gouarderes et al. 1981, Traynor and Rance 1984). This low level of kappa binding agrees with the findings of Lahti et al. (1985) who were unable to detect binding of the kappa selective [^{3}H]U69593 to rat cord membranes.

TABLE 3. Binding of [^{3}H]ligands to rat lumbo-sacral spinal cord membranes.

[^{3}H]ligand	K_D (nM)	Bmax (fmols.mg^{-1} protein)
Bremazocine	0.47±0.05	89.6±5.3 (3)
Bremazocine with 10μM DADLE*	0.20±0.02	11.9±0.4 (3)
Dynorphin(1-9) with mu and delta suppression**	0.28±0.03	10.1±0.8 (4)
GLYOL+	1.95±0.2	24.2±1.3 (3)
DADLE+	1.92±0.3	11.1 ±0.6 (3)

* 10μM DADLE to 1 x K_D of the [^{3}H]ligand and ** 100 μM each of GLYOL and DADLE to 1 x K_D of the [^{3}H]ligand.
+ Taken from Traynor and Rance, 1984.

It is well known that apparently heterogeneous binding may be a consequence of using non-physiological conditions (Creese 1985). To determine if this could explain our findings we have used the largely kappa containing guinea-pig cerebellum (Robson et al. 1984). In this tissue, in Hepes buffer, [^{3}H]Bremazocine (0.2nM) is displaced by U-50488H with an IC_{50} of 10.3nM but a Hill coefficient of only 0.59 which was similarly shallow even in the presence of 3μM DADLE. Using Hepes buffered Krebs containing the stable GTP analogue GppNHp the dose-response curve for U-50488H is shifted twenty-fold to the right but the slope remains at 0.59, suggestive of true heterogeneity. Further studies are in progress to confirm whether the nature of this heterogeneity is the same as that seen in the rat cord.

The results demonstrate that the rat spinal cord contains a small population of classical kappa sites, similar to that reported in rat brain (Gillan and Kosterlitz 1982), and that [^{3}H]bremazocine, but not the peptide [^{3}H]Dynorphin(1-9), labels a site which is apparently not of mu, delta or kappa specificity. It is unlikely that this should be considered as a sub-type of the kappa site in view of its inability to reccgnise Dynorphin(1-9). It will be of interest to determine what role, if any, this site plays in spinally-mediated analgesia.

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OPIOID RECEPTORS OF BOVINE POSTERIOR PITUITARY NEUROSECRETOSOMES ARE EXCLUSIVELY KAPPA

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ABSTRACT

Intact neurosecretosomes (NSS) from bovine posterior pituitary were prepared and characterized. Ligand binding studies were performed, using ^{3}H-[D-Ala^{2}-D-Leu^{5}]enkephalin (DADL), ^{3}H-etorphine and ^{3}H-ethylketocyclazocine (EKC). The absence of specific binding of ^{3}H-DADL and the inability of DADL to displace ^{3}H-etorphine, implies the absence of mu, delta, or DADL-suppressible benzomorphan ("kappa-2") sites. Self- and cross displacement studies for etorphine and EKC suggested receptor heterogeneity. EKC fails to displace - 20% of specific binding of etorphine. Mathematical modelling indicates the presence of three classes of sites. The present findings imply that bovine posterior pituitary opioid receptors are exclusively of the kappa type.

INTRODUCTION

Opioid peptides are present in the neural lobe of the pituitary. Modulation of the secretion of oxytocin and vasopressin by opioid peptides directly at the neural lobe level has been widely studied. Although the coexistance of vasopressin and oxytocin with opioid peptides in neurosecretory vesicles of nerve terminals themselves is now accepted, the underlying mechanisms of the modulation of the secretion and the type or subtype of opioid receptors involved remains unclear. Several reports suggested that opioid receptors are present primarily in the pituicytes (astrocytoglia), rather than on the nerve terminals (Bunn et al. 1985; Lightman et al. 1983). The present study was undertaken to determine whether opioid binding sites are also present on neurosecretosomes (i.e., nerve terminals) and to characterize the types of these receptors. We used DADL as a probe of mu and delta receptors. Etorphine and EKC were selected, following protocols similar to that used by another laboratory (Castanas et al. 1985) and ourselves to characterize 3 subtypes of kappa receptors in bovine adrenal medulla.

MATERIALS AND METHODS

Neurosecretosome preparation: Fifteen to twenty bovine posterior pituitaries were suspended in 0.25 M sucrose, 10 mM HEPES-Tris buffer (pH 7.3) and homogenized with a teflon-glass homogenizer. The homogenate was centrifuged at 750 x g for 2 min and the

supernatant was centrifuged at 5.000 x g for 20 min. The pellet was resuspended in sucroserpercoll (30%) medium and centrifuged at 50,000 x g for 45 min. The particulate band at the 1.045-1-055 g/ml density region of the gradient was collected as purified neurosecretosomes (NSS). The purity of the preparation was assayed both by enzyme analysis and electron microscopy (Russell, in preparation).

Opiate binding assay: Binding studies were performed in isotonic sucrose solution with the following characteristics: sucrose 0.25 M; HEPES-Tris, 10 mM (pH 7.3); PMSF, 3 pg/ml; aprotonin, 1000 U/ml; bacitracin, 0.02% (w/v). The samples were incubated at 37°C for 30 min and then filtered through Whatman GF/B filters.

Mathematical and statistical analysis: Data were analyzed with the computer program ALLFIT to estimate IC_{50} values and pseudo-Hill coefficients, as described elsewhere (De Lean et al. 1978). Binding parameters were obtained using program LIGAND, employing a weighted non-linear regression analysis (Munson and Rodbard 1980; Cruciani et al. in press).

Materials: ^{3}H-etorphine (40 Ci/mmol) was obtained from Amersham, Arlington Heights, IL. ^{3}H-EKC (24 Ci/mmol) and ^{3}H-DADLE (45 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA.

RESULTS AND DISCUSSION

Competition studies for ^{3}H-etorphine by DADL were performed (fig. 1).

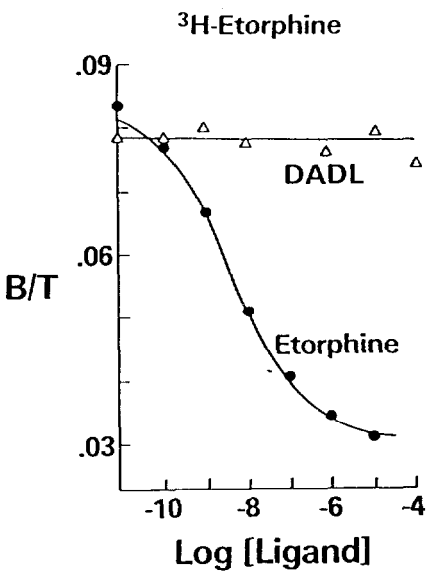

Fig. 1. Displacement of ^{3}H-etorphine from neurosecretosomes of bovine posterior pituitary by unlabeled etorphine and DADL.

DADL failed to displace specific binding of etorphine even when 10^{-4}M unlabeled ligand was used as displacer, implying the absence of mu, delta, and benzomorphan binding sites. This observation was further confirmed by the absence of specific binding for ^{3}H-DADL.

Self-displacement curves for etorphine and EKC, using a fixed concentration of labeled ligand showed a complex isotherm that suggested hetereogeneity of binding sites. When each of these curves were analyzed individually or by pooling results from several experiments, one could reject a model involving a single class of sites ($p < 0.01$). In each case a model involving two classes of sites was sufficient, and further complexity was not supported by analysis of data from homologous displacement curves considered alone. Etorphine showed apparent Kd's of 1 and 100 nM; EKC showed Kd's of 5 nM and > 100 µM.

Complete self- and cross-displacement study: We next sought to study whether the two sites for etorphine and EKC were independent or whether the two ligands shared the high affinity site. Accordingly, a complete self- and cross-displacement study for labeled etorphine and EKC was performed (table 1). Mathematical modelling of the data with program LIGAND indicated that a model involving three classes of sites was significantly better than a model involving two classes of sites ($p < 0.01$). Parameters of the "3-site" model are shown in Table 1. The data did not support a more complex model with 4 classes of sites. R_1 showed high affinity for both etorphine and EKC, with approximately two-fold higher affinity for etorphine. R_2 has a 750-fold higher binding capacity than R_1, with low affinity for etorphine (320 nM) and negligible affinity for EKC. The third site, R_3 showed an even larger binding capacity but with an extremely low affinity for both ligands (Kd's in the µM range).

TABLE 1. Binding parameters of opioid binding to NSS. Kd and binding capacities for a model involving three classes of sites (R_1-R_3. A complete set of four self- and cross-displacement curves for labeled and unlabeled etorphine and EKC was analyzed simultaneously using the computer program LIGAND.

Class of site	Receptor concentration (pmol/gr tissue)	Etorphine K_D (nM)	EKC K_D (nM)
R_1	4	0.9	2
R_2	320	150	> 10000
R_3	1700	7300	2300

The present studies have shown that the neurosecretosomes from the bovine posterior pituitary have kappa opioid receptors characterized by high affinity for etorphine and EKC and complete lack of affinity for the relatively delta/mu selective ligand DADL. It is possible that R_1 of the NSS (Table 1) corresponds to the "kappa-1" or "kappa-3" subtypes of bovine adrenal medulla which have high affinity for both etorphine and EKC, but are not suppressible by DADL (Castanas et al. 1985). In contrast, in bovine adrenal medulla, 80% of kappa receptors are present as "benzomorphan sites," also designated as the "kappa-2 subtype." This receptor was defined operationally as sites with Kd - 1 nM for both etorphine and EKC, but which could be suppressed by 1-100 µM DADL. The failure of 10^{-4}M DADL to displace labeled etorphine from NSS implies that the kappa receptor of bovine posterior pituitary NSS differs from the major form in membranes from bovine adrenal medulla.

The present findings are in agreement with recent reports of autoradiographic data (Herkenham et al. 1985, Gerstgerger and Bardin 1986) and with other radioreceptor binding studies (Martin and Falke 1985) that indicate predominance of kappa binding sites in the neural lobe.

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THE PHARMACOLOGICAL PROFILE OF BAM 18

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ABSTRACT

The opioid receptor selectivity of BAM 18 was determined by radioligand binding and peripheral tissue bioassay. Using selective radioligand binding conditions, BAM 18 bound to the μ opioid receptor with an affinity twice that of the K receptor and over 10 times that of the δ opioid receptor (K_i = 0.29, 0.84 and 3.9 nM, respectively). K_e values for naloxone antagonism of BAM 18 activity in the electrically stimulated guinea pig ileum and the mouse vas deferens were 4.3 and 9.9 nM respectively. The pharmacological profile of BAM 18 was similar to that of metorphamide.

INTRODUCTION

BAM 18 is an 18 amino acid peptide derived from proenkephalin A. Although it is present in rat and guinea pig CNS in relatively high concentrations (Evans et al. 1985), the pharmacological properties and physiological function of this peptide are presently unknown. In the present study we have compared the pharmacological properties of BAM 18 to that of another proenkephalin A-derived peptide, metorphamide. Our data indicate that the pharmacological profile of BAM 18 is similar to that of metorphamide, in that both peptides display high affinity for the μ opioid receptor.

MATERIALS AND METHODS

Radioligand binding.

Male guinea pigs (350-400 g) were obtained from Simonsen Labs., Gilroy, CA. Brain homogenates were prepared as described previously (Leslie et al. 1982). Brain membranes (10 mg original tissue wet weight) were incubated for 90 min at 22°C in 1 ml 50 mM Tris-HCl buffer (pH 7.4) containing radioligand, appropriate competing ligand and enzyme inhibitors (bestatin, 10 μM, and thiorphan, 0.1 μM). Selective binding conditions for each receptor subtype were utilized. The μ binding site was labelled with [^{3}H] DAGO, the δ binding site with [^{3}H] DADLE in the presence of 300 nM D-Pro^4-morphiceptin (PLO17) (to saturate all μ sites), and the K binding site with [^{3}H] EKC in the presence of 100 nM DSLET and 300 nM PLO17 (to saturate all δ and μ binding sites, respectively). Specific binding was defined as the difference in bound radioactivity in the absence and presence of 1 μM levallorphan. Following incubation, membranes were filtered through GF/C paper and rinsed with 16 ml of ice cold buffer. Saturation curves were constructed using increasing concentrations of radioligand. The equilibrium dissociation constant (K_d) and binding capacity (B_{max}) of each radioligand were derived by non-linear, least squares regression analysis using the computer program LIGAND (Munson and

Rodbard 1980). Both BAM 18 and metorphamide were used to competitively displace radioligand binding. The dissociation constant of each inhibiting ligand (K_i) was determined using the Cheng-Prusoff equation (Cheng and Prusoff 1973).

Bioassay

Male guinea pigs and Balb C mice were obtained from Simonsen Labs., Gilroy, CA. Myenteric plexus-longitudinal muscle strips (GPI) and mouse vas deferens (MVD) were prepared as described previously (Leslie et al. 1980). Enzyme inhibitors (bestatin, 1 μM, and thiorphan, 0.1 μM) and drugs were added to the tissue baths at 20 min intervals for the GPI, and at 8 min intervals for the MVD. Dose response curves were constructed by adding increasing concentrations of the test drug to the organ bath and IC_{50} values determined. K_e values for naloxone antagonism of agonist activity were determined by the 'single dose' method of Kosterlitz and Watt (1970).

RESULTS

Affinity constants for inhibition of radioligand binding are shown in table 1. BAM 18 displaced the binding of all three radioligands. Although it exhibited hi hest affinity for the μ binding site, it also potently displaced [^{3}H] EKC binding from the K site. In contrast, BAM 18 had a 10-fold lower affinity for the δ | binding site. Metorphamide displayed a similar receptor selectivity profile. It was a potent displacer of both [^{3}H] DAGO and [^{3}H] EKC binding, with a lower affinity for the δ binding site. IC_{50} values

TABLE 1. Potencies of opioid peptides for displacement of radioligand binding to guinea pig brain homogenates.

DRUG	K_i (nM)		
	[^{3}H] DAGO	[^{3}H] EKC	[^{3}H] DADLE
BAM 18	0.29 ± 0.063	0.75 ± 0.26	3.17 ± 0.60
Metorphamide	0.06 ± 0.005	0.23 ± 0.08	1.76 ± 0.07

Values represent the mean ± SEM of 3-8 observations.

determined in bioassay preparations are shown in table 2. Both BAM 18 and metorphamide were more potent inhibitors of twitch contraction in GPI than MVD. In both tissues, K_e values for naloxone antagonism were intermediate between those previously reported for antagonism of μ and K agonists (table 3; Lord et al. 1977).

TABLE 2. Potencies of opioid peptides for inhibition of stimulated twitch contraction in GPI and MVD.

DRUG	IC_{50} (nM) GPI	MVD
BAM 18	1.37 ± 0.30	26.2 ± 6.34
Metorphamide	0.51 ± 0.08	6.53 ± 2.50

Values represent the mean ± SEM of 3-6 observations.

TABLE 3. Naloxone antagonism of BAM 18 and Metorphamide agonist activity in GPI and MVD.

DRUG	Naloxone K_e (nM) GPI	MVD
BAM 18	4.34 ± 1.31	9.86 ± 2.17
Metorphamide	4.88 ± 1.43	10.6 ± 0.97

Values represent the mean ± SEM of 3-6 observations.

DISCUSSION

Data from both receptor binding and bioassay indicate that BAM 18 has high affinity for μ and K opioid receptors, with somewhat lower affinity for the δ opioid receptor. This receptor selectivity profile is similar to that of metorphamide (present study; Weber et al. 1983). In contrast, met-enkephalin has a different pattern of pharmacological activity, exhibiting the highest affinity for the δ receptor, with lower affinities for μ and K receptors.

BAM 18 is present in higher concentrations in the CNS of rat than is metorphamide (Evans et al. 1985; Weber et al. 1983). Its regional distribution throughout brain is similar to that of enkephalin (Evans et al. 1985; Sanders et al. 1984; Hong et al. 1977; Zamir et al. 1985). It has previously been noted that the anatomical distribution of proenkephalin-derived peptides does not correspond precisely to that of δ receptors (Moss et al. 1986). In several brain regions, however, there is a significant overlap between the pattern of enkephalin immunoreactivity and that of μ receptors (Laughlin et al. 1985). Based on our present findings that BAM 18 has highest affinity for the μ binding site, we propose that this peptide may mediate its physiological actions in certain brain regions via the μ opioid receptor. The functional significance of BAM 18 remains to be determined.

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CROSS-PROTECTION OF μ AND δ OPIOID RECEPTORS IN THE MOUSE VAS DEFERENS.

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ABSTRACT

Opioid receptors in the C57/BL mouse vas deferens can be irreversibly blocked by pre-treatment with β-chlomaltrexamine. Pre-incubation with the highly selective δ receptor antagonist ICI 174864 concentration-dependently protected δ-receptors from inactivation, but the same concentrations similarly protected μ receptors. Pre-incubation with selective μ-receptor agonist, [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin, also protected both μ and δ receptors to the same degree. Neither ligand protected k- opioid receptors. The results suggest that there may be a structural interaction between μ and δ receptors in the mouse vas deferens.

INTRODUCTION

Much effort has been expended in demonstrating the existence of multiple types of opioid receptor (Martin et al. 1976, Lord et al. 1977). There is, nevertheless, a growing body of evidence for interaction between the types, particularly μ and δ receptors in vitro (Rothman and Westfall 1981) and in vivo (Holaday and D'Amato 1983). It has even been suggested that the two receptors may be interconvertible forms of a single macromolecule (Bowen et al. 1981). In this study we have taken advantage of the presence in the mouse vas deferens of all three established types of opioid receptor (μ, δ and k : Lord et al. 1977; Miller and Shaw 1984) to-attempt to selectively protect one opioid receptor type from inactivation while irreversibly blocking the remainder. The irreversible antagonist used was β-chlornaltrexamine (β-CNA), an alkylating agent selective for opioid receptors but which does not discriminate between the three types (Ward et al. 1982). The protecting ligands used were the highly selective δ-receptor antagonist ICI 174864 (Cotton et al. 1984) and the selective μ-agonist, [D-Ala^2, MePhe^4, Gly(ol)^5] enkephalin (DAGO; Corbett et al. 1984). The results are interpreted as supporting an interaction between μ and δ receptors in this tissue.

MATERIALS AND METHODS

Vasa deferentia from mature Glaxo-bred C57/BL mice were mounted and field-stimulated as described in Hayes et al. (1985). Cumulative concentration-response curves were constructed in random order for the μ-selective agonist DAGO, the k-selective agonist U50488 (trans-(±)-3, 4-dichloro-N-methyl-[2-(1-pyroll-

idinyl) cyclohexyl] benzene acetamide methanesulphonate), and a δ-selective a onist, [D-Pen^2, L-Pen^5] enkephalin (DPLPE) or [D-Pen^2, D-Pen^5] enkephalin (DPDPE). Tissues being treated with irreversible antagonist were incubated with β-CNA for 15 min, after which any non-covalently bound antagonist was removed by extensive washing at 5 min intervals for 90 min. For protection experiments, the protecting ligand was incubated with the tissue for 10 min (DAGO) or 30 min (ICI 174864) before and during the β-CNA treatment. Control experiments showed that the 90 min washout period was also enough to remove any remaining ICI 174864. Concentration-response curves for each agonist were re-determined in the treated tissues and, where a parallel shift was obtained, a dose-ratio was calculated.

DPLPE and DPDPE were supplied by Peninsula and DAGO by Cambridge Research Biochemicals. U50488 was a gift from Dr. P.F. von Voigtlander of the Upjohn Company, ICI 174864 was a gift from Dr. R. Cotton of ICI. β-CNA was synthesised by Dr. A. McElroy of the Chemical Research Dept., Glaxo Group Research Ltd.

RESULTS

In the absence of any protecting ligand, β-CNA (10^{-8}M - 10^{-7}M, 15 min) caused a concentration-dependent antagonism of the responses to DAGO, DPLPE and U50488 which was not significantly reversed by 3hr of extensive washing of the tissues. The receptor reserve for each agonist can be estimated by the extent to which an irreversible antagonist can produce a parallel rightward shift of the concentration-response curve without diminution of the maximum available response (Furchgott 1966); these shifts were about 30-fold for DAGO and 10-fold for DPLPE, while a parallel shift for U50488 was rarely obtained.

Pre-incubation with the selective δ antagonist ICI 174864 (10^{-7}-$5x10^{-5}$M) protected the response to DPLPE in a concentration-dependent manner, but also protected the response to DAGO to a similar extent (table 1). There was no significant protection of the response to U50488 at any but the highest concentration of ICI 174864 (data not shown).

Similar experiments were performed using DAGO (10^{-6}-$3x10^{-5}$M) to protect μ-opioid receptors. As before, there was little protection of the response to U50488 (data not shown) but their responses to DAGO and DPDPE were protected to a similar extent (table 2).

In order to investigate whether there was an interaction between the agonist effects mediated by μ and δ receptors, concentration-response curves were constructed to one agonist in the presence of low concentrations of the other.

The concentrations used were either just sub-threshold, causing no inhibition of twitch by itself, or a concentration causing about 10% inhibition of twitch. When tested thus, DAGO had no significant effect on the concentration-response curve to DPDPE, and was itself unaffected by low concentrations of DPDPE.

TABLE 1 Protection against ß--CNA by ICI 174864 in the mouse vas deferens

The values given are the dose ratios (mean ± S.E.M., n = 4-8) for the parallel rightward shifts in the concentration-response curves for the μ-selective agonist, DAGO, and the δ· selective agonist, DPLPE. Below it is given the ratio of the μ-shift/δ--shift. The larger this figure, the greater the selective protection of d-receptors. NT, not tested.

Conc. of ß-CNA(M)	Protecting concentration of ICI 174864 (M)							
	$1x10^{-7}$		$3x10^{-7}$		$1x10^{-6}$		$5x10^{-6}$	
	μ	δ	μ	δ	μ	δ	μ	δ
$1x10^{-8}$	NT		NT		4.2 ±1.3 ratio= 3.8	1.1 ±0.2	3.8 ±1.0 ratio	0.5 ±0.2 7.6
$3x10^{-8}$	33.7 ±7.2 ratio=	14.9 ±3.3 2.3	3.9 ±0.6 ratio=	1.2 ±0.3 3.4	5.3 +1.2 ratio=	1.2 ±0.4 4.4	3.5 +1.3 ratio=	1.5 ±0.4 2.3
$1x10^{-7}$	116 ±32 ratio=	613 ±150 0.2	25 ±6.5 ratio=	9.6 ±1.9 2.6	3.7 ±0.9 ratio=	2.8 +1.5 1.3	NT	

TABLE 2 Protection against ß·-CNA by DAGO in the mouse vas deferens

Details are as in the legend to Table 1. In this context the ratio of the μ-shift/δ-shift is smaller for greater degrees of μ-receptor protection.

Conc. of ß-CNA(M)	Protecting concentration of DAGO (M)							
	$1x10^{-6}$		$3x10^{-6}$		$1x10^{-5}$		$5x10^{-5}$	
	μ	δ	μ	δ	μ	δ	μ	δ
$1x10^{-8}$	3.5 0.5 ratio=	2.3 ±0.7 1.5	5.4 +2.3 ratio=	3.6 ±1.1 1.5	6.4 +3.1 ratio	3.6 ±0.9 1.8	0.7 ±0.2 ratio=	1.3 ±0.3 0.5
$3x10^{-8}$	NT		27.4 ±4.5 ratio=	7.6 ±2.0 3.6	27.3 ±8.1 ratio=	35.9 ±19.0 0.8	NT	
$1x10^{-7}$	NT		41.4 ±11.8 ratio=	12.0 ±7.1 3.5	87.9 ±7.4 ratio=	153 +49.8 0.6	NT	

DISCUSSION

The δ·-receptor antagonist ICI 174864 was able to protect the response to DPLPE from blockade by ß-CNA in the C57/BL mouse vas deferens, but it also protected the response to DAGO to a similar

extent, even at concentrations which occupy only a small proportion of μ-receptors (Ki at δ-receptors = 30nM and at μ-receptors > 5000nM : Cotton et al. 1984). It thus appears that occupation of δ-receptors by ICI 174864 can make μ-receptors inaccessible to B-CNA. Similarly, DAGO was able to protect its own agonist effect from β-CNA but often produced an even greater degree of protection of the response to DPDPE. The K_i value for DAGO in displacing [^{3}H]-naloxone from μ-receptors in a physiological medium is 1300 nM (calculated from Carroll et al. 1984); the comparable value at δ-receptors has not been reported but the binding selectivity of DAGO for μ-over δ-receptors in Tris buffer is 185-fold (Corbett et al. 1984). Ward et al. (1982) selectively protected δ-receptors from B-CNA in the mouse vas deferens using an agonist, [D-Ser^2, Leu^5] enkephalinyl-Thr, as protecting ligand. Our own studies using this ligand did not improve upon the low degree of selective δ-receptor protection seen with some of the treatments shown in table 1 (unpublished observations).

Our results suggest that there may be a structural interaction between μ- and δ-opioid receptors in the C57/BL mouse vas deferens, but not between k-opioid receptors and either of the other types. However, since there was no synergism or antagonism between DAGO and DPDPE in this tissue, this putative interaction does not appear to alter the transduction of the agonist effects mediated by μ and δ receptors.

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AFFINITY CROSSLINKING OF ^{125}I-HUMAN BETA-ENDORPHIN TO CELL LINES POSSESSING EITHER MU OR DELTA TYPE OPIOID BINDING SITES

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ABSTRACT

Affinity crosslinking of human ^{125}I-β-Endorphin to cell lines possessing either mu or delta binding sites was carried out. Autoradiography of SDS-PAGE gels from these crosslinked cell lines revealed that these two sites contain major peptide subunits that differ in molecular size. This confirms our earlier finding in mammalian brain which demonstrated separate and distinct subunits for mu and delta opioid receptors.

INTRODUCTION

To contribute to our understanding of the molecular basis of opioid receptor heterogeneity our laboratory has used affinity crosslinking of iodinated human beta-endorphin (^{125}I-β-End$_H$) to membrane preparations. In previous studies (Howard et al. 1985) two major peptide bands were identified by autoradiography of SDS-PAGE gels namely a 65 kDa peptide which displayed mu characteristics and a 53 kDa peptide which displayed delta characteristics. The assignment of mu or delta characteristics was based on differential displacement by the highly mu-selective ligand Tyr-D-DAla-Gly-MePhe-Gly-ol(DAGO) or by the highly delta- selective ligand [D-Pen^2-D-Pen^5]-enkephalin (DPDPE) and on the distribution and levels of the 65 kDa and 53 kDa peptides in membranes having various mu:delta ratios (Howard et al. 1985, 1986).

In order to further support these findings, we have studied the specific labeling pattern of opioid receptor-related peptides in two cell lines, NG-108-15 which contains exclusively delta binding sites (Klee and Nirenberg 1974) and SK-N-SH which contains mainly mu binding sites (Yu et al. 1986).

MATERIALS AND METHODS

The SK-N-SH cell line was a gift of Dr. W. Sadee (University of California, San Francisco). The NG-108-15 cell line was a gift of Dr. A.J. Blume (Hoffman-La Roche). ^{125}I-β-End$_H$ (2,000 Ci/mmol) was purchased from Amersham and ^{3}H-bremazocine (30 Ci/mmol) was obtained from NEN. Bis [2-(succinimidooxycarbonyloxy)ethyl]sulfone (BOSCOES) was from Pierce Chemical Corp, electrophoresis chemicals were from Bio-Rad.

Cell cultures - SK-N-SH cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 40 U/ml penicillin and 40 U/ml streptomycin. NG-108-15 hybrid cells were grown in Dulbecco' s modified Eagle's medium (DMEM) containing HAT (0.1 mM hypoxanthine, 1 uM aminopterin, 16 uM thymidine), supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 40 U/ml penicillin and 40 U/ml streptomycin.

Membrane preoaration At confluency the SK-N-SH cells were washed and harvested by mechanical agitation in a Ca++ Mg^{++}-free phosphate buffered saline containing 0.04% EDTA. After 10 min centrifugation at 1,000 x g, the cells were homogenized in 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA. The homogenate was spun at 20,000 x g for 20 min at 4°C and the resulting pellet was resuspended in 0.32 M sucrose and stored at -70°C. At confluency, the NG-108-15 cells were washed and then harvested by mechanical agitation in DMEM medium. After centrifugation at 1,000 x g for 10 min, the cell pellet was washed with 25 mM Tris-HCl buffer containing 290 mM sucrose, 1 mg/ml BSA and 0.1 mg/ml bacitracin and stored at -70°C. Membranes were prepared, as described above, freshly before each experiment, in K_2HP0_4 buffer pH 7.4 containing 1 mM EDTA, 0.2 mM PMSF, 10 ug/ml leu-peptin and 50 ug/ml bacitracin.

Affinity crosslinkinq - Membranes prepared from both cell lines were diluted 4 fold (approx. 0.5 mg/ml) in K_2HP0_4 buffer pH 7.4 containing protease inhibitors (see above). ^{125}I-β- End_H (1 nM) was bound and crosslinked to the membranes by the crosslinking reagent BOSCOES (1 mM) as dessolubilized membranes were subjected to SDS-polyacrylamide by Howard et al. (1985). The crosslinked gel electrophoresis (PAGE) and autoradiography of the gels was performed [Howard et al. 1985).

RESULTS

Yu et al. (1986) have shorn by saturation binding experiments that the human neuroblastoma cell line SK-N-SH contains mainly mu opioid binding sites (mu:delta ratio 5:l). Similar results were obtained by us in competition experiments with the mu-selective ligand DAGO and the delta-selective ligand, DPDPE against ^{3}H-bremazocine. When crosslinking of ^{125}I-β-End_H to membranes prepared from SK-N-SH cells was performed, 3 major bands were visible after gel-electrophoresis of the solubilized membranes and autoradiography of the gel, a large peptide band which corresponds to molecular weight 65 kDa and two narrow bands of 92 kDa and 25 kDa (Fig. 1).

All three peptide bands are opioid receptor related since they are eliminated when binding of ^{125}I-β-End_H to the membranes is conducted in the presence of 3 uM naloxone.

Crosslinking of ^{125}I-β-End$_H$ to membranes prepared from NG-108-15 cells, which contain exclusively delta opioid binding sites, revealed a different labeling pattern of peptides. Two major peptide-bands were observed, corresponding to molecular weights of 53 and 25 kDa (fig.1). Both peptides are opioid receptor-related, as their labeling was reduced when binding of ^{125}I-β-End$_H$ was conducted in the presence of 3 uM naloxone.

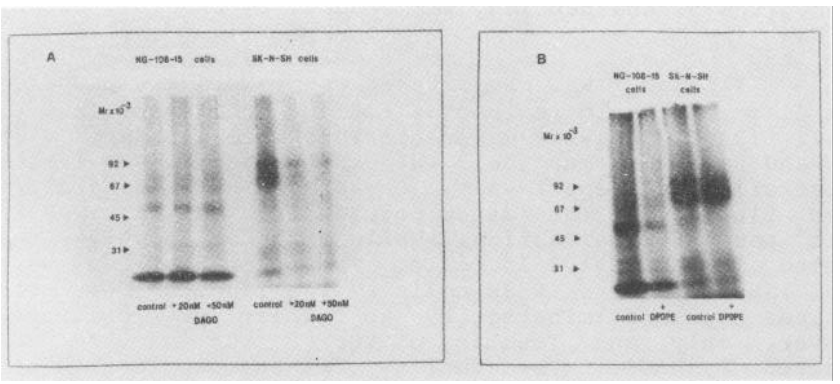

FIGURE 1. SDS-PAGE of NG-108-15 or SK-N-SH cell membranes crosslinked with ^{125}I-β-End$_H$. A. Binding of 125I- -End$_H$ was carried out in the absence or presence of DAGO (20 nM, 50 nM). B. Binding of 125I-β-End$_H$ was carried out in the absence or presence of 20 nM DPDPE.

In order to label selectively peptides containing mu or delta opioid binding-sites, membranes prepared from both cell lines were bound with ^{125}I-β-End$_H$ in the presence of 20 nM and 50 nM of the mu-selective ligand DPDPE. Fig. 1A presents the labeling patterns observed when binding of ^{125}I-β-End$_H$ was carried out in the presence of DAGO. While DAGO, up to a concentration of 50 nM, had no effect on the labeling of the peptides derived from the NG-108-15 cells, labeling of the 65 kDa peptide, and to a lesser extent, the 92 kDa peptide, derived from SK-N-SH cells was reduced. Fig. 1B presents the labeling patterns observed when binding of ^{125}I-β-End$_H$ was performed in membrane preparations from both cell lines in the presence of DPDPE. As shown, DPDPE displaced the labeling of both 53 kDa and 25 kDa peptides, derived from NG-108-15 cells, while it had no effect on the labeling of peptides derived from SK-N-SH cells.

DISCUSSION

The labeling patterns observed after crosslinking of these two cell lines with ^{125}I-β-End$_H$ were different. In NG-108-15 cells, two major peptides were labeled, corresponding to molecular weights of 53 kDa and 25 kDa. Both peptides displayed delta characteristics, i.e., their labeling was reduced by DPDPE but not by DAGO. In SK-N-SH cells, three major peptides were labeled by ^{125}I-β-End$_H$ corresponding to molecular weights of 92, 65 and 25 kDa: The labeling of the 65 kDa peptide, and to a lesser extent, the 92 kDa peptide, was reduced by DAGO but not by DPDPE.

When ^{125}I-β-End$_H$ was crosslinked to membranes prepared from different brain regions of various species by Howard et al. (1985,1986), 2 major peptides were labeled, a 65 kDa and a 53 kDa peptide, which displayed mu and delta characteristics respectively. Our present results confirm these findings. The major peptide labeled in SK-N-SH cells was a 65 kDa peptide, while a 53 kDa peptide was labeled in the NG-108-15 cells. Two other peptides were also labeled in SK-N-SH cells. The labeling of both peptides was eliminated by an excess of naloxone. However, only the labeling of the 92 kDa peptide was affected by DAGO. The identity of these peptides is not clear. The 92 kDa peptide may represent a lower-affinity binding site for mu-selective ligands. In agreement with previous results (Howard et al. 1985), in NG-108-15 cells a 25 kDa peptide was labeled in addition to the 53 kDa peptide. Preliminary results suggest that this peptide may be a breakdown product of the 53 kDa peptide, but more experiments are needed to confirm this finding.

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UPREGULATION OF THE MU-NONCOMPETITIVE DELTA BINDING SITE BY CHRONIC MORPHINE ADMINISTRATION: EFFECT OF PREINCUBATING MEMBRANES IN 400 nM SODIUM CHLORIDE

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ABSTRACT

Rats treated chronically with morphine were found to have an increased number of mu-noncompetitive delta binding sites. The increase in the B_{max} was 39% and the enhancement was blocked hy pre-incubation in 50 mM Tris. pH 7.4 with 400 mM NaCl for one hour at 25°C. These findings are' consistent with the removal of newly manifested binding sites being a specific property of sodium, by analogy with previous studies where sodium has been shown to extract extrinsic proteins from red cell ghosts.

INTRODUCTION

We have previously shown that mu-noncompetitive delta binding sites were upregulated after chronic morphine treatment (Rothman et al. 1986). These results were obtained in membranes that were not pre-incubated in high concentrations of sodium, a technique reported by Pasternak et al. (1975) to remove any endogenous ligand bound to the opiate receptor.

The present studies were performed to investigate the possible effect of pre-incubation, in 50 mM Tris, pH 7.4 with 400 mM NaCl for one hour at 25°C, on the binding parameters of the two delta binding sites in rats treated chronically with morphine. In these studies pretreatment with the site-directed acylating agents, BIT (1-[2-(diethylamino)-ethyl]-2-(4-ethoxybenzyl)-5-isothiocyanato--benzi-midazole) and FIT (N-[1-[2(4-isothiocyanatophenyl]-4-piperi-dinyl]-N-phenyl-propanamide) were used to enrich membranes with the mu-competitive or mu-noncompetitive delta binding sites respectively (Rothman et al. 1984).

MATERIAL AND METHODS

Male Sprague-Dawley rats (200-250 g; Zivic-Miller Labs., Allison Park, PA) were acclimatized for six days. They were then implanted with 2 morphine pellets (75 mg) on day 1 and 4 morphine pellets on day 2 (Holaday et al. 1982). The rats were rapidly decapitated on day 5, exactly 96 hours after the first implantation. A control group of rats were implanted with placebo pellets with precisely the same protocol.

A lysed mitochondrial P_2 membrane preparation was made, by differential centrifugation, using the whole rat brain (minus cerebellum) following a modified method of Gray and Whittaker (1962).

The mitochondrial pellet was split into two aliquots; one aliquot was incubated in 50 mM Tris, pH 7.4 with 400 mM NaCl for one hour at 25°C, while the other was kept on ice. The pre-incubated pellet was centrifuged at 4825 xg for 15 minutes and the pellet resuspended in ice-cold Tris, pH 7.97. This washing step (resuspension and centrifugation) was repeated three times, in total, to ensure that all sodium ions had been removed. After this point both aliquots were treated identically.

The pellets were then treated with the acylating agents, BIT and FIT (1 µM) for one hour at 25°C in 0.01 M MOPS (3-(N-Morpholino)propanesulphonic acid) and 3 mM $MnCl_2$. The pellets were centrifuged and resuspended, as they were after the sodium pre-incubation. The final pellet was resuspended in 0.75 ml/g original weight of Tris, pH 7.97.

Tritiated D-ala^2-D-leu^5-enkephalin was used as the ligand in the binding assays. The incubation medium contained 10 mM Tris, pH 7.4, 100 mM choline chloride and 3 mM $MnC1_2$. [^{3}H]DADL was added to the assay in a protease inhibitor cocktail, which consisted of bacitracin (0.1 mg/ml), Leupeptin (0.004 mg/ml), chymostatin (0.002 mg/ml), bestatin (0.01 mg/ml), Captopril (1 µg/el), 2-mercaptoethanol (1 mM) and phosphoramidone (1 pM).

'Binding surfaces' were used (Rothman et al. 1985), to describe the binding sites. For both washed and control membranes, complete saturation curves (0.25 nM to 95.6 nM) were constructed using isotopic dilution; the K_d and B_{max} were determined using computer curve fitting methods.

RESULTS

The results set out in table 1 are from studies carried out in membranes that had been pre-incubated in 50 mM Tris, pH 7.4 with 400 mM NaCl at 25°C for one hour. It should be noted that there is no increase in the B_{max} in either of the delta binding sites when rats receive chronic morphine treated (n=3), although there were significant increases in the dissociation constants of both the mu-noncompetitive delta binding sites.

TABLE 1: The effect of Morphine tolerance on the two [^{3}H]DADL. binding sites in membranes that have been pre-incubated with 50 mM Tris, pH 7.4 and 400 mM NaCl for 1 hr at 25°C.

		$K_d \pm$ SD (Nm)	$B_{max} \pm$ SD (fmol/mg prot)
MU-COMPETITIVE			
(BIT-treated)	Placebo	0.74±(0.08	72±‘4
	Tolerant	1.02±(0.09	72±‘4
	% of Control	158*	100
MU-NONCOMPETITIVE			
(FIT-treated)	Placebo	4.3±(0.2	86±,7
	Tolerant	6.8±(0.4	97±14
	% of Control	158*	113

*P <0.05, n=3

The results of the studies with membranes that were not greincubated in 50 mM Tris, pH 7.4 with 400 mM NaCl for one hour at 25°C are shown in table 2. The B_{max} of the mu-noncompetitive delta binding sites in morphine pre-treated rats was significantly elevated (39%) above the controls. In contrast, the B_{max} of the mu-competitive delta binding site was not significantly altered. The dissociation constants of both the mu-competitive and the mu-noncompetitive delta binding sites were significantly increased (52% and 111%, respectively).

TABLE 2: The effect of chronic Morphine treatment on the two [^{3}H]DADL binding sites in membranes that have not been pre-incubated with 50 mM Tris, pH 7.4 and 400 mM NaCl for 1 hr at 25 C.

		$K_d \pm$ SD (nM)	$B_{max} \pm$ SD (fmol/mg prot)
MU-COMPETITIVE			
(BIT-treated)	Placebo	0.92±0.08	64±4
	Tolerant	1.4±0.1	55±5
	% of Control	152*	86
MU-NONCOMPETITIVE			
(FIT-treated)	Placebo	3.6±0.2	76±5
	Tolerant	7.6±0.4	106±11
	% of Control	211*	139"

*P <0.05, n=3

DISCUSSION

The findings of this study demonstrate that our regime of chronic morphine administration results in a significant increase in the number of mu-noncompetitive delta binding sites, an increase which is not seen when brain membranes are pre-incubated in 50 mM Tris, pH 7.4 with 400 mM NaCl for one hour at 25°C. In contrast, the elevation in the dissociation constant for both delta binding sites was present, regardless of whether or not the membranes were pre-incubated.

Our previous work (Rothman et al. 1986) indicated there was an increase in the number of mu-noncompetitive binding sites after-chronic morphine treatment compared with rats not so treated. While earlier studies (Klee and Streaty 1974) failed to find any significant alteration in opioid binding parameters following chronic morphine treatment, these studies used mu-selective radioligands, and therefore the findings primarily concern the mu binding sites. There are also reports in which [^{3}H]DADL, the specific delta ligand, was used in studies on membranes from chronically morphinized animals (Holaday et al. 1982). These authors found that there was an increase in the number of delta binding sites.

The experimental finding that pre-incubation in high sodium for one hour was not followed by an increase in the B_{max} of the mu-noncompetitive delta binding site appears worthy of discussion. Since the report by Pasternak et al. (1975) that pre-incubation of membranes in sodium removed endogenous ligand bound to the opiate

receptor, this step has become an integral part of the preparation of brain membranes for radioligand assay, despite the fact that the range of radioligands have increased. The decrease in the number of binding sites with pre-incubation may be explained by the finding that the sodium ion reportedly extracts extrinsic membrane proteins as has been shown for red cell ghosts (Capaldi 1972). This effect of pre-incubation in high sodium upon delta opiate binding sites will thus be investigated further, using appropriate biochemical techniques. It is possible that these sodium-labile binding sites may be different biochemically from those unaffected by the sodium preincubation.

In summary, our findings are that chronic morphine treatment upregulates the mu-noncompetitive delta binding site, and that this upregulation can be blocked by pre-incubation in a high sodium medium.

ACKNOWLEDGEMENT

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INTERACTION BETWEEN HIGHLY SELECTIVE MU AND DELTA OPIOIDS IN VIVO AT THE RAT SPINAL CORD

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ABSTRACT

Possible interactions between μ- and δ-receptors in the rat spinal cord were studied using the radiant-heat-induced tail flick response and the highly selective μ- or δ- ligands: [NMePhe^3,D-Pro^4] morphiceptin(PL-17) and cyclic[D-Pen^2,D-Pen^5]enkephalin(DPDPE). Intrathecal infusions of PL-17(0.5μg/hr) for 5 days caused 61- and 6-fold shifts to the right of the dose-response curves for PL-17 and DPDPE, respectively. Since PL-17 and DPDPE are highly selective agonists for μ- and δ-receptors, the partial cross-tolerance to the δ-agonist induced by PL-17 is probably not resultant from a cross-reactivity between these ligands. These data suggest that there may be an interaction between μ- and δ-receptors in the rat spinal cord.

INTRODUCTION

Several opioid receptor types and subtypes as well as their endogenous opioid peptide ligands comprise complex pharmacologic systems (Chang 1986). Despite the heterogeneity of these opioid systems and a wide spectrum of biologic activities ascribed to them, clinical interactions with opioid receptors are predominantly for analgesia. The speculated roles of the endorphins in a variety of physiologic and disease states are becoming more easily testable with the development of highly receptor-specific ligands.

Such ligands are also powerful tools for examining the biochemical nature of opioid receptor systems. For example, interactions between opioid receptor types have recently been suggested by data from D'Amato and Holaday (1984) and Rothman et al. (1985). Such interactions, if present, may complicate the pharmacology of opioid agonists' cross-reactivities. In order to relate the issues of tolerance and cross-tolerance to the concept of μ- δ receptor interactions we used two highly specific μ- or δ-agonists: PL-17 (Chang et al. 1983) and DPDPE (Mosberg et al. 1983).

This study examines the cross-reactivity between these two highly receptor-selective opioids by employing an assay of spinal analgesic activity and a paradigm for inducing chronic opioid tolerance at the rat spinal cord level. The μ- versus δ-selectivity of our ligands has allowed us to speculate regarding the mechanisms of ligand cross-reactivities.

MATERIALS AND METHODS

Materials

The following ligands were used: [N-Methyl-Phe^3,D-Pro^4]morphiceptin (PL-17), a highly specific μ-agonist (Chang et al. 1983); cyclic [D-Penicillamine^2, D-Penicillamine^5]enkephalin(DPDPE), a highly specific δ- agonist (Mosberg et al. 1983); β- funaltrexamine(R-FNA), an irreversible μ-antagonist (Takemori et al. 1981) and naloxone HCl, a competitive, relatively selective μ-antagonist. Ligands were prepared in normal saline and diluted such that bolus doses equalled twenty microliters.

Methods

400 gram male Sprague-Dawley rats had two polyethylene lo-gauge catheters passed to the lumbar enlargement via the atlanto-occipital membrane, following the technique of Yaksh and Rudy (1976). The nociceptive assay was the tail-flick response to a radiant-heat source beneath the tail (D'Armour and Smith 1941). The mean baseline latency response was 4 seconds: the cut-off time was 10 seconds. Each rat was tested 4-6 times over 6 minutes--beginning 30 minutes after intrathecal test doses. Tail-flick data were converted to maximum percent effects (MPE): (latency-response - baseline/cut-off - baseline) x 100. Responses of each rat were averaged and a mean MPE was calculated based upon N=6-8 rats at each dose.

Spinal opioid tolerances were induced by infusing PL-17 (0.5 μg/μl) intrathecally at 1 μl/hr for 5 days using subcutaneously implanted osmotic pumps. Tolerance assessments were performed using the second catheter while pumps were still attached. The dose-response curve for PL-17 following a 5-day infusion of normal saline was not shifted from the baseline curve for PL-17 (Figure 1). Scarcity of materials precluded a similar vehicle-infusion control for DPDPE.

ED_{50}'s were calculated from the linear regression equations. Dose-response curve shifts are reported as the ratios of after-to-before treatment ED_{50} values. ED_{50}'s whose 95% C.I.'s do not overlap are significantly different at the $P<.05$ level. Tests of the null hypothesis, H_o=homogeneity of slopes, (conducted at $P<.05$) for before- and after-treatment dose-response curves indicated that all dose-response curve shifts were parallel.

RESULTS

Both PL-17 and DPDPE given as acute lumbar-intrathecal bolus doses elicited antinociceptive effects. However, DPDPE was much less potent and exhibited a flatter dose-response curve than PL-17 (Figure 1). The ED,, values were 0.06 μg (95% C.I., 0.05-0.07) and 3.56 μg (2.64-4.90) for PL-17 and DPDPE, respectively. Their μ- and δ- receptor selectivities were confirmed by the relative potencies of naloxone or β- FNA in antagonizing their analgesic effects. The doses of i.p. naloxone required to reduce the effects of an ED,, dose of PL-17 or DPDPE to 50 percent effectiveness were 0.09 (0.08-0.11) and 2.71 (1.81-4.34) mg/kg, respectively, This difference is about 30-fold.

Sixty hours following a 2.3 µg intrathecal β-FNA pretreatment (Figure 1) the dose-response curve for PL-17 was shifted parallel and to the right 25-fold. PL-17-ED_{50} values before and after β-FNA were 0.06 µg (0.05-0.07) and 1.50 µg (1.09-1-98), respectively. β-FNA pretreatment had no significant effect upon the DPDPE dose-response curve. DPDPE-ED_{50} values before and after β-FNA were 3.56 µg (2.64-4.90) and 5.65 µg (4.04-8.64), respectively. These data suggest that PL-17 and DPDPE elicit antinociceptive effects through µ- and δ-receptors, respectively.

A chronic PL-17 infusion of 0.50 µg/hr for 5 days (Figure 2) effected a parallel and right shift of the PL-17 dose-response curve: ED_{50} after infusion, 3.65 µg (2.71-5.19)/ED_{50} before infusion, 0.06 µg (0.05-0.07)--a 60.8-fold parallel shift. Cross-tolerance to the highly selective δ-agonist DPDPE was smaller but significant at the P<.05 level: ED,, after infusion, 21.00 µg (17.88-26.57)/ED_{50} before infusion, 3.56 µg (2.64-4.90)--a 5.9-fold parallel shift.

DISCUSSION

The existence of some cross-tolerance between opioid agonists with differing receptor-selectivities may be explained by cross-reactivities of the compounds examined. However, the affinity of PL-17 to δ-receptors is 2000 times less than to µ-receptors (Chang et al. 1983). The hourly infusion dose of PL-17 in this experiment was only eight times a baseline PL-17 bolus-ED,, dose. It appears unlikely that the partial cross-tolerance to DPDPE induced by PL-17 resulted from cross-reactivity of PL-17 to δ- receptors. Furthermore, PL-17 has been shown to exert its activity via the µ-receptor even in a δ- receptor dominated tissue, the isolated mouse vas deferens (Chang et al. 1983). Since the affinity of DPDPE to δ- receptors is at least 300 times greater than to µ-receptors (Mosberg et al. 1983) and there is no β-FNA treatment effect upon DPDPE-induced analgesia and the potency of naloxone against DPDPE is 30-times lower than that against PL-17, it is unlikely that DPDPE interacts with µ-receptors at doses employed in this study.

These data suggest that the PL-17 infusion caused an interaction between µ- and δ- receptors resulting in a partial cross-tolerance to DPDPE. This finding supports similar µ-δ receptor interactions described in rat brain membrane receptor-binding studies by Rothman et al. (1985) and postulated for an in vivo system from endotoxic shock experiments in rats by D'Amato and Holaday (1984). The nature of this µ- δ receptor interaction remains to be explored. The suitability of spinal tissues for use in biochemical studies and the in vivo nature of this model offer significant opportunities for correlating the cellular biology of opioid receptor systems with physiologic parameters.

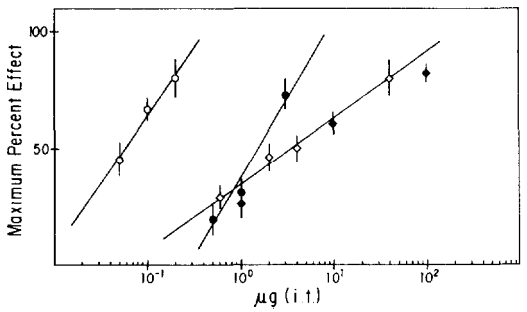

FIGURE 1. Analgesic dose-response curves for PL-17 (○) and DPDPE (◇). Depicted is the curve for PL-17 (●) and the data points for DPDPE (◆) 60 hours following μ-receptor antagonism with a 2.3μg i.t. bolus dose of β-FNA.

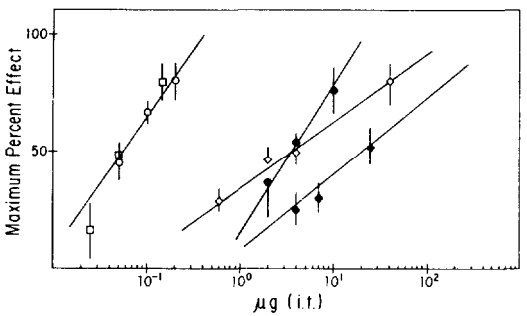

FIGURE 2. Analgesic dose-response curves: baseline curves for PL-17 (○) and DPDPE (◇). Depicted are the curves for PL-17 (●) and DPDPE (◆) following 5 day i.t. infusions of PL-17, 0.5 μg/hr and the data points for PL-17 following a vehicle-control infusion (□).

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OPIOID RECEPTOR ACTIVITY IN THE DENTATE REGION OF THE RAT HIPPOCAMPUS

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ABSTRACT

The electrophysiological actions of normorphine and dynorphin-A were compared in the dentate gyrus and CA1 regions of the rat hippocampus. In both regions, these opioids increased cell excitability and induced afterpotentials following electrical stimulation of synaptic afferents. Based upon apparent naloxone dissociation constants, we conclude that normorphine activated μ receptors in both regions. However, dynorphin-A appears to act via μ receptors in CA1, but κ receptors in the dentate gyrus.

INTRODUCTION

High concentrations of opioid peptides have been detected in the dentate region of the hippocampus, suggesting that this brain region is a likely site of endogenous opioid action (McGinty et al. 1983, Chavkin et al. 1985b). μ, δ, and κ binding sites have been detected throughout the hippocampus (Zamir et al. 1985), and opioid receptor mediated actions in the different hippocampal regions are beginning to be described. In CA1, opioids have been found to increase the excitability of pyramidal cells, probably by reducing the inhibitory effects of GABAergic interneurons (Zieglgansberger et al. 1979). The role of opioids in the dentate region was previously studied (Tielen et al. 1981; Linseman and Corrigall 1982). To further characterize the potential sites of endogenous opioid action, we have compared the receptor types mediating the effects of opioids in the CA1 and dentate regions of the rat hippocampus.

MATERIALS AND METHODS

Hippocampal slices (500 μm) were prepared from Sprague-Dawley rats (150-250g) using methods previously described (Siggins and Schubert 1981). Extracellularly recorded responses were evoked by stimulation of the dorsal blade of the dentate gyrus with a concentric bipolar electrode placed in the center of the outer molecular layer at least 400 μm away from the recording electrode and recorded in the stratum granulosum using glass microelectrodes (1-2 μm tip, 4-10 MΩ). Healthy responses in the dentate granule cell layer were typically 3-5 mV without afterpotentials even at supramaximal stimuli (figure 1A). CA1 pyramidal cell responses were similarly evoked and recorded as described (Chavkin et al. 1985a). Responses recorded in the cell body layers are likely to be due to the orthodromic activation of presynaptic inputs as the evoked responses were blocked by the addition of 10 mM $MgCl_2$ to the superfusion buffer (data not shown).

RESULTS AND DISCUSSION

Dynorphin-A(1-17) (DYN) and normorphine (NM) were applied to the tissue either by bath superfusion (0.01-10 µ) or by a pressure micropipette (100 µM in 10 mM HEPES buffered saline, pH 7.4) placed near the recording site. The effect of DYN delivered by pressure pulse in the dentate region is shown in figure 1B. DYN and NM had identical actions.

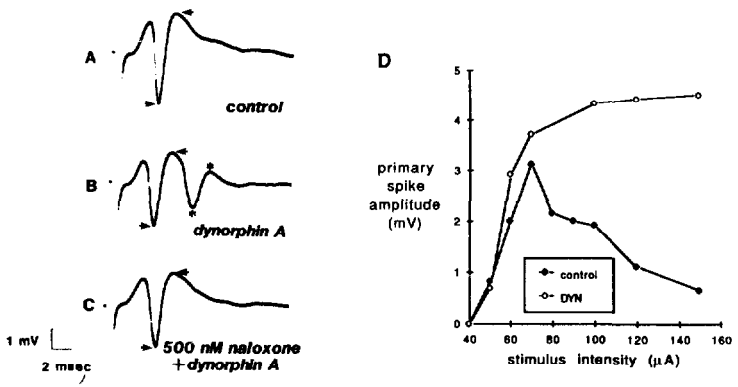

Figure 1. DYN increased cell excitability in the dentate granule cell layer. Each response in A, B, and C was evoked by a near maximal stimulus; arrows mark the primary spike and asterisks mark the afterpotential. A) control. B) 100 µM DYN was delivered by pressure pipette (80 msec, 275 KPa) 10 sec prior to electrical stimulation. C) DYN was delivered (as in B) in the presence of 500 nM NAL. D) stimulus-response curve of the primary population response before and during DYN exposure (1 sec, 275 KPa).

The response to opioids was similar but not identical in the CA1 and dentate regions. As has been previously reported, opioids induced both afterpotentials (APs) and a profound reduction in the threshold for electrically stimulated primary spikes in the CA1 region. In the dentate region opioids did not effect the threshold for primary spikes, but consistently induced APs (figure 1A-C). In CA1 only sigmoidally shaped stimulus-response (S/R) curves were observed; whereas, dentate S/R curves were either sigmoidal or biphasic, depending on the precise placement of the stimulating electrode. An example of a biphasic S/R curve is shown in figure 1D. When the dentate responses demonstrated biphaaic S/R curves, opioids induced a change from a biphasic to a sigmoidal shape (figure 1D). We presume that the biphasic response is due to the activation of inhibitory pathways at higher stimulation intensities. Differences in the nature of the opioid responses between the CA1 and dentate region are likely to reflect differences in synaptic circuitry.

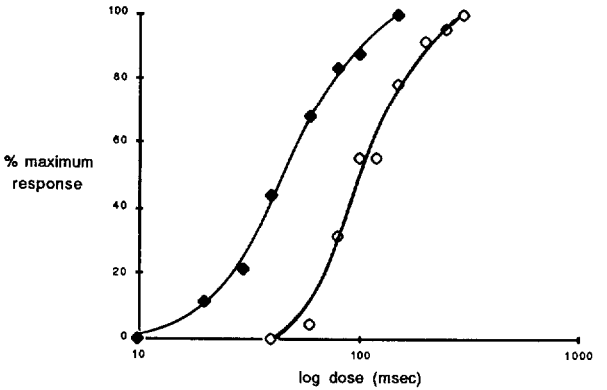

Figure 2. The amplitude of the afterpotential induced by DYN was measured in the dentate region. Log dose-response curve for DYN (●) (100 µM, 275 KPa) and DYN in the presence of 100 nM NAL (◇). In this example the NAL K_i value was 55 nM.

NAL (1 µM) completely blocked DYN and NM effects in both regions. Apparent equilibrium dissociation constants (K_i) for NAL were determined by Schild analysis at several NAL concentrations (Arunlakshana and Schild 1959). We used bath application of NAL (10-100 nM) while applying DYN or NM by pressure pipette (100 µM, 140-275 KPa) and measured the change in AP amplitudes. NAL caused a parallel shift in the DYN sensitivity (figure 2); DYN sensitivity returned to control levels following washout of NAL for 30-60 min. The NAL K_i value for NM in CA1 was 2.7 $\pm$ 0.3 nM (n=3) and 2.2 $\pm$ 1.6 nM (n=4) in the dentate region (figure 3). These values are not significantly different ($p>0.5$, Wilcoxon rank sum test), and suggest the presence of µ receptors in both regions (Lord et al. 1977). The NAL K_i value for DYN in CA1 was 5.2 $\pm$ 1.8 nM (n=3) and 32 $\pm$ 11 (n=4) in the dentate gyrus. The NAL K_i value for DYN in CA1 is not statistically different from the value for NM, suggesting that DYN may also act at µ receptors in CA1. This result is in agreement with our previous finding that ß-funaltrexamine, a µ-selective irreversible antagonist, inhibits NM and DYN but not DADLE action in CA1 (Chavkin et al. 1985a). In contrast, the NAL K_i value for DYN is significantly larger than the value for NM in the dentate region ($p<0.05$), suggesting that DYN acts at different receptors than NM in this region, presumably via κ receptors. Therefore, although DYN is active in both the CA1 and dentate regions, it appears to act via different receptors in these regions. Preliminary studies using the δ-selective peptides [D-Ser^2,Leu^5,Thr^6]enkephalin and [D-Pen^2,L-Pen^5]enkephalin show that δ receptors exist in both the CA1 and dentate region and cause very similar responses to those produced by NM and DYN.

The demonstration of opioid receptor mediated effects and the detection of endogenous opioids in the dentate region support the

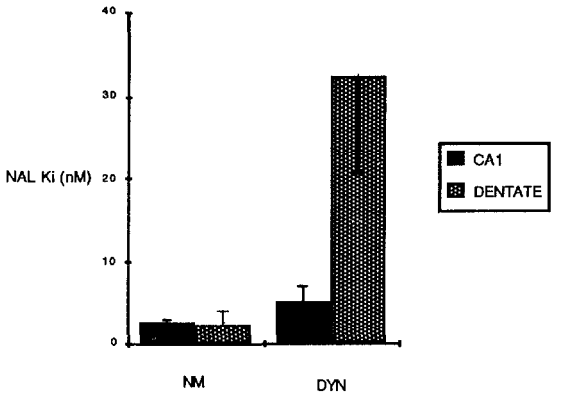

Figure 3. NAL K_i values for NM and DYN in the CA1 and dentate regions.

hypothesis that endogenous opioids may be neurotransmitters at this site. In addition, the presence of κ receptors suggests that dynorphin may be released locally in the dentate gyrus. The excitatory actions of opioids in the dentate gyrus imply that opioids act via presynaptic disinhibition as has been shown in the CA1 region, although the synaptic circuitry is likely to differ.

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DELTA AND KAPPA OPIOID RECEPTORS IN THE RABBIT EAR ARTERY

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ABSTRACT

Delta and kappa opioid receptors have been characterized in the central ear artery of the rabbit. The δ selective D-Pen^2,D-Pen^5-enkephalin and the highly selective δ antagonist ICI 174864 have been used for the δ opioid receptors. K_e values were determined and compared to that of the electrically stimulated mouse vas deferens preparation. In case of κ opioid receptors, Dynorphin (1-13) and Ethylketazocine were used as agonists and Mr 2266 as κ antagonist. Because of the relatively low selectivity of the κ antagonist (it is only twice as active at the κ than at the μ receptor sites) the K_e values obtained in the rabbit ear artery were compared to both the normal and beta-funaltrexamine treated vasa deferentia. In the rabbit ear artery, the K_2 values of Mr 2266 were also determined in the presence of the δ selective antagonist ICI 174864. The K_e values for the δ receptors were close in the two assay systems, suggesting that the δ receptors have similar properties in rabbit ear artery and mouse vas deferens. As for the κ receptors, the K_e values in the rabbit ear artery were not significantly different in the presence or absence of the δ antagonist compound, nor were they different from the K_e values obtained on the normal mouse vas deferens. However, they were significantly different from that of the β-funaltrexamine treated mouse vas deferens. Further experiments need to be done to interpret the meaning of these results.

INTRODUCTION

Since the introduction of the isolated central ear artery of the rabbit (De la Lande and Harvey 1965) it has been widely used as a pharmacological tool to study the perivascular sympathetic neurotransmission. After the discovery that some opioid peptides inhibit the electrically induced vasoconstriction of the rabbit ear artery (REA) (Knoll 1976) much work has been done to investigate the mode of action and characterize the opioid receptors present in this tissue (Illes et al. 1983, Illes et al. 1985, Illes and Bettermann 19861. It has been established that the postganglionic sympathetic neurons innervating the rabbit ear artery possess inhibitory presynaptic receptors of the δ and κ but not of the μ subtypes. In our present study we characterized the δ and κ opioid receptors with the help of selective agonists and antagonist and the results obtained were compared to that of the mouse vas deferens (MVD).

METHODS

Rabbit ear artery (REA). Male rabbits of 2.5-3 Kg weight were decapitated, and the proximal 3 to 4 cm of the central ear artery was dissected. The arteries were cleaned with the help of a dissecting microscope and cannulated at both ends. To prevent longitudinal displacement, both cannulae were tightly fixed. The vessels were perfused with Krebs solution at a steady rate of 2.4-2.6 ml/min by means of a multichannel peristaltic pump. The perfusion pressure was determined with a Statham P23 Db transducer and potentiometric recorder. The preparation was equilibrated for 60 min before the beginning of electrical stimulation. The platinum electrodes were placed at the top and the

bottom of the tissue-bath. A Grass S-88 electrostimulator was used for field stimulation. The parameters of stimulation were as follows: short trains (5 Hz, 5 shocks) of supramaximal rectangular pulses of 1 msec duration were delivered every minute. The composition of Krebs solution used was as follows (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; $MgSO_4$, 1.2 and glucose 11.5. It was saturated with 95% O_2 5% CO_2 and kept at 37°C. The agonists were added extraluminally into the tissue-bath, while the antagonists were present both extra and intraluminally. The tissue was incubated for at least 60 min with the antagonist.

Mouse vas deferens (MVD). Swiss Webster mice of 30-35 g weight were used. The vasa deferentia were prepared according to Hughes et al. (1975) and bathed at 31°C in Mg^{2+}-free Krebs solution, bubbled with a mixture of 02 and CO_2 (95:5). An initial tension of 100-150 mg was used. The experiments were carried out in an organ-bath of 10 ml capacity. The parameters of field stimulation were slightly modified (Ronai et al. 1977). Paired shocks with 100 msec delay between the supramaximal rectangular pulses of 1 msec duration were used, delivered at a rate of 0.1 Hz. Contractions were recorded using an isometric transducer (Statham UC2) and potentiometric recorder. In order to eliminate the μ receptors present in this preparation, the highly selective non-equilibrium μ antagonist, β-funaltrexamine (Takemori et al. 1981, Ward et al. 1.982) was added into the tissue-bath for 30 min in the concentration of 2×10^{-7} M.

In kinetic studies complete dose-response curves were determined in the absence and presence of antagonist. The antagonist activities were expressed in terms of Ke (dissociation equilibrium constant) values (Kosterlitz and Watt 1968). The antagonism was designated as competitive if it met the criteria proposed by Arunlakshana and Schild (1959).

RESULTS AND DISCUSSION

K_e values are listed in table 1 for different agonists with the selective δ opioid receptor antagonist ICI 174864 in rabbit ear artery and mouse vas deferens preparations. The results show that Leu-enkephalin and D-Pen^2, D-Pen^5-enkephalin (DPDPE) act on relatively homogenous receptor population in both systems and that the δ receptors are similar in these two tissues.

TABLE 1. Comparison in MVD and REA of K_e values for the δ selective antagonist, ICI 174864, using three different agonists

AGONISTS	MOUSE VAS DEFERENS K_e (nM)	RABBIT EAR ARTERY K_e (nM)
MET-ENKEPHALIN	43.3 ± 7.1 (16)	40.0 ± 12.0 (11)
LEU-ENKEPHALIN	17.5 ± 3.1 (9)	12.3 ± 2.5 (9)
DPDPE	17.0 ± 1.8 (10)	12.9 ± 3.5 (10)

To characterize the κ opioid receptors, K_e values were determined in "normal" and β-funaltrexamine (β-FNA) treated vasa deferentia for Dynorphin (1-13) and Ethylketazocine with the non-selective κ receptor antagonist Mr 2266. The significantly different K_e values (table 2) in the "normal" and "μ-less" MVD are in good agreement with results of binding studies (Kosterlitz et al. 1981, Paterson et al. 19831, which proved that Dynorphin (1-13) and Ethylketazocine as well as Mr 2286 partially act on μ receptor sites.

TABLE 2. Comparison of K_e values of κ opioid agonists with non-selective κ antagonist compound (Mr 2266) on "normal" and β-funaltrexamine treated mouse vas deferens preparations.

AGONISTS	K_e (nM) and Schild slope on "normal" MVD		on β-FNA MVD	
DYNORPHIN (1-13)	3.86 ± 0.4 (12)	-0.875	1.94 ± 0.2 (8)	-0.979
ETHYLKETAZOCINE	2.28 ± 0.1 (6)	-0.925	0.63 ± 0.1 (6)	-0.766

According to the results obtained in the β-FNA treated vasa deferentia, we expected similar data on REA, which is known to contain only δ and κ receptor subtypes. Surprisingly enough, the K_e values were (table 3) similar to the ones obtained in the "normal" MVD. To eliminate the possible interaction of the compounds with the δ receptors, the experiments were repeated in the presence of 2×10^{-7} M ICI 174864.

TABLE 3. Characterization of the κ opioid receptor in the rabbit ear artery with Mr 2266 in the presence and absence of ICI 174864.

AGONISTS	K_e(nM)	Schild slope	K_e (nM) (in the presence of 2×10^{-7}M ICI 1748641	Schild slope
DYNORPHIN (1-13)	3.88 ± 0.5 (8)	-0.98	3.49 ± 1.1 (6)	-1.08
ETHYLKETAZOCINE	2.91 ± 0.2 (3)	-0.97	2.62 ± 0.2 (5)	-1.02

Further experiments need to be done to understand and correctly interpret the data obtained for the κ1 receptors.

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A COMPARISON OF MICROSOMAL AND SYNAPTIC δ SITE BINDING KINETICS

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ABSTRACT

High-affinity binding of the agonist, [^{3}H]D-ala^2-D-leu^5-enkephalin (DADLE), to δ sites on bovine hippocampal synaptic plasma membranes (SPM) entails a multi-step association process. Microsomal binding sites, which are thought to originate from internal membranes (golgi, ser, etc.), display kinetic patterns that differ from SPMs. This is evidenced by the absence of an association time dependent rate of dissociation from microsomal binding sites. Although high affinity steady state binding of agonists to microsomes occurs, kinetic analysis indicates little or no formation of the high affinity slowly dissociating complex. This slowly dissociating complex of SPMs is most sensitive to guanine nucleotides. Consequently, the effect of Gpp(NH)p on dissociation is significantly less for microsomes.

INTRODUCTION

We had previously demonstrated that agonist binding to μ and δ opioid receptors displays a multi-step association while antagonists appear to undergo simple bimolecular association (Schiebe et al. 1984). It was determined that the dissociation rate for agonist binding is association time dependent, i.e., a high affinity slowly dissociating state is formed over time. The occurrence of high and low affinity states for agonist binding was later corroborated by others using different techniques (Law et al. 1985; Costa et al. 1985). We postulated that the high affinity state may be linked to some aspect of the signal-transduction functionality of the receptor such as receptor-effector coupling. The hypothesis might be tested if a preparation could be obtained which is composed of uncoupled receptors.

A body of evidence has accumulated to suggest that opioid receptors exist primarily in two subcellular pools; SPMs and microsomes (Pert et al. 1974; Simantov et al. 1976; Smith and Loh 1976; Glasel et al. 1980; Roth et al. 1981; Klein et al. 1986). Despite their early recognition, the functional significance of intracellular sites is still not completely clear. We had shown that the binding characteristics of opioid receptors on purified SPMs and microsomes differ (Roth et al. 1981; Roth and Coscia 1984) with microsomes being less sensitive to the effects of guanine nucleotides. These results suggest that microsomal sites may be only partially coupled to a guanine nucleotide binding protein. We now report an impaired formation of a high affinity agonist state by microsomal receptors. It is this slowly dissociating state which is most sensitive to guanine nucleotides in SPMs and consequently there is a decreased effect on microsomal dissociation rate.

METHODS

Subcellular fractions were prepared by sucrose density gradient as previously described (Roth et al. 1981). Rapid filtration was used to-quantify bound ligand while δ site selectivity for ^{3}H-DADLE was ensured by a 40 min pre-incubation of membranes with 20 nM d-Ala^{2}-mePhe^{4}-Glyo1^{5}-enkephalin prior to addition of radioligand (Spain et al. 1985). Dissociation was initiated by 1 µM DADLE.

RESULTS

As reported previously, the binding kinetics of ^{3}H-DADLE to the δ site in bovine hippocampal SPMs display characteristics which suggest a multi-step association (Schiebe et al. 1984). During the association process, there is an association time dependent formation of a high affinity slowly dissociating state. GTP and its analogs have been shown to increase the rate of agonist dissociation (Blume et al. 1978; Roth et al. 1981). The effect of guanine nucleotides appears to be limited to a transformation of the slowly dissociating state to a lower affinity state (fig. 1A). This is demonstrated by the profound effect of 50 µM Gpp(NH)p on the off-rate when added following a 60 min incubation (fig. 1A) while there is little effect on rate following a 7 min association (data not shown).

In contrast, the rate of ^{3}H-DADLE dissociation from δ receptors present on highly purified microsomes is not dependent upon association time (fig. 1B) suggesting a less complex association process. Although high affinity agonist binding to microsomes is still detected (K_D=0.86±0.09 nM), the formation of the high affinity slowly dissociating state occurs to a much lesser extent, resulting in a rate of dissociation which is similar to that seen from SPMs after a 7 min. association period. The addition of 50 µM Gpp(NH)p at the onset of dissociation from microsomes resulted in a modest acceleration of the off-rate (fig. 1B).

DISCUSSION

Past work had demonstrated that differences exist between the receptors of SPM and microsomal origin (Roth et al. 1981; Roth and Coscia 1984). In this study, we have found that the kinetics of agonist binding to microsomal receptors also differ from SPMs. An association time dependency for the rate of dissociation that is readily demonstrated for SPM receptors was not detected with microsomes. This suggests that the association process to the δ site of microsomes does not procede in the same multi-step manner which has been shown to occur to SPMs. In specific, the time dependent formation of the high affinity slowly dissociation state does not occur with microsomal receptors. Interestingly, in a previous study (Moudy et al. 1985) we suggested that crude membranes (polytron, P_{20}) contain vesicles which are a mixture of internal and synaptic sites. A single receptor type found in crude membranes can, therefore, be a heterologous population of receptors and result in confused analysis.

Several studies have reported a decreased formation of the high affinity state of opioid receptors in preparations which have become expermentally uncoupled from guanine nucleotide binding protein, N_i.

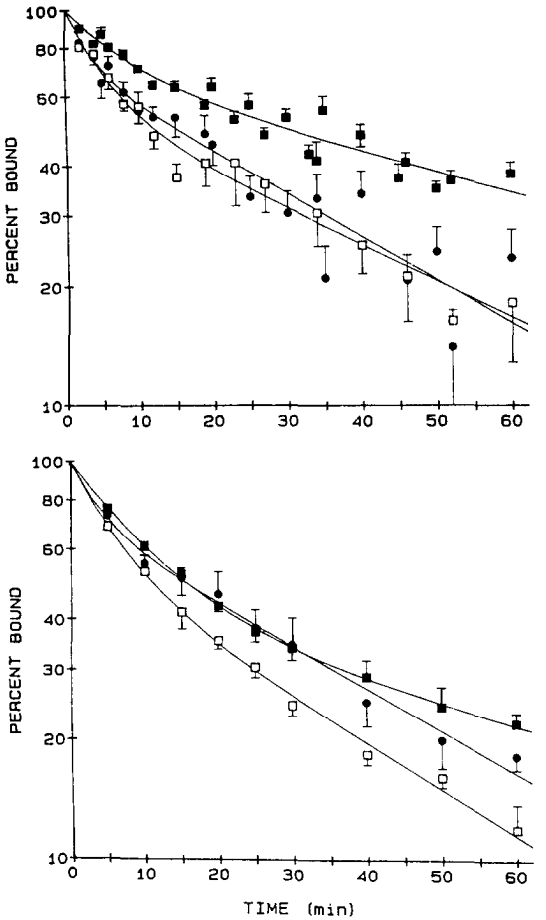

FIGURE 1. Dissociation of DADLE from SPMs and microsomes. (A) SPMs were incubated with 1 nM ^{3}H-DADLE for 7 min (●) or 60 min (■) prior to dissociation with 1 µM DADLE. Binding at 7 min was 46.0±2.6 % of the 60 min value (942±86 specific dpm/tube). (B) Microsomes were incubated with radioligand for 6 min (●) or 60 min (■) prior to dissociation with 1 µM DADLE. Binding at 6 min was 68.9±10.2 % of the steady state value (60 min, 652±100 specific dpm/tube). 50 µM Gpp(NH)p (□) was added to each preparation following a 60 min incubation. Each line is the best fit for a bi-exponential decay determine by non-linear regression.

Pertussis toxin (IAP) acts through ADP-ribosylation (Katada and Ui 1982) of Ni to uncouple receptors from the inhibition of adenylate cyclase. The uncoupling procedure can result in a δ receptor population which is much less able to form agonist high affinity binding (Hsia et al. 1984) as determined by displacement of antagonist. Similarly, desensitization by chronic agonist treatment can produce a uncoupled receptor population (Sharma et al. 1975; Blasig et al. 1979) and has been shown to decrease formation of the agonist high affinity state (Law et al. 1983).

The decreased effect of guanine nucleotides on binding to microsomes suggests that an interaction with a GTP binding protein is less effective in this preparation and is analogous to membranes treated with pertusis toxin or desensitized receptors. These results argue for the existence of at least a three step association for agonists to SPM δ opioid receptors whereas microsomes display one less step, i.e., there is no formation of the slowly dissociating high affinity complex which is most sensitive to guanine nucleotides. Studies are now in progress to examine this hypothesis further.

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MULTIPLE SITES OF DIVALENT CATION MODULATION OF δ OPIOID RECEPTOR BINDING

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ABSTRACT

Having established differential ontogeny for rat brain μ,δ, and κ opioid receptors (Spain et al. 1985) we have examined the effect of divalent cations on δ receptors during postnatal development. The pronounced inhibition of the binding of 1 nM [^{3}H]D-Ala^2-D-Leu^5-enkephalin (DADLE) by 100 mM Na was reversed by Mn to the levels seen in the absence of ions both in adults and in lo-day-old neonates. Mg^{++} and Ca^{++} on the other hand were much less potent in adults, and ineffectjve in neonates. These results suggest that, in the presence of Na^+, regulation of δ binding with Mg^{++} or Ca^{++} emerges subsequent to Mn^{++} modulation in postnatal development. Dose-dependency studies in adults performed with each ion in the presence of fixed concentrations of either of the other ions revealed that while Mg^{++} or Ca^{++} antagonized stimulation of δ binding by Mn^{++} and vice versa, Mg^{++} and Ca^{++} displayed no antagonism toward each other. Our data suggest the existence of at least two divalent cation regulatory sites for δ opioid receptors, one of which appears to be specific for Mn^{++} and can readily be detected in the presence of Na^+.

INTRODUCTION

Previous studies from our laboratory (Wohltmann et al. 1982; Spain et al. 1985) and from others (Coyle and Pert 1976; Leslie et al. 1982; Tsang et al. 1982) revealed that while high affinity μ and κ opioid receptors are present in rat brain at birth, δ receptors appear later. Using the bis-penicillamine enkephalin derivative, [^{3}H]DPDPE (thought to be highly δ specific, Mosberg et al. 1983) authentic δ binding sites in low levels were detected in the 5-day-old neonates (Szücs et al. 1986). In this report, divalent cation modulation of neonatal δ opioid receptors in the presence and absence of Na^+ is described and compared with that of adult rat brain. In adults, Na^+ inhibits opioid agonist binding and divalent cations (Me^{++}) reverse this effect (Pasternak et al. 1975; Simon et al. 1975; Simantov et al. 1976). Divalent cations are also known to play an important role in the physiological action of morphine (for a review see Chapmann and Way 1980). The results presented in this paper suggest the development of two divalent cation regulatory sites for the δ receptors.

METHODS

Rat forebrain membranes (20,000 g x 25 min) were prepared as previously described (Spain et al. 1985). Binding assays were erformed with 1 nM [^{3}H]DADLE (in the presence of 10 nM D-Ala^2-MePhe^{4}Glyo1^5-enkephalin) in a final volume of 1 ml for 1 hr at 25°C. Nonspecific binding was determined with 10 nM unlabeled DADLE and subtracted.

RESULTS AND DISCUSSION

Delta receptors present in 10-day-old neonates were slightly, but significantly, more inhibited (62±6% of control binding) by 100 mM Na^+ than those of adults (46±2%). figure 1. Manganese ion reversed

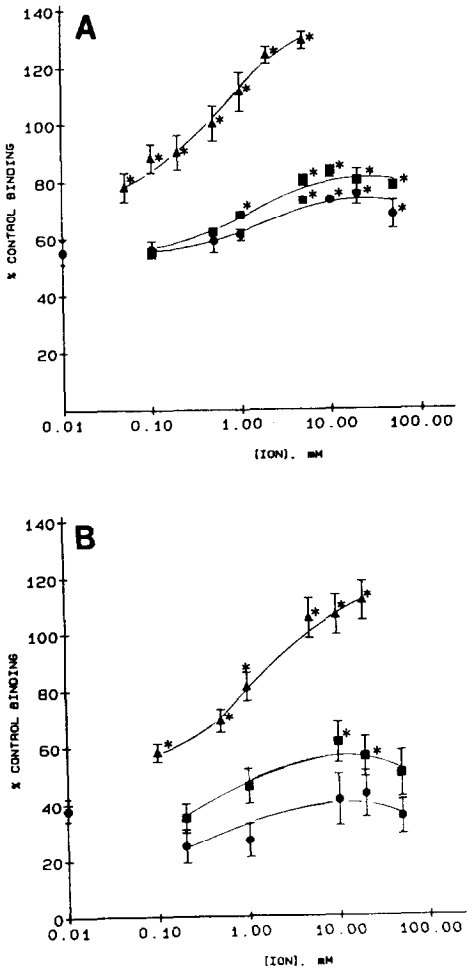

FIGURE 1: Divalent cation reversal of Na^+ inhibition of 6 binding in adult (A) and 10-day-old neonate (B). Membranes were incubated with 1 nM [3H]DADLE, 100 mM NaCl alone (o), or in the presence of Mn^{++}(▲); Mg^{++}(■); or Ca^{++}(●). Specific control binding (binding in the absence of ions) was 2214±157 cpm x mg protein $^{-1}$ in adult, and 763±283 cpm x mg protein $^{-1}$ in neonate. Each point is the mean±S.E. of 3-7 experiments.

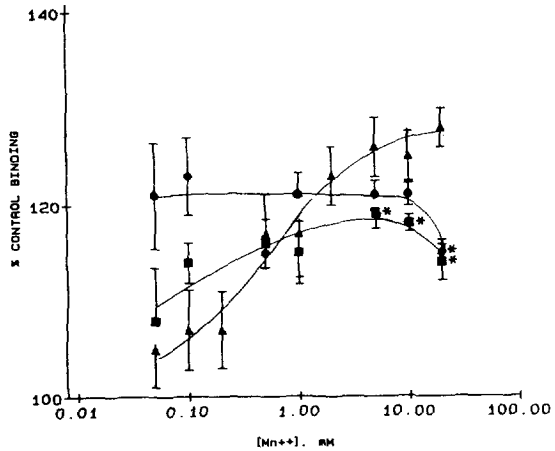

FIGURE 2: Effect of Mn^{++} in the presence of Mg^{++} on 1 nM [^{3}H]DADLE binding. Membranes were incubated with varying concentrations of Mn^{++} alone (▲), or in the presence of 2 mM (■), or 20 mM Mg^{++} (●). Statistically significant ($p<0.05$, T-test) inhibition of the Mn effect is shown by *. Each point is the mean ±S.E. of 3 experiments.

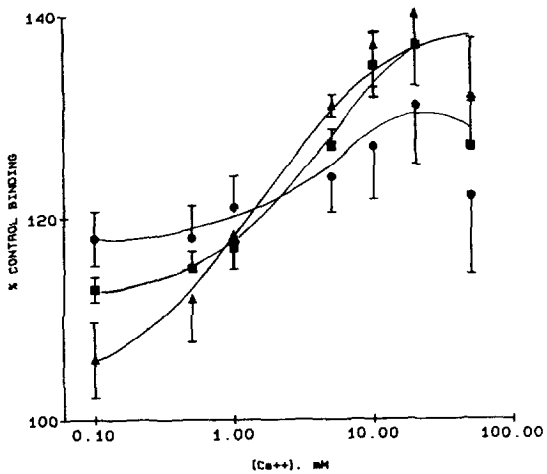

FIGURE 3: Effect of Ca^{++} in the presence of Mg^{++} on 1 nM [^{3}H]DADLE binding. Membranes were incubated with Ca^{++} alone (▲), or in the presence of 2 mM (■), or 20 mM Mg^{++} (●). Each point is the mean ±S.E. of 3 experiments.

the Na^+-induced decrease of binding to the level seen in the absence of ions in adults (fig. 1A); as well as in neonates (fig. 1B). Mg^{++} and Ca^{++} were much less potent than Mn^{++} in reversing inhibition of binding by Na^+ in adults. These results are in a good agreement with those of Pasternak et al. (1975) obtained for a μ selective ligand, [^{3}H]-dihydromorphine. In contrast, our data show that Mg^{++} or Ca^{++} are not able to reverse Na^+ inhibition of [^{3}H]DADLE binding in 10-day-old neonate (fig. 1B). Thus, these results suggest that regulation of δ binding with Mg^{++} or Ca^{++} emerges later than Mn modulation during postnatal development.

To elaborate on these results, dose-dependency studies in adult rat brain membranes for each ion were performed in the presence of fixed concentrations of either of the other Me^{++}. The fixed ion concentrations were chosen so that one represented the maximal, while the other a submaximal effect. The results of these experiments are summarized in Figs. 2-3. Ca^{++} or Mg^{++} antagonized the effect of Mn^{++} and vice versa, especially at higher concentrations (>5 mM). Mg++ and Ca^{++} alternatively displayed no antagonism toward each other. Thus, data presented in this report suggest the existence of two divalent cation regulatory sites for δ opiod receptors: one site binds Mg^{++} and Ca^{++}, a distinct one binds Mn^{++} with an allosteric interaction between the two.

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ACKNOWLEDGEMENTS

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EFFECTS OF MORPHINE AND B-ENDORPHIN ON Ca^{2+}-ATPASE ACTIVITY OF SYNAPTIC PLASMA MEMBRANES

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ABSTRACT

Morphine and B-endorphin were found to inhibit the high calcium -affinity Ca^{2+}-ATPase of synaptic plasma membranes which is believed to be involved in the regulation of intracellular Ca^{2+} that triggers the release of neurotransmittera. The inhibition was blocked by naloxone and reversed by calmodulin. Kinetic analyses revealed that opioida decreased the affinity of Ca^{2+}-ATPase for Ca^{2+} and calcium bound to the enzyme decreased the affinity of the enzyme for opioida.

INTRODUCTION

It is well established that calcium antagonizes morphine analgesia, but the mechanism of calcium-morphine interaction is still unknown. However, morphine action has been hypothesized to be caused by morphine's interference with neurotransmission through the disturbance of intracellular calcium disposition.

The high Ca^{2+}-affinity Ca^{2+}-ATPase of the synaptic plasma membrane (SPM) is believed to be involved in the regulation of intracellular Ca^{2+} (Lin and Way 1982a) which triggers the release of neurotransmittera from synaptic vesicles (SV) (Miledi 1973). This process involves the fusion of the SV with the SPM (Palade 1975). Nevertheless, the protein components for the attachment of the two membranes have not been identified. The high Ca^{2+}-affinity Mg^{2+}-independent Ca^{2+}-ATPases of SPU and SV are *of* particular interest because their K_m values for Ca^{2+} assayed either in the absence or presence of Mg^{2+} are close to the concentration of the intracellular Ca^{2+} (Lin and Way 1982) and because Ca^{2+} binding is required for membrane attachment.

It should be noted that although the high Ca^{2+}-affinity Ca^{2+} activity can be measured both in the absence and th presence of Mg^{2+}, Mg^{2+} is not required for the activation by Ca^{2+}(Lin 1991; Lin and Way 1984). This differentiates it from the Ca^{2+}/Mg^{2+}-ATPases reported by others (Schatzmann and Rossi 1971; Duncan 1976) which require the simultaneus presence of Mg^{2+} for stimulating ATP hydrolysis by Ca^{2+}. The Ca^{2+}-ATPase of SPM is found to be localized on the cytoaolic aide (Lin and Way 1982) and that on SV, dispersed in the cytosol near SPM. The localization of the Ca^{2+}-ATPases in the two organelles would appear to provide the proper condition for membrane attachment. The present study was designed to examine the effects of morphine and B-endorphin on SPM Ca^{2+}-ATPase activity.

MATERIAL AND METHODS

Morphine hydrochloride was purchased from Merck Chemical Division, Rahway, New Jersey. All chemicals were of the highest grade available and used without further purification. Calmodulin were gifts from Dra. W.Y. Cheung and Jim Potter, B-endorphin, from Dr. C.H. Li and naloxone, from Du Pont, Welmington, Delaware.

Synaptosomes from brain of male ICR mice were prepared by differential and Ficoll gradient centrifugations as described previously (Lin and Way 1984). SPM were fractionated by sucrose gradient centrifugation of lysed synaptosomes and collected between 0.6M-0.8M sucrose-HEPES (SPM,) and 0.8M-1.1M sucrose-HEPES (SPM_2) solutions.

Ca^{2+}-ATPase activity was determined by measuring colorimetrically the inorganic phosphate (P_i) liberated from ATP by Ca in imidazole buffer, pH 7.5, at 37°C in 15 min (Lin and Way 1984). In experiments with morphine (or B-endorphin), naloxone, calmodulin, alone or in combination, the enzyme preparation in HEPES buffer was first mixed with the compound(a) and allowed to stand in ice-bath for 30 min before adding other constituents.

The double reciprocal plots were drawn by programmed least squares linear regression. K_i and K'_i represent respectively the dissociation constants for morphine (or B-endorphin) complexes of enzyme and of enzyme-Ca^{2+} complex and were calculated according to a model for a simplest mixed-type inhibition system (see Segel 1975). It was considered that the enzyme-substrate-inhibitor complex was non-productive.

RESULTS

Ca^{2+}-ATPase activity in SPM was decreased in the presence of 10^{-6} morphine as shown in fig. 1A. The double reciprocol plots intersect at a point at the left aide of vertical-axis above the $1/Ca^{2+}$-axis. Morphine increased K_m and decreased V_{max} values. This suggests that the binding of morphine to the enzyme reduced the affinity of the enzyme for Ca^{2+} and the enzyme-morphine-Ca^{2+} complex is less or non-productive. The morphine inhibition was reversed by naloxone and calmodulin. The addition of 10^{-7}M naloxone restored the K_m and V_{max} to near control values. Calmodulin, on the other hand, lowered K_m and increased V_{max} to exceed their control values.

Similarly in SPM_2, morphine was also shown to inhibit Ca^{2+}-ATPase activity. Again, the inhibition was reversed by naloxone and calmodulin (fig. 1B) .

Fig. 1C shows the inhibition of Ca^{2}+-ATPase activity of SPM_2 by B-endorphin (10^{-6}M). The addition of 10^{-7}M naloxone reversed the K_m and V_{max} values of the enzyme in the presence of B-endorphin to near control values and $6X10^{-7}$M calmodulin reversed the K_m and V_{max} further beyond the controls.

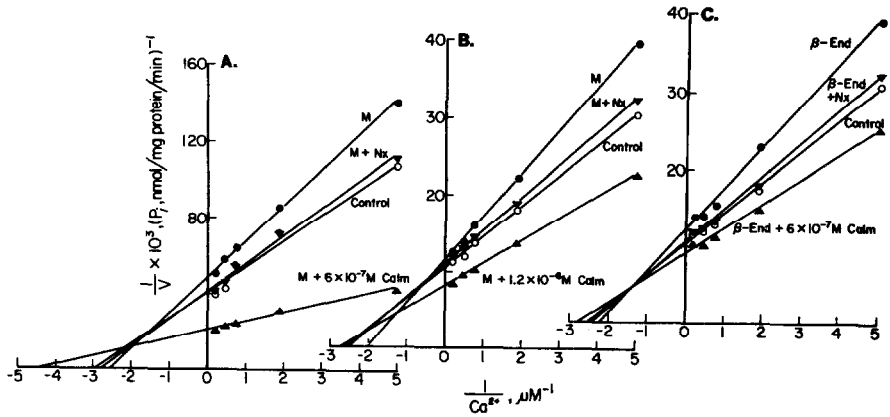

FIGURE 1. Effect of morphine and B-endorphin on Ca^{2+}-ATPase activity in synaptic plasma membranes. (A), SPM_1, (B) and (C), SPM_2. The incubation medium contained membrane protein of about 100 ug, 1mM ATP, 1mM $MgCl_2$, various $CaCl_2$ (0 to 950 uM) -EGTA (1.0mM) mixtures without (○) or with 10^{-6}M morphine (or B-endorphin) (●), 10^{-6}M morphine (or B-endorphin)+ 10^{-7}Mnaloxone (▼) or 10^{-6}M morphine (or B-endorphin) + $6X10^{-7}$M or $1.2X10^{-6}$M calmodulin as indicated (▲). Incubation was at 37°C for 15 min. P represents the difference between P_i liberated by (Ca^{12+} + Mg^{2+}) and by Mg^{2+} alone.

DISCUSSION

SPM Ca^{2+}-ATPase is believed to be of special importance in synaptic transmission because the enzyme has binding sites for both Ca^{2+} and ATP (Lin and Way 1982a). Moreover, Ca^{2+}-binding is required for membrane fusion and ATP is required for secretion (Woodin and Wienieke 1964; Douglas 1968). It has been shown that SPM Ca^{2+}-ATPase activity is regulated by calmodulin both in the absence (Lin, unpublished data) and the presence (see Roufogalis 1979) of Mg^{2+} and that calmodulin decreases K_m and increased V_{max} values as compared to control values.

The mode of morphine (or B-endorphin) inhibition of Ca^{2+}-ATPase suggests that morphine can bind to the enzyme simultaneously with Ca^{2+} at distinctly different sites, hence morphine cannot be considered to interact with the catalytic site. The dissociation constants K_i and K'_i calculated (see Methods) from the slopes and intercepts in fig.1 are, respectively, 2.84 and 5.05 (M-SPM_1), 2.56 and 9.09 (M-SPM_2) and 2.88 and 5.68 (B-endorphin -SPM_2). Thus, morphine and B-endorphin are less firmly bound to the enzyme-Ca^{2+} complex than to the free enzyme. However, the precise site for morphine (or B-endorphin) binding remains unresolved at present.

There are several possibilities that may explain the morphine and B-endorphin effect. The opioid may interfere with the Ca^{2+} binding of calmodulin to the enzyme subunit and result in a decrease of the amount of active form of the enzyme, or the opioid may distort the enzyme conformation and render the enxyme-substrate-opioid complex non-productive (or less productive).

In conclusion, the present results demonstrate that opioids decrease the affinity of SPM Ca^{2+}-ATPase for Ca^{2+}, and Ca^{2+} decreases the binding affinity of the enzyme for opioids. Since SPM Ca^{2+}-ATPase is believed to be regulated by intracellular Ca^{2+} which triggers the release of neurotransmitter through Ca^{2+}-dependent membrane fusion. The present findings may provide the underlying basis for opioid action as well as the antagonistic effect between calcium and opioids.

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OPIOIDS AND RAT ERYTHROCYTE DEFORMABILITY

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ABSTRACT

In previous studies from this laboratory, it was noted that opioids in vitro reduced human red blood cell deformability. The effect was found to be dose-dependent, naloxone reversible and preferentially selective kappa ligands exhibited the highest potency. To extend these findings studies were carried out using rat erythrocytes. The time required for erythrocytes to pass through a 5.0 um pore membrane was determined and used as an index of deformability. Opioids added in vitro produced inhibition of deformability in a dose-dependent, naloxone reversible manner. Injecting naive animals with morphine or nalbuphine also produced dose related reductions in red cell deformability. The degree of inhibition produced by nalbuphine correlated well with its plasma concentrations as measured by high performance liquid chromatography (HPLC). Chronic morphine treatment by pellet implantation resulted in-the development of tolerance as evidenced by a loss in the ability of morphine in vitro to inhibit red cell deformability. Addition of naloxone resulted in a decrease in filtration time. Thus, the data confirm and extend previous findings on human red blood cells. In as much as previous data from this laboratory demonstrated that opioids inhibit calcium flux from erythrocytes by inhibiting calcium-ATPase and calcium efflux is necessary for normal deformability, it is concluded that opioids act to reduce red cell deformability by inhibition of the calcium pump.

INTRODUCTION

Opioids cause a reduction in the ability of human red blood cells to deform in a dose dependent, naloxone reversible, stereoselective manner. Relatively selective kappa ligands are more potent in eliciting this effect and extended opioid incubation with erythrocytes produces tolerance and a rebound phenomenon upon exposure to naloxone (Rhoads et al.1985). To extend these findings studies were carried out using rat erythrocytes.

MATERIALS AND METHODS

Heparinized blood wa_s obtained from Sprague Dawley rats (200-300g) under CO_2 anesthesia by cardiac puncture. The time necessary for 1 ml of blood to pass through a 5 um pore Nucleopore membrane under 5 ml water pressure was measured and used as an index of the ability of the erythrocytes to deform

(Reid et al 1976).

The effects of chronic opioid treatment on deformability were studied by making rats morphine tolerant and dependent. One 75 mg morphine-base pellet was implanted on day 0 and 2 pellets on days 1 and 2. Pellets were removed on day 3 and with some rats naloxone was either injected intraperitoneally (5 mg/kg) or added in vitro (0.2 uM) immediately afterwards.

In order to study the correlation between drug concentration and deformability, rats were injected with nalbuphine HCl intraperitoneally and the plasma concentration of nalbuphine was determined according to the HPLC method described by Lo et al. (1984). Nalbuphine was extracted from plasma and separated using a C8, 5 um (4.6 x 250 mm) column, with a mobile phase of 27% CH_3CN, 0.035% H_3PO_4 at a flow rate of 1.4 ml/min using an amperometric detector.

RESULTS

All opioids used in the study inhibited rat erythrocyte deformability dose-dependently. Dynorphin A(1-13) and 1-pentazocine showed the highest potency, the IC_{50} being 0.3 nM and 0.5 nM respectively (figure 1). The lowest potency was observed for D-ala-D-leucine enkephalin (800 nM) and d-pentazocine (925 nM).

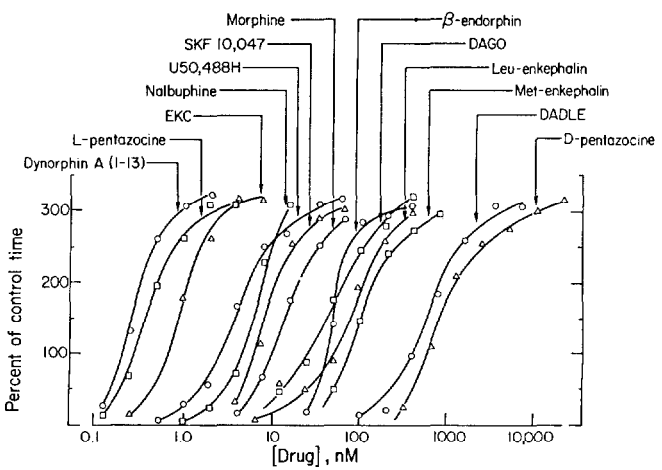

FIGURE 1. Dose response curves of opioids increasing erythrocyte filtration time. Erythrocytes were incubated with opioids in vitro at 37°C for 15 minutes prior to the measurement of filtration time.

In erythrocytes obtained from morphine tolerant/dependent rats, morphine added in vitro exhibited reduced ability to inhibit deformability when compared to those from naive animals (figure 2). An increasing shift of the dose response curve to the right occured with increasing pellet implantation time and when the pellets were removed after the third day of implantation the curves shifted back toward the naive state (figure 2). Addition of naloxone resulted in a rebound effect with an increased deformability of the red cells, as evidenced by a reduction in the filtration time to one half (data not shown).

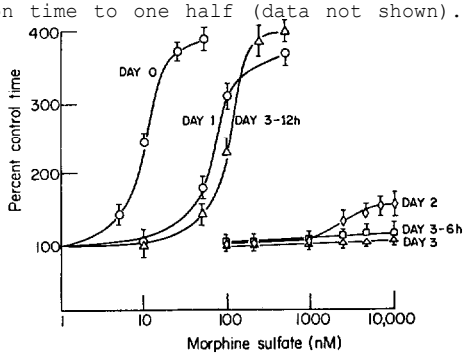

FIGURE 2. Chronology of development and loss of tolerance to morphine inhibition of erythrocyte deformability. Rats were implanted with one pellet of morphine base on day 0, two pellets on days 1 and 2. Blood was withdrawn and the filtration time of the erythrocytes in the presence of varying concentrations of morphine was measured on day 0, 1, 2, 3 and on day 3, six and 12 hours after pellet removal.

HPLC measurement of the plasma concentrations of nalbuphine yielded values that correlated directly with the degree of inhibition of rat erythrocyte deformability (figure 3).

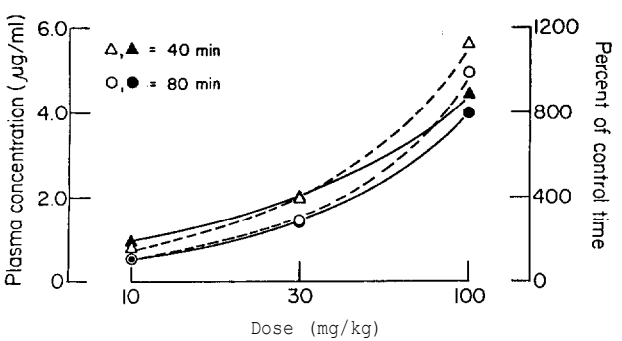

FIGURE 3. Nalbuphine plasma concentrations (left y-axis, open symbols and dotted lines) and changes in red cell deformability (right y-axis, closed symbols and solid lines) 40 and 80 minutes after 10, 30, and 100 mg/kg intraperitoneally injected nalbuphine HCl.

DISCUSSION

Our results demonstrate that opioids inhibit rat erythrocyte deformability in a dose dependent, stereoselective manner with relatively selective kappa ligands exhibiting the highest potency. Chronic treatment of rats with morphine resulted in tolerance and a rebound effect occured upon addition of naloxone. These results extend and confirm similar results with human erythrocytes (Rhoads et al. 1985). The HPLC measurements revealed that the deformability changes correlated well with the amount of opioid available in plasma; higher nalbuphine plasma concentrations produced a higher inhibition of deformability.

The change of deformability in erythrocytes is often postulated as a symptom for an existing pathological condition and many agents can increase or decrease the ability of erythrocytes to deform. Intracellular calcium is believed to play a piv tol role in the deformability of red cells and because Yamasaki and Way (1983) found that the calcium-ATPase of rat red cells can be inhibited by opioids, we suggest that such an action inhibits calcium efflux and decreases erythrocyte deformability.

Endogenous peptides with opioid activity have been reported to be released during stress and naloxone to be efficacious in reversing some of debilitating effects of stress (Bernton et al.1985). Since reduced red cell deformability is known to be deleterious to systems by decreasing blood flow, we are presently examining what role a reduced red cell deformability might play under these circumstances.

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Calmodulin Content in Rabbit Reticulocyte and the Influence of Opioid Peptides on Cslmodulin Activity in its Membrane.

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ABSTRACT

Calmodulin (CaM) content in rabbit reticulocyte and the influence of opioid peptides on CaM activity in its membrane were studied by a highly sensitive assay of CaM activity based on the stimulation of Calcium-dependent phosphodiesterase activity. The CaM contents in reticulocytes were higher than those in normal erythrocytes, both in the cytosol fraction and in the membrane fraction. Among the opioid peptides, β-endorphin (β-EP) and dynorphin-A-(1-13) (dyn) had a significant inhibitory effect on CaM activity in reticulocyte membrane. The effect was not antagonized by naloxone or Mr. 2286, nor influenced by increase of Ca^{2+} concentration, but was reversed by the addition of exogenous CaM. This implies that the action of β-EP and dyn on reticulocyte membranes probably involves an non-opioid mechanism, in which CaM may be an important key of linkage.

INTRODUCTION

Calmodulin (CaM) has a wide distribution in the body. While calcium is important in the stimulation-activation coupling of the central nervous system or hormonal effects, CaM plays an important role in these calcium-dependent processes. It has been reported that EGTA, the $Ca2^{+}$ chelating agent, inhibited phosphodiesterase (PDE) activity as a result of CaM inactivation (Lin et al. 1975, Teo and Wang 1973). Neuropeptides or opioid peptides like ACTH, dynorphin, and beta-endorphin were also found to have an inhibitory effect on CaM activity (Simantov et al. 1982, Barnette and Weiss 1983, 1984). Among them, β-EP was the most potent, and its IC_{50} was 3 μM (Clouet et al. 1983).

Rat reticulocytes have been used as a convenient model for the study of the interaction of receptor and second messenger systems. In the course of maturation, they showed a marked loss of adenylate cyclase responsiveness to catecholamines and guanine nucleotides without significant loss of basal enzyme activity (Limbird et al. 1984). So we tried rabbit reticulocyte membrane to study the molecular basis of the functional activity of opioid peptides.

MATERIALS AND METHODS

1. Chemicals:

   ^{3}H-cGMP (23 ci/mmole) was obtained from Institute of Atomic Energy, Chinese Academy of Science; β-endorphin, dynorphin-A-(1-13), met-enkephalin, leu-enkephalin, were from Sigma; QAE-Sephadex A-25 was from Pharmacia; CaM-deficient PDE was extracted from bovine heart (Liu et al 1985) at a concentration of 0.5 unit/μl; and CaM was prepared from rat testes.

2. Solutions for CaM assay:

   Assay buffer consisted of Tris-HCl 45 mmol/L, Imidazole 45 mmol/L, Mg-acetate 5.5 mmol/L, pH 7.5. All the following solutions were prepared with assay buffer: CaCl, 4.5 mmol/L, EGTA 4.5 mmol/L, cobra venom 10 u/ml, and ^{3}H-cGMP 100,000 cpm / 50 μl (~250 pmole / 50 μl).

3. Generation of rabbit reticulocytes and the preparation of membranes (Tucker and Young 1982):

Healthy male New Zealand white rabbits, about 2 kg in body weight, were used. Subcutaneous injection of vitamin B_{12} 1 mg / 1 ml and folic acid 10 mg/l ml were given on the first day, followed by subcutaneous injection of phenylhydrazine HCl in saline (10 mg/kg) once daily for 5 days. On the 6th day, examination of blood sample taken from the ear vein and stained on slide with brilliant cresyl blue showed that more than 98% of the red blood cells were reticulocytes. The rabbit was bled from the carotid artery on the 7th day, and blood collected and heparinized (1 mg heparin / 100 ml blood), centrifuged under 1,000 x g for 15 minutes. After washing thrice with normal saline, the blood cells were suspended in normal saline, counted and made up the EGTA-buffer to concentration of 10^9 cells / ml. The cells were then homogenized under supersonic treatment (15 sec x 4) at 4° C, and centrifuged under 40,000 x g for GO minutes to yield sediments of cell membranes, which were then kept, in aliquots and frozen under -65°C before use.

4. Determination of CaM activity in rabbit reticulocyte membrane:

CaM activity was determined by a highly sensitive assay based on the stimulation of calcium-dependent phosphodiesterase activity (Liu et al. 1985). Rabbit reticulocyte membrane preparations (10^9 cells / ml) were serially diluted into five different dilutions of 1:5, 1:10, 1:20, 1:40, and 1:80. The CaM content in 20 µl of each were measured according to the methods of Thompson et al. (1971). The activity of CaM in the rabbit reticulocyte membrane which activated 20 µl of PDE from bovine heart to 50% of its full activity was designated as 1 unit (Thompson and Appleman 1971). It can be seen clearly from fig. 1 that in our experimental conditions, the CaM activity in 20 µl of 1:15 dilution of membrane. preparation was equivalent to 1 unit.

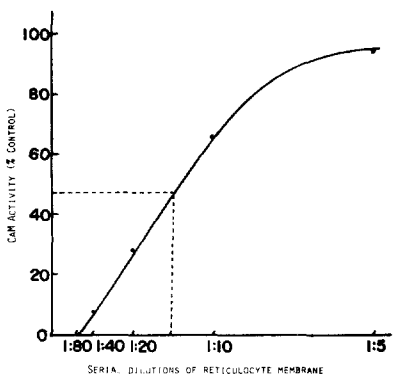

FIGURE 1. CaM activity in rabbit reticulocyte membrane.

RESULTS

1. CaM content in the cytosol and membrane fractions of normal rabbit erythrocyte and reticulocyte:

The separated whole cells, suspended in EGTA-buffer and homogenized by supersonic treatment, were kept in 100°C water bath for 5 minutes and centrifuged under 40,000 x g for GO minutes. Assay of the supernatants (cytosols) as compared to the membrane fractions showed that most of the CaM content was found in the cytosol and the contents of CaM in reticulocytes were much higher than those in normal erythrocytes both in the cytosol fraction or within the cell membrane (table 1).

2. The effect of dyn on CaM activity in rabbit reticulocyte membranes:

Different amounts of dyn were added into reticulocyte cell membranes (1:15 dilution) to make up a serial final concentrations of 0.5, 1.0, 2.0, 4.0, and 16.0 µM in the presence of Ca^{2+} 2 mM, with 10 units (20 µl) of PDE extract from bovine heart and ^{3}H-cGMP (100,000 cpm / 50 µl) as substrate. Dose-dependent inhibition of PDE activation in reticulocyte membranes was observed (fig. 2). The inhibitory effect of dyn was not reversed by 50 µl of naloxone, and Mr. 2266 (Vonvoigtlander et al. 1984), the κ- antagonist, up to a maximal concentration of 100 µM.

TABLE 1. CaM content of normal rabbit erythrocyte and and reticulocyte ($ng/10^9$ cells)

Sample		CaM ($\bar{x} \pm SD$)	
Erythrocyte	Cytosol	6.05 ± 2.37	(N=5)
	Membrane	0.26 ± 0.09	(N=2)
Retieulocyte	Cytosol	37.40 ± 8.69	(N=6)
	Membrane	1.71 ± 0.56	(N=6)

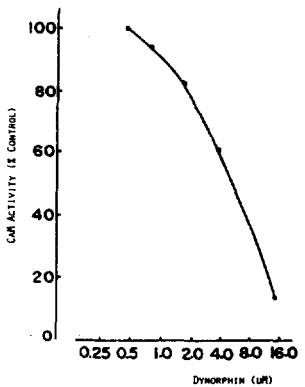

FIGURE 2. Effect of dyn on the activity of CaM in rabbit reticulocyte membrane.

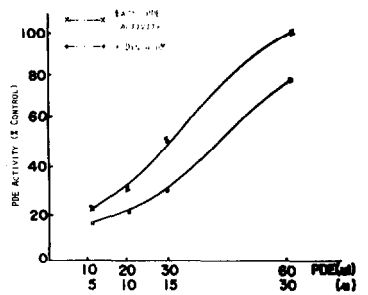

FIGURE 3. Activation of PDE by rabbit reticulocyte membrane in the presence and the absence of dyn.

T)yn at 4 μM also exerts a significant inhibitory effect on the stimulation of PDE activity by CaM present in reticulocyte membrane preparation across various amounts of PDE from 10 to 60 μl, corresponding to 5 to 20 units. The curve of PDE activity shifts parallelly to the right in the presence of dyn (fig. 3). Similar results were obtained as the experiment was repeated three times. The inhibitory effect of dyn on CaM-stimulated PDE activity was not influenced by the addition of Ca^{2+} to 4 mM, but reversed by CaM in a dose-dependent manner (fig. 4).

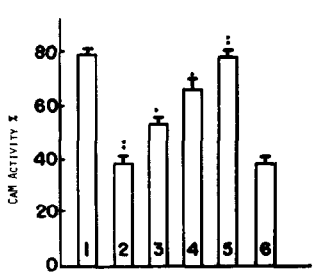

FIGURE 4. Effect of dyn on CaM activity in rabbit reticulocyte membrane. 1: Control. 2: 4 μl dyn. 3,4,5: CaM 0.2,0.8,3.2 ng. 6: Ca^{2+}added to 4 mM.

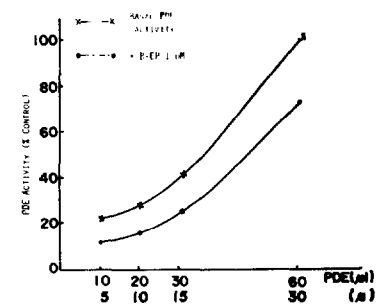

FIGURE 5. Activation of PDE by rabbit reticulocyte membrane in the presence and the absence ofβ-]EP.

3. The effect of β-EP on CaM activity in rabbit reticulocyte membranes:

β-]EP had a similar and even stronger antagonistic effect on the activity of CaM in rabbit reticulocyte membranes than dyn. β-]EP, like dyn, does not affect PDE activity directly, but inhibited the CaM PDE activating effect in 20 μl reticulocyte membranes preparation (1:15 dilution). The effect of 1 μM of β-EP corresponded to 4 μM of dyn and caused a

parallel shift of the curve of CaM-activated PDE activity to the right (fig. 5). The effect of β-EP was not antagonized by 50 nM of naloxone, not affected by an increase of Ca^{2+} concentration to 4 mM, but was reversed by the addition of CaM. The results implied that β-EP antagonized the activating effect of CaM probably by competition with PDE for CaM.

Besides, opiate alkaloids and endogenous opioid peptides like met-enkephalin, leu-enkephalin, morphine, and etorphine were all not effective up to 10 μM. Naloxone had also no effect on CaM activated PDE activity up to 50 μM.

DISCUSSION

CaM was assayed according to its ability to activate PDE and hydrolyze cGMP. Our experimental results indicate that the CaM content in the cytosol and membranes of reticulocyte were much higher than those in normal erythrocytes. This is probably related to the maturation process of red cells and coincides with the findings of Limbird who reported a marked loss of adenylate cyclase responsiveness to catecholamines and guanine nucleotides in the course of maturation (Limbird et al. 1984). High CaM content in reticulocyte facilitates its applications in the study of the mechanism of drug action upon second messenger systems. Reticulocyte could be easily obtained in practically pure form and changes involved during its maturation could be followed in cell culture.

Dyn at a concentration of 4 μM, which did not affect PDE basal activity, already showed a significant inhibitory effect (around 50%)on the CaM activity of rabbit reticulocyte. The effect was dose related (between 0.5 - 16.0 μM) and was not blocked by an increase of Ca^{2+} concentration, but could be reversed by adding CaM. Naloxone and Mr 2266 did not block the inhibitory effect of dyn on rabbit reticulocyte CaM. These implied that dyn probably acts directly upon CaM and inhibits the activity of CaM in activating PDE.

β-EP, similar to but stronger than dyn, also inhibits CaM activity at a lower concentration of 1 μM. Although as opioid ligands, β-EP and dyn, which belonged to different classes, had similar effect on CaM in cell membranes; morphine, met-enkephalin and leu-enkephalin showed no significant effect. These are additional proofs to the fact the β-EP and dynorphin act directly through CaM irrelevant to opiate receptors.

Known antipsychotics, e.g. fluphenazine, show a strong specific binding to CaM and competes with dyn and β-EP in binding with CaM (Prozialeck and Weiss 1982, Weiss et al. 1982). It is interesting to explore further the mechanism of action of β-EP and dyn on CaM and its relation to anti-psychotic therapy.

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ADRENAL MEDULLARY OPIOID RECEPTORS ARE LINKED TO GTP-BINDING PROTEINS, PERTUSSIS TOXIN SUBSTRATES

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ABSTRACT

Effects of Gpp(NH)p and pretreatment of membranes with pertussis toxin (PT) were studied on opioid binding to bovine adrenal medullary opioid receptors. Gpp(NH)p inhibited [^{3}H]DADLE binding by increasing the dissociation constant. PT treatment reduced [^{3}H]DADLE binding and almost abolished the Gpp(NH)p inhibition of [^{3}H]DADLE binding. Levorphanol did not show any significant effect on bovine adrenal medullary adenylate cyclase activity. These results suggest that bovine adrenal medullary opioid receptors are linked to PT-sensitive GTP-binding proteins which are not directly coupled to adenylate cyclase.

INTRODUCTION

At the present time, molecular mechanisms underlying opioid inhibition of hormone or neurotransmitter release is still obscure. Recently, GTP-related processes of signal transduction have been suggested for the possible molecular mechanism of opioid action. These suggestions are based on findings that GTP or its stable analogs inhibits binding of opioid agonists to receptors. It has been also shown that opioid receptors in neuroblastoma-glioma hybrid cells (NG 108-15 cells) (Kurose et al. 1983) and-rat caudate nucleus (Kamikubo et al. 1981, Law et al. 1981. Abood et al. 1985) are coupled to inhibition of adenylate cyclase through a pertussis toxin (PT)-sensitive GTP-binding protein.

In also adrenal medulla, it has been shown that opioids inhibit hormone (catecholamine) secretion from chromaffin cells (Kumakura et al. 1980, Barron and Hexum 1986). Because of the relative homogeneity of composing cells, adrenal medulla may be a suitable model system to study post-receptor mechanisms of opioid action. In the present study, we have investigated the possible coupling of bovine adrenal medullary opioid receptors to PT-sensitive GTP-binding proteins and adenylate cyclase. The results suggest that adrenal medullary opioid receptors are coupled to PT-sensitive GTP-binding proteins but not to inhibition of adenylate cyclase.

MATERIALS AND METHODS

[^{3}H]D-Ala^2-D-Leu^5-enkephalin ([^{3}H]DADLE) (49.3 Ci/mmol) was from Amersham. Guanylyl imidodiphosphate (Gpp(NH)p) was from Sigma. PT was a generous Dr. Ui (Hokkaido University) and Kaken Pharmaceuticals. [^{125}I]cAMP and anti-CAMP antibody were obtained from Yamasa.

Pretreatment of membranes with PT

Bovine adrenal medullary crude plasma membranes, prepared as described elsewhere (Kamikubo et al. 1986b), were incubated in 2 mM NAD, 8 mM DTT, 1 mM ATP, 2 mM MgCl2, 10 mM thymidine and 50 mM Tris-HCl (pH 7.4) with or without PT (20 pg/ml) at 37°C for 30 min and washed twice with ice-cold 50 mM Tris-HCl (pH 7.4).

Opioid binding assay

Binding of [^{3}H]DADLE to membranes was assayed in 50 mM Tris-HCl (pH 7.4) at 25°C for 30 min. Bound and free ligands were separated by rapid vacuum filtration through Whatman GF/C filters. Non-specific binding was determined in the presence of 1 pM DADLE. Saturation binding data were analyzed by the computerized non-linear least squares curve fitting procedure (Kamikubo et al. 1986a).

Adenylate cyclase assay

Adenylate cyclase activity was measured at 37°C for 10 min by the method of Katada and Ui (1981). Incubation mixture contained 0.5 mM ATP, 5 mM MgCl2, 0.1 mM EGTA, 0.5 mM isobutyl methylxantine, 7.2 mM phosphocreatine, 40 U/ml creatine phosphokinase, 0.1% BSA, 10 μM GTP, 0.1 M NaCl and 25 mM Tris-HCl (pH 7.4). Cyclic AMP produced was measured by sensitive and specific radioimmunoassay.

RESULTS AND DISCUSSION

Gpp(NH)p inhibited [^{3}H]DADLE binding to bovine adrenal medullary membranes in a concentration-dependent manner. The primary action of Gpp(NH)p on the [^{3}H]DADLE binding was an increase in the dissociation constant (from 2.9 to 3.9 nM).

Pretreatment of the membranes with PT resulted in a 29% reduction of [^{3}H]DADLE binding (Table 1). In addition, the pretreatment abolished almost completely the Gpp(NH)p inhibition of [3H]DADLE binding.

TABLE 1 Effects of pertussis toxin treatment on [^{3}H]DADLE binding to bovine adrenal medullary membranes

	[^{3}H]DADLE binding (%)	
Addition	PT (-)	PT (+)
None	100	71 ± 5
GPP(NH)p (10 uM)	36 ± 3	65 ± 3

Specific binding of 5 nM [^{3}H]DADLE to bovine adrenal medullary membranes treated with (PT (+)) or without (PT (-))PT was shown. The control binding (100%) was 21 ± 1 fmole/mg protein.

These results suggest that adrenal medullary opioid (DADLE) receptors are regulated by Gpp(NH)p and that the Gpp(NH)p regulation may be mediated by PT-sensitive GTP-binding proteins. The existence of PT-sensitive GTP-binding proteins in bovine adrenal medulla was

demonstrated by PT-catalyzed [^{32}P]ADP-ribosylation of the proteins (Kamikubo et al. manuscript in preparation). In several receptor systems, it has been known that PT inhibits agonist binding to hormone receptors and reduces GTP inhibition of agonist binding by uncoupling PT-sensitive GTP-binding proteins from receptors (Kurose et al. 1983, Hsia et al. 1984, Cote et al. 1984). From the present observations, therefore, it seems likely that the PT-sensitive and ADP-ribosylatable GTP-binding proteins mediate the Gpp(NH)p regulation of adrenal medullary opioid receptors.

In NG 108-15 cells and rat caudate nucleus, it is likely that opioid receptors are coupled to inhibition of adenylate cylase through the PT-sensitive inhibitory GTP-binding protein (Ni) (Kurose et al. 1983, Abood et al. 1985). In membranes from rat brain caudate nucleus, levorphanol inhibited adenylate cyclase activity as previously shown by us (Kamikubo et al. 1981) and by others (Law et al. 1981)(Table 2). However, in bovine adrenal medullary membranes, levorphanol showed no clear inhibitory action on adenylate cyclase activity. PT

TABLE 2 Effects of levorphanol on adenylate cyclase activity in rat caudate nucleus and bovine adrenal medulla

	Adenylate cyclase activity (% of control)	
Addition	Rat caudate nucleus	Bovineadrenal medulla
None	100	100
Levorphanol 1 nM	92 ± 4	96 ± 5
Levorphanol 10 nM	83 ± 3	98 ± 4
Levorphanol 100 nM	76 ± 5	97 ± 3
Levorphanol 1 uM	74 ± 5	93 ± 6

Data are the means ± SD of triplicates. The control activity was 148 ± 9 pmole/mg protein/min for rat caudate nucleus and 83 ± 6 pmole/mg protein/min for bovine adrenal medulla.

treatment showed only modest. if any, enhancing effect on GTP activation of adrenal medullary adenylate cyclase.

The reason for the discrepancy between the actions of levorphanol in rat caudate nucleus and in bovine adrenal medulla is not known. However, it may be possible that adrenal medullary opioid (DADLE) receptors are coupled mainly to other PT-sensitive GTP-binding proteins than Ni. In addition to Ni, there are several functionally or molecularly distinct classes of PT-sensitive GTP-binding proteins, such as the stimulatory GTP-binding protein of phospholipase C in certain cells (Nakamura and Ui 1985) and the GTP-binding protein with 39,000 dalton A subunit (No) (coupled to potassium channel?) (Sternweis and Robinshaw 1984). PT treatment of adrenal medullary membranes did not significantly modified the GTP activation of adenylate cyclase. This finding may suggests that activity or content of Ni in adrenal medulla is not high to significantly inhibit adenylate cylase. Functional linkage of opioid (DADLE) receptors to Ni may be scarce in adrenal medulla.

The present observations provide indirect evidence for the functional linkage of adrenal medullary opioid (DADLE) receptors to PT-sensitive GTP-binding proteins which are not directly coupled to adenylate cyclase. Post-receptor mechanisms of signal transduction other than adenylate cyclase regulation, such as K channel regulation etc., may be involved in opioid (DADLE) action in bovine adrenal medulla (Figure 1).

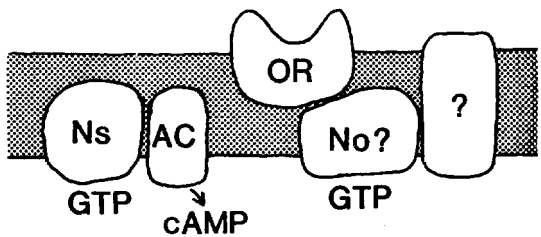

FIGURE 1 Linkage of bovine adrenal medullary opioid (DADLE) receptors (OR)to pertussis toxin-sensitive GTP-binding protein (No?) not directly coupled to adenylate cyclase (AC).

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VARYING EXPRESSION OF OPIOID RECEPTORS AND GTP-BINDING PROTEINS IN HUMAN PHEOCHROMOCYTOMAS

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ABSTRACT

The expression of opioid receptors and GTP-binding proteins was studied in 14 pheochromocytomas. The amount of [^{3}H]diprenorphine bound to membranes varied from 13 to 62 fmole/mg protein, but significantly higher in adrenaline-secreting tumors than in noradrenaline-secreting tumors. None of [^{3}H]DADLE, [^{125}I]β-endorphin or [^{3}H]ethylketocyclazocine binding was correlated with [^{3}H]diprenorphine binding. Gpp(NH)p inhibition of [^{3}H]DADLE binding was evident in all four normal human adrenal medullae but in only 8 out of 14 pheochromocytomas. The extent of Gpp(NH)p inhibition was not correlated with the amount of pertussis toxin (PT)-sensitive GTP-binding proteins as measured by PT-catalyzed [^{32}P]ADP-ribosylation. The present findings suggest that opioid receptors and PT-sensitive GTP-binding proteins are variously expressed in transformed chromaffin cells, pheochromocytoma.

INTRODUCTION

It has been shown that various amounts of opioid peptides derived from proopiomelanocortin, proenkephalin A or proenkephalin B are produced in transformed chromaffin cells, pheochromocytoma (Imura et al. 1984). In addition to the production of opioid peptides, expression of opioid receptors and GTP-binding proteins and their functional coupling may be modified in pheochromocytomas. The present study was undertaken to demonstrate such modification of the opioid receptor system in pheochromocytomas. In addition, possible correlation between the modification of opioid receptor system and the clinical feature of pheochromocytoma has been studied.

MATERIALS AND METHODS

[^{3}H]Diprenorphine (41 Ci/mmol). [^{3}H]D-Ala^2-D-Leu^5-enkephalin ([^{3}H]DADLE) (49.3 Ci/mmol), and [^{125}I] human β-endorphin (approximately 2000 Ci/mmol) were from Amersham. [^{3}H] (±)Ethylketocyclazocine ([^{3}H]EKC) (23 Ci/mmol) was from New England Nuclear. Pertussis toxin (PT) was a generous gift from Dr. Ui (Hokkaido University) and Kaken Pharmaceuticals.

Subjects

In 7 out of 14 patients with pheochromocytoma, urinary excretion of only noradrenaline was increased (Noradrenaline type). In other 7 patients, urinary excretions of both noradrenaline and adrenaline were increased (Adrenaline type). Pheochromocytomas were obtained surgically and kept -80°C until use. Four normal human adrenal medullae were obtained at autopsy and kept -80°C until use.

Preparation of membranes

Pheochromocytomas were homogenized in 0.3 M sucrose and 10 mM Tris-HCl (pH 7.4) and centrifuged at 800 g for 10 min. The supernatant was centrifuged at 30,000 g for 20 min. The pelleted membranes were washed with 50 mM Tris-HCl (pH 7.4) and resuspended in 50 mM Tris-HCl (pH 7.4).

Opioid binding

The binding of [^{3}H]diprenorphine. [^{3}H]DADLE and [^{3}H]EKC was assayed in 50 mM Tris-HCl (pH 7.4) at 25°C for 30 min. The binding of [^{125}1]β-endorphin was assayed in 0.1% BSA, 0.01% bacitracin and 50 mM Tris-HCl (pH 7.4) at 25°C for 30 min (Kamikubo et al 1986). Bound and free ligands were separated by rapid vacuum filtration through Whatman GF/C filters. Non-specific binding was determined in the presence of 1 μM of each unlabelled ligand.

Quantitation of PT-sensitive GTP-binding proteins

The amount of PT-sensitive GTP-binding proteins in pheochromocytoma membranes was measured by the method of Pobiner et al. (1985) by using the ability of PT to catalyze [^{32}P]ADP-ribosylation of the proteins. GTP-binding proteins were extracted from membranes by incubating membranes in 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1% (w/v) sodium cholate and 20 mM Tris-HCl (pH 8.0) at 4°C for 60 min. The extract was centrifuged at 12,000 g for 15 min, and the solubilized proteins were used as the source of GTP-binding proteins. PT-catalyzed [^{32}P]ADP-ribosylation was performed in 100 μl of solution containing PT (25 μg/ml), 10 μM [^{32}P]NAD (5-10 μCi), 10 mM DTT, 1 mM ATP, 2 mM $MgCl^{2}$, 10 mM thymidine and 50 mM Tris-HCl (pH 7.4) at 35°C for 30 min. The reaction was terminated by adding 1 ml of 10% trichloric acid. Proteins were precipitated by centrifugation at 12,000 g for 5 min and solubilized in Laemli's sample buffer. SDS-polyacrylamide gel electrophoresis was run on 10% polyacrylamide gel. Autoradiography was performed at -80°C using Kodak XO-AR film. Gel was cut, and radioactivity incorporated into proteins was counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

In all normal human adrenal medullae and pheochromocytomas examined, specific binding sites for [^{3}H]diprenorphine, [^{3}H]DADLE, [^{125}I]β-endorphin and [^{3}H]DADLE were demonstrated in the membrane fraction. The amount of [^{3}H]diprenorphine bound to normal adrenal medullary membranes was 40.5 ± 5.5 fmole/mg protein (mean ± SD). The amount of [^{3}H]diprenorphine bound to pheochromocytoma membranes varied from 13

to 62 fmole/mg protein. As shown in figure 1, the adrenaline type pheochromocytomas showed higher [^{3}H]diprenorphine binding (40.6 ± 11.7 fmole/mg protein) than the noradrenaline type (25.0 ± 10.7 fmole/mg protein). These findings suggest that adrenaline-secreting

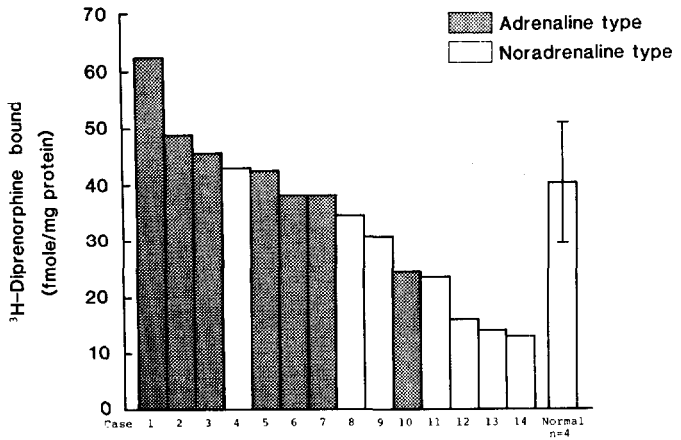

FIGURE 1 [^{3}H]Diprenorphine binding to pheochromocytoma membranes.

pheochromocytoma sells bear more [^{3}H]diprenorphine-binding sites than noradrenaline-secreting cells. Considering the relative non-selectivity of diprenorphine to opioid receptor subtypes, it seems likely that the total content of opioid receptors is also higher in adrenaline-secreting pheochromocytoma cells than in noradrenaline-secreting cells.

The amount of [^{3}H]DADLE, [^{3}H]EKC or [^{125}I]β-endorphin bound to pheochromocytoma membranes was not correlated with that of [^{3}H]diprenorphine bound. These findings suggest that the subtype composition of opioid receptors varies considerablely in pheochromocytomas.

Effects of Gpp(NH)p on [^{3}H]DADLE binding were examined to study the possible coupling of opioid receptors to GTP-binding proteins in human adrenal medulla. Gpp(NH)p inhibited [^{3}H]DADLE binding to normal adrenal medulla, suggesting the coupling of opioid (DADLE) receptors to GTP-binding proteins. The extent of Gpp(NH)p inhibition of [^{3}H]DADLE binding was 37 ± 8% in normal adrenal medulla. In pheochromocytomas, Gpp(NH)p inhibited [^{3}H]DADLE binding in only 8 out of 14 tumors. Apparent Gpp(NH)p inhibition of [^{3}H]DADLE binding varied from 19 to 76% inhibition in these 8 tissues.

PT-sensitive GTP binding proteins were demonstrated in normal human adrenal medulla by using the ability of PT to [^{32}P]ADP-ribosylate the proteins (1.05 ± 0.24 pmol/mg protein). In pheochromocytomas, the amount of PT-sensitive GTP-binding proteins was not correlated with the extent of Gpp(NH)p inhibition of [^{3}H]DADLE binding.

In the present study, the presence of opioid receptors and PT-

sensitive GTP-binding proteins were demonstrated in normal and transformed human adrenal medullae. Our results suggest that the subtype composition of opioid receptors varies considerablely in pheochromocytoma. The amount of [^{3}H]diprenorphine bound, as a measure of total opioid receptor content, was higher in adrenaline type than in noradrenaline type pheochromocytomas. These findings may be in line with the observations that naloxone increases plasma adrenaline but not noradrenaline in the normal human (Mannelli et al. 1984) and increases plasma catecholamines in adrenaline type but not in noradrenaline type pheochromocytomas (Maggi et al. 1985). Interestingly, enkephalins are more rich in adrenaline cells than in noradrenaline cells of bovine (Livett et al. 1982) and hamster adrenal medulla (Pelto-Huikko et al. 1982). In addition, tissue content of opioid peptides is higher in adrenaline type than in noradrenaline type pheochromocytomas (Imura et al. 1984). Taken together, these findings may suggest that opioid peptides and opioid receptor systems are located mainly in adrenaline-secreting chromaffin cells in adrenal medulla and that this preferential localization is maintained in neoplasmic adrenal medulla.

The Gpp(NH)p inhibition of opioid binding was modifed variously in pheochromocytomas. These findings suggest that functional aspects of opioid receptor system is also important as the quantitative one to consider modulatory action of opioid peptides in pheochromocytoma. In addition. the observation that the extent of the Gpp(NH)p inhibition was not correlated with the amount of PT-sensitive GTP-binding proteins may suggest the uncoupling of opioid receptors from PT-sensitive GTP-binding proteins. In conclusion, it was shown that adrenal medullary opioid receptor-GTP-binding protein system is variously modified by neoplasmic changes of chromaffin cells.

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PERTUSSIS TOXIN TREATMENT RESULTS IN TOLERANCE TO THE DEPRESSANT EFFECTS OF OPIOID, MONOAMINERGIC, AND MUSCARINIC AGONISTS ON DORSAL-HORN NETWORK RESPONSES IN MOUSE SPINAL CORD-GANGLION CULTURES

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ABSTRACT

After treatment of mouse spinal cord-ganglion explants with pertussis toxin (PTX), the acute depressant effects of opioids on sensory-evoked dorsal-horn network responses are markedly attenuated, and characteristic cord discharges can then occur even in the presence of >100-fold higher opioid concentrations, as observed after chronic exposure to opioids. The usual acute depressant effects of serotonin, norepinephrine, and oxotremorine on dorsal-horn discharges are similarly attenuated in PTX-treated cultures. These results together with our previous physiologic and biochemical analyses of adenylate cyclase (AC) and cyclic AMP (cAMP) activities in cord-ganglion cultures suggest that the neuromodulatory effects of opioid, monoaminergic and muscarinic agonists on primary afferent networks in the spinal cord may be mediated by binding to neuronal receptor subtypes that are negatively coupled via G_i to a common pool of AC.

INTRODUCTION

Exposure of cord-DRG explants to opiates or opioid peptides results in stereospecific, naloxone-reversible, dose-dependent depression of sensory-evoked dorsal-horn synaptic-network responses within a few minutes (Crain et al.1978). When cord-DRG explants are treated briefly with cAMP analogs or forskolin (a selective activator of AC; Seamon & Daly 1981), the usual depressant effects of opioids, as well as serotonin and norepinephrine, on sensory-evoked dorsal-horn responses are markedly attenuated (Crain et al.1986), resembling some aspects of the tolerance that develops in cord-DRG explants after chronic exposure to opioids (Crain 1984; Crain et al.1979; 1982). PTX is known to interfere with the guanine nucleotide protein G_i. that is required for opioid, α_2-adrenergic, and muscarinic receptor-mediated inhibition of AC in various cells (Katada & Ui 1982; Kurose et al. 1983; Ui 1984). The present study shows that PTX selectively blocks the depressant effects of opioid and other G_i mediated agonists on dorsal-horn responses in cord-DRG explants, consonant with PTX effects on guinea pig ileum (Lujan et al.1984; Tucker 1984) and isolated DRG neurons (Holz et al.1986).

MATERIALS AND METHODS

Spinal cord cross-sections with attached DRGs from 13-day fetal mice were explanted onto collagen-coated coverglasses and incubated at 35°C in Maximow slide culture chambers. Extracellular recordings

were made with Ag-AgCl electrodes via micropipettes (3-5 um tips) filled with isotonic saline. Electric stimuli were applied through pairs of similar pipettes with 10 um tips. Recordings were made in 0.5 ml of BSS (pH 7.2; temp., 33°C; see technical details in Crain 1976; Crain et al.1978,1982). Pertussis toxin was obtained from List Co., DADLE from Peninsula Co., oxotremorine from Research Biochemicals Co., and other chemicals from Sigma Co.

RESULTS AND DISCUSSION

Focal DRG stimuli elicit negative slow-wave potentials restricted to dorsal regions of spinal cord explants, arising abruptly after latencies of 2-3 msec, and often lasting more than 500 msec. These extracellularly recorded field-potentials resemble sensory-evoked synaptic-network responses in dorsal spinal cord in situ (Crain 1976). Introduction of morphine or DADLE into these cultures regularly led to sustained, dose-dependent, naloxone-reversible depression of DRG-evoked dorsal cord responses within 3-10 min (table 1). Measurements of the opioid sensitivity of dorsal cord responses in these explants were made systematically in the present study (n>40), (see also Crain et al.1978,1979,1982,1986). Similar dose-dependent depressant effects on these cord responses were produced by brief exposures (<10 min) to serotonin, norepinephrine, carbamylcholine and the muscarinic agonist, oxotremorine (table 1). The depressant effects of both cholinergic agonists were reversed or prevented by atropine (1-10 uM), but not naloxone, whereas the opioid-depressant effects were not antagonized by atropine.

In contrast, after pretreatment of cord-DRG explants with PTX (10 ug/ml for >1-2 days or 1 ug/ml for >4-5 days), the depressant effects of all of these opioid, monoaminergic and cholinergic agents failed to occur, and DRG-evoked cord responses were stably maintained during 10-30 min tests, often even with concentrations >100-fold -higher than levels which strongly depressed control explants (table 1). On the other hand, agents which produce depressant effects by more direct actions on ionic channels were still as effective on PTX-treated explants as in controls, e.g. Mg^{++} (5 mM, GABA (1 mM), xylocaine (ca. 100 uM), and ethanol (ca. 1-2%).

The blockade by PTX of the depressant effects of opioid, monoaminergic and muscarinic agonists on dorsal-horn responses in cord-DRG explants is consonant with our demonstration that elevation of intracellular cAMP levels produces similar, though less effective, interference with these depressant actions (Crain et al. 1986). The electrophysiologic data, together with correlative assays of AC activity (see below), suggest that opioid, serotonergic, noradrenergic and muscarinic agonists may all act on receptor subtypes on DRG and cord neurons that are negatively coupled via G_i to a common pool of AC. The resulting decrease in AC/cAMP levels may, in turn,

TABLE 1

PTX-Blockade of Acute Depressant Effects of Opioid, Monoaminergic, and Muscarinic Agonists on Dorsal-Horn Responses

	Effective Depressant Concentration (uM)[a]		
Agonist	Control	PTX-Treated[b]	n*
morphine	0.1 - 1	>10 - 100	10
DADLE	0.03 - 0.3	>1 - 10	4
serotonin	0.01 - 0.1	>10	6
norepinephrine	0.1 - 1	>30 - 50	4
carbachol	0.3 - 3	>50	2
oxotremorine	0.1 - 1	>10 - 25	5

[a] Concentration required to depress sensory-evoked dorsal-horn response in DRG-cord explant by >50% (Crain et al. 1978).

[b] DRG-cord explants (ca. 2 wks in vitro) were pretreated with 10 ug/ml PTX for 1-2 days (see text).

* Number of PTX-treated explants tested; 108 for each agent in tests on control cultures.

regulate phosphorylation-dependent gates in K^+ (e.g. Siegelbaum et al.1982) and/or Ca^{++} channels (Reuter 1983) of DRG neurons, so that Ca^{++} influx in presynaptic terminals as well as in the perikarya is attenuated (Crain et al.1986). Our data do not preclude possible PTX interference with G_i. coupling to other second messengers in these neurons (Holz et al. 1986; Miller 1985).

The >100-fold tolerance to the depressant actions of opioids that develops in cord-DRG explants after chronic exposure to PTX, as well as to opioids (Crain et al.1979; Crain 1984), correlates well with the stable increase in AC activity observed after similar treatments of these explants (Crain & Makman 1986; Dvorkin et al.1985). In addition, PTX-treatment attenuated the inhibition of AC produced in control cord-DRG explants by levorphanol, and in preliminary assays, by serotonin and norepinephrine (Crain & Makman 1986). Our studies are consonant with the hypothesis that neurons may develop tolerance and/or dependence during chronic opioid exposure by a compensatory enhancement of their AC/cAMP system following initial opioid depression of AC activity (e.g. Collier 1980; Crain et al.1986; Klee et al.1984; Sharma et al.1977). Further studies with this in vitro model may help to determine the degree to which alterations in opiate receptor linkages to AC/cAMP and other second messenger systems are involved in the expression of physiological tolerance in the CNS.

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INHIBITION OF ADENYLATE CYCLASE AND INDUCTION OF HETEROLOGOUS DESENSITIZATION BY KAPPA AGONISTS IN RAT SPINAL CORD.

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ABSTRACT

Using crude P_2 membranes of adult rat spinal cord we were able to show that the κ opiate agonist U50488 significantly and dose-dependently inhibited the basal cyclase activity, while μ (DAGO) and δ (DADL) agonists were ineffective. The regulatory action was stereospecific and required the presence of GTP plus Na^+ as well as Ca^{2+} ions. This inhibitory effect of K agonists was also observed when the cyclase activity was stimulated by forskolin. Similar inhibition was observed in spinal cord-dorsal root ganglion cocultures. Following chronic exposure of cultured cells to etorphine or U50488, the κ agonists lost their ability to inhibit the cyclase. Furthermore, the desensitization process appeared to be. heterologous, since the α_2 adrenergic agonist, norepinephrine and the muscarinic agonist, carbachol exhibited significant lawer potency for inhibiting cyclase activity when compared to control cultures. These data suggest that in spinal cord, opiate receptors of the K type are negatively coupled to adenylate cyclase and the induction of tolerance produced by K agonists is related to alterations of post-receptor regulatory components.

INTRODUCTION

Many lines of evidence suggest that the spinal cord is an important site for the mediation of the antinociceptive action of opiates and endogenous opioids (Yaksh 1983). Pharmacological studies showed antagonism of opiate analgesia by injections of cAMP (Ho et al. 1973) or inhibitors of cAMP phosphodiesterase (Juma 1984). Furthermore, biochemical studies demonstrated that opiate agonist binding in rat spinal cord membranes is regulated by GTP and Na^+ (Kelly et al. 1982) suggesting a possible coupling to the adenylate cyclase system. The rat spinal cord contains high concentrations of K receptor sites (Gouarderes et al. 1985). However, contrary to the δ receptors very little is known about the modulation of adenylate cyclase activity by κ opiate ligands. The present communication shows that K opiate receptors are negatively coupled to adenylate cyclase in rat spinal cord and that K agonists induce heterologous desensitization upon chronic treatment.

METHODS

Rat (Sprague Dawley, male adult) spinal cords were homogenized in 10% sucrose (wt/wt) containing 1mM DTT and 1M Tris-HCL (pH 7.4). Nuclei and cell debris were discarded by centrifugation (800xg, 10min) end the supematant was centrifuged at 27,500xg for 3min. The crude P_2 membranes were washed twice and assayed for cyclase activity

according to the procedure of Salomon et al. (1974). Rat dorsal root ganglion-spinal cord primary cocultures were prepared according to routine methods (Ransom et al. 1977). After 3 weeks in culture the cells were washed twice with PBS and homogenized in 1mM DTT, 5mM $MgCl_2$ and 10mM Tris-HCL (pH=7.4).

RESULTS

We found that the K selective opiate agonist U50488 significantly and dose-dependently inhibited the basal cyclase activity of membranes from adult rat spinal cord (table 1), while μ (DAGO 10 µM) and δ (DADL10µM) agonists were ineffective. Etorphine (1µM) and levorphanol (1µM), two non-selective opiate agonists significantly affected the enzyme activity as well. The regulatory action was stereospecific since dextrorphan was unable to inhibit the basal cyclase activity. In all cases GTP (10μM) and NaCl(100mM) were required for the expression of agonist inhibitory action (data not shown). Naloxone, a non selective opiate antagonist fully antagonized etorphine inhibition whereas it partially reversed the U50488 response.

TABLE 1. Effect of opiates on basal adenylate cyclase activity in adult rat spinal cord membranes.

	Untreated membranes (% of control)	EGTA-treated membranes (% of control)
etorphine 1µM	80±3**	99±4
etorphine 1µM +naloxone 10µM	97±2	109±9
U50488 10µM	69±5 *	102±3
U50488 10µM +naloxone 10µM	87±4	
naloxone 10 µM	102±3	101±3
Levorphanol 1µM	79±2**	91±5
dextrorphan 10µM	103±5	95±5

The experiments were performed in the presence of 10µM GTP and 100mM NaCl. When EGTA(was included, it was present at 1mM concentration throughout the membrane preparation and the assay. The adenylate cyclase activity of EGTA-treated membranes and untreated membranes was 0.81±0.03 and 3.89±0.01 nmoles cAMP/15 min/mg protein, respectively. The results are expressed as percent of control. The data represent the mean +SEM of 2-6 separate experiments performed in triplicate. The significance of the data was calculated by unpaired student t tests (*p<0.01; **p<0.001).

When the membranes were treated with 1mM EGTA, the basal cyclase activity decreased by 4-fold and the opiate inhibition was no longer observed. To study opioid regulation of cyclase following chronic agonist treatment, we used homogenates of rat spinal cord-dorsal root ganglia primary cocultures (3 weeks in vitro). In control cultures, we found that the K agonist inhibitory action exhibited similar properties to those displayed in adult spinal cord preparations.

TABLE 2. Effect of chronic treatment of rat spinal cord-dorsal root ganglion primary cultures with etorphine on adenylate cyclase inhibition by opiates, carbachol and norepinephrine.

	untreated cells (% activity)	Etorphine treated cells (% activity)
basal activity	100 (4.97±0.14)	100 (4.87±0.33)
+U50488 10µM	86±2**	102±7+
+U50488 10µM +naloxone 100µM	98±1	100±1
+naloxone 100µM	99±2	102±1
Forskolin stimulated activity (25µM)	100 (13.76±0.84)	100 (13.66±0.43)
+U50488 10µM	78±10*	101±4++
+U50488 10µM +naloxone 100µM	94±7	97±4
+naloxone 100µM	98±3	97±6
+carbachol 1mM	79±3	97±7++
+norepinephrine 1mM	57±5	76±2+++

Cells were grown for 3 weeks in vitro, the last 4 days in the presence of etorphine (1µM). Assays were performed in the presence of 10µM GTP, 100mM NaCl and included the opiates, forskolin, carbachol and norepinephrine in the concentration stated. Cells remained in the. presence of etorphine. until the assay. Then numbers in parentheses correspond to the adenylate cyclase activity in moles/15 min/mg protein. Results are expressed as percent of activity. The data correspond to the mean±SEM of 2-6 separate experiments performed in triplicate. For each experiment, at least 2 different cultures were used. The significant of thedatawas calculated by unpaired student t test (significance versus basal or forskolin stimulated activities in control cells*, *p<0.01; **p<0.001; significance versus untreated cells in etorphine treated cells +p<0.05, ++p<0.02, +++p<O.OO1).

In addition, as with the spinal cord tissue the forskolin stimulated cyclase was inhibited by norepinephrine and carbachol (43 and 21% respectively). Following chronic exposure of cells to U50488 (10µM) or etorphine (1µM) for 4 days, the K agonist U50488 lost its ability to inhibit the cyclase, while the basal as well as the forskolin-stimulated enzyme activities remained identical to those of paired control cultures (table 2). Furthermore, the addition of opiate antagonists such as naloxone (100µM) did not produce any overshoot of adenylate cyclase activity. Interestingly, the desensitization of the agonist action on cyclase activity was found to be heteroloqous since norepinephrine and carbachol exhibited significant lower potency for inhibiting spinal adenylate cyclase following chronic treatment by the opioid ligands as compared to control untreated cultures.

DISCUSSION

Our biochemical data demonsirate that in the spinal cord, opiate receptors are negatively coupled to adenylate cyclase. The ineffectiveness of μ and δ ligands as well as the ability of K agonists to modulate the enzyme activity suggest that the receptors involved are likely of the K type. The lack of effect of K agonists on basal enzyme level upon EGTA treatment of membranes suggests the participation of Ca^{2+}-calmodulin in K opiate-dependent inhibitory circuits. such involvement of Ca^{2+}-Calmodulin has been suggested for adenosine and enkephalin inhibition of rat hippocampal cyclase (Girardot et al. 1983). Interestingly, the desensitization process was shown to be heteroloqous, since the α_2 adrenergic agonist norepinephrine and the muscarinic agonist carbachol exhibited lower potency on cyclase activity after chronic exposure of cultured cells to opiate agonists. This is in good agreement with recent electrophysiologic studies showing that cAMP or forskolin not only attenuate the depressant effects of opioids on sensory-evoked dorsal horn responses in mouse spinal cord-ganglion explants but antagonized as well the depressant action of norepinephrine and serotonin (Crain et al. 1986). Such a pattern of regulation completely differs from that observed in NG108 15 cells, where chronic agonist treatment leads to homologous desensitization with a concomittant increase in adenylate cyclase activity and subsequent receptor down-regulation (Law et al. 1983). In contrast, K receptors in spinal cord cultures only poorly down-regulate (Attali et al. this volume). Furthermore, the lack of compensatory increase in adenylate cyclase activity as well as the heterologous pattern of desensitization rather suggest that the induction of tolerance produced by K opiates in the spinal cord is related to alterations of post-receptor regulatory mechanisns such as changes in the balance of Gi/Gs or in the functional state of Gi.

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SPARE LIGAND BINDING SITES IN THE COUPLING OF OPIOID RECEPTOR TO BRAIN GTPase

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ABSTRACT

Receptor alkylation with 10 μM superfit (SF), an irreversible delta-specific opioid ligand, in rat brain membranes abolished 73% of available binding sites for ^{3}H-DSLET without affecting the stimulation of high affinity GTPase by DSLET. Alkylation with 25 μM SF further decreased the number of low affinity sites and revealed a slight inhibition of GTPase stimulation. Membrane treatment with 75 μM SF abolished high affinity ^{3}H-DSLET binding and reduced GTPase stimulation to below 50% of control accompanied by a 10-fold decrease in the potency of DSLET. Protective alkylation with 25 μM β-CNA in the presence of DSLET yielded similar results. The findings describe spare delta receptors in their coupling to the GTPase effector component in brain, and reveal possible functional significance of the low affinity ligand binding sites.

INTRODUCTION

In rat brain membranes opioid agonists stimulate high affinity GTPase (Barchfeld et al. 1984), recently identified as the inhibitory guanine nucleotide binding protein in brain (Milligan and Klee 1985). The stereospecific effect is blocked by opioid antagonists, and the stimulation by mu, kappa, or delta ligands can be prevented by alkylating the respective opioid receptors (Clark and Medzihradsky 1985, Clark et al. 1986). In the present study, we have investigated the correlation between delta receptor occupancy and GTPase stimulation by gradual alkylation of the receptor with superfit, a delta-selective (Smith et al. 1985) irreversible ligand.

MATERIALS AND METHODS

Membrane preparation (Fischel and Medzihradsky 1981). Membranes from rat cerebrum were isolated bv differential centrifuaation as described. Aliquots of the membrane suspension were kept frozen until use.

Receptor alkylation (Clark et al. 1986). Membranes were incubated at 25" with different concentrations of SF (methylfentanrlisothiocyanate) and subsequently washed by centrifugation and resuspension. Alternatively, the membranes were incubated with β-CNA in the presence of protecting concentrations of DSLET.

Binding assay (Fischel and Medzihradsky 1981). After incubation at 25° to reach binding equilibrium, the suspension of brain membranes

in the assay medium containing 120 mM Na^+ was quickly filtered and the bound radioactivity determined. Opioid receptor binding of ^{3}H-DSLET was determined as the difference between binding in the absence and presence of 50 µM unlabeled DSLET. Kd and Bmax were estimated from Scatchard plots after resolving the high and low affinity binding components according to Neal (1972). Final values for the binding parameters were derived through the use of computer analysis and statistical evaluation as described (Fischel and Medzihradsky 1981 1986). Presented are the means of results obtained in 3-5 experiments, with each sample carried out in duplicate.

GTPase assay (Clark et al. 1986). The assay is based on the release of inorganic phosphate from (gamma-^{32}P)GTP in the presence of different concentrations of opiates (and 120 mM NaCl). The released ^{32}P-phosphate was separated from the nucleotides by adsorption on charcoal, and the radioactivity determined by liquid scintillation counting. The results are expressed as concentration of a compound to produce half-maximal stimulation of GTPase (Ks) and as maximal stimulation of basal rate (Smax). Shown are the means of data from 3-5 experiments, each sample run in triplicate

RESULTS AND DISCUSSION

Pretreatment with 10 µM SF markedly reduced ^{3}H-DSLET binding to opioid receptor (fig. 1). Scatchard analysis showed the presence of both high and low affinity sites with little effect on the respective Kd's (table 1). In these membranes the ratio of Kd/Ks for the high affinity binding component was essentially unchanged from that in control membranes (table 1), suggesting the importance of these binding sites for coupling to the effector component (Fantozzi et al. 1981). However, following alkylation with 75 µM SF, high affinity ^{3}H-DSLET binding was undetectable but GTPase stimulation, although impaired relative to control, still occurred. Work is in progress to elucidate the role of the high and low affinity opioid receptor sites in the interaction with the guanyl nucleotide binding protein and adenylate cyclase.

The possibility was considered that at the higher concentration of SF, partial alkylation of the mu receptor could interfere with the interpretation of our results. However, membrane treatment with 25 µM of the nonspecific alkylator β-CNA in the presence of 400 µM DSLET (Clark et al. 1986) reduced ^{3}H-DSLET binding by 72% without affecting GTPase stimulation by the delta opiate (Ks 6.5 µM, Smax 33%).

In the receptor alkylated membranes GTPase stimulation by DSLET was blocked by ICI 174,864, a delta specific antagonist. The treatment of membranes did not increase the basal rate of GTPase activity, and similar correlations between occupancy of delta receptor and GTPase stimulation were observed if ^{3}H-DSLET binding was determined in the GTPase assay medium. It should also be emphasized that the treatments with superfit did not significantly alter the Kd values of either the high or low affinity receptor binding sites for ^{3}H-DSLET (table 1), thus indicating the lack of allosteric binding of the alkylator.

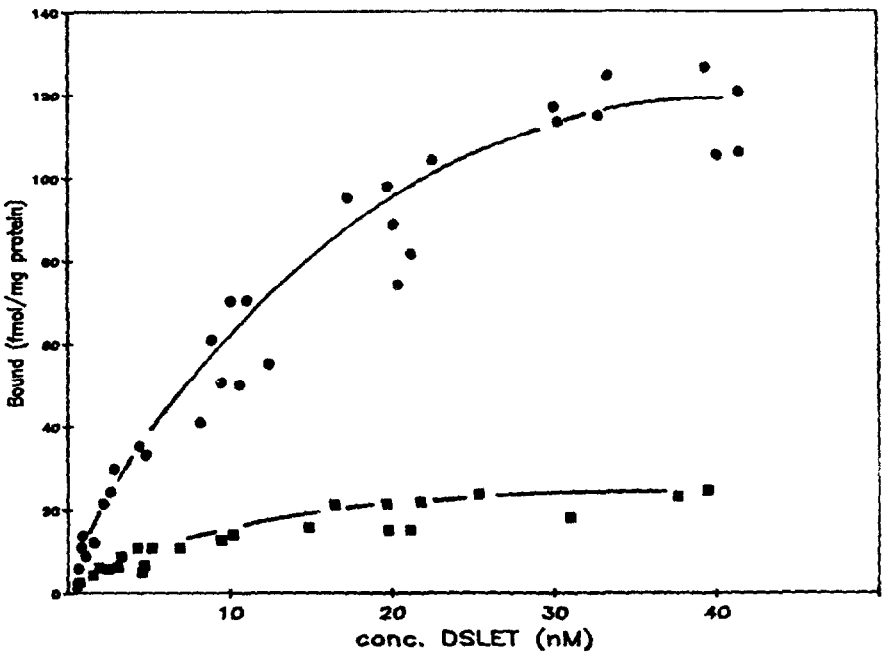

FIGURE 1. Opioid receptor binding of ^{3}H-DSLET in rat brain membranes. Cerebral membranes were incubated with (■) and without (●) 10 µM superfit as described under MATERIALS AND METHODS. Subsequently, the membranes were incubated with different concentrations of ^{3}H-DSLET to reach binding equilibrium at 65 min. Plotted is specifically bound radiolabeled ligand against the concentration of free DSLET in the medium.

TABLE 1

Parameters of opioid receptor binding and GTPase stimulation in brain membranes

	Receptor Binding (^{3}H-DSLET)					GTPase stimulation (DSLET)	
Membrane treatment (µM SF)	Kd1 (nm)	Kd2 (nm)	Bmax1 (fmol/mg)	Bmax2 (fmol/mg)	Available receptor sites (% control)	Ks (µM)	Smax (%)
0	1.43	19.3	1 5	122	100	5.6	27
10	1.37	19.2	5	34	27	7.5	2 5
10	1.20	13.5	6	23	21	11.0	19
75		18.3	*	18	13	57.0	1 3
100	1 0 0	-	*	*	*	-	*

* undetectable

To what extent maximal GTPase stimulation reflects inhibition of adenylate cyclase, the putative effector, is uncertain at this time. It is of interest to note the similar magnitude of Smax (table 1) and of maximal inhibition of adenylate cyclase in rat brain membranes (Law et al. 1981). Spare receptors have been implicated in the coupling of opioid receptor to adenylate cyclase in NG108-15 hybrid cells (Fantozzi et al. 1981, Law et al. 1983), and as determinants of dynorphin potency in smooth muscle preparation (Cox and Chavkin 1983).

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COMPARATIVE PROPERTIES OF SH-GROUPS AT MU. DELTA AND KAPPA OPIOID RECEPTOR

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ABSTRACT

Crude synaptosomal membrane from rat brain was treated with three kinds of DTNB analogs. The receptor bindings of opioids were inhibited on a concentration dependent manner by 5-nitro-2-PDS and DTNHEB, regardless of the receptor types. DTNB showed complete inhibition of the bindings of mu and delta agonists. But, the effect of DTNB on the binding of kappa agonist was incomplete, resulting in approximately 50% inhibition at the maximal effective concentration. Treating the membrane with DTNB, but not with DTNHEB and 5-nitro-2-PDS. completely eliminated inhibitory effects of guanine nucleotides on agonist binding. These results suggest that one SH-group, being sensitive to DTNHEB, exists at an active center of the receptor binding site(R) and another SH-group, blocked by DTNB, forms a binding site for GTP in GTP-binding protein(Ni), and that a coupling mechanism between R and Ni at kappa receptor differs from those at mu and delta receptor.

INTRODUCTION

It is well known that sulfhydryl covalent linking agents such as NEM and DTNB discriminate between agonist and antagonist bindings to membrane bound and partially purified opioid receptor, particularly when DTNB is used (Pasternak et al. 1975, Nozaki and Cho 1985). DTNB specifically reacts with all the surface SH-groups of protein which is not influenced by negative charge of DPNB. A SH-group in the opioid receptor binding site should interact with positively charged N-atom of opiate. Therefore, without too great difficulty, DTNB can react with the SH-group in the binding site. We compared the effects of differently charged DTNB analogs on the opioid receptor binding.

MATERIALS AND METHOD

DTNB and 5-nitro-2-PDS, positively charged analog of DTNB, were obtained from Dojindo Laboratories (Kumamoto, Japan). DTNHEB was newly synthesized. Tritiated DHM(80Ci/mmol), DADLE(45Ci/mmol), EKC(23.3Ci/mmol) and phencyclidine(43.5Ci/mmol) were purchased from New England Nuclear. Tritiated naloxone(45Ci/mmol). diprenorphine (25Ci/mmol) and dynorphin-A(1-9)(25.3Ci/mmol) were obtained from Amersham.

Abbreviations used: NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTNHEB, 5,5'-dithiobis-(2-nitro-n-2'-hydroxyl ethylbenzamide); 5-nitro-2-PDS, 2.2'-dithiobis-(5-nitropyridine); DHM, dihydromorphine; EKC, ethylketocyclazocine.

Crude synaptosomal membrane from rat brain without cerebellum was preincubated at 20°C for 20 minutes with DTNB analog. After washing the membrane by centrifugation, the membrane was incubated at 20°C for 30 minutes with ^{3}H-opioid ligand and filtered over glass filter (GF/B). In the case of dynorphin-A(1-9). the binding assay was carried out with incubation at 4°C for 90 minutes. All assay tubes were coated with silicon to prevent adsorption of dynorphin. The filter was pre-soaked with pH 7.4, 50 mM Tris-buffer containing 0.4% BSA and 0.1% polylisine. The filtrated residue was washed with the ice-cold buffer containing 0.1% BSA and 100 mM cholin chloride.

RESULTS AND DISCUSSION

The P2-membrane was pretreated with varying concentrations of DTNB, DTNHEB or 5-nitro-2-PDS. The inhibition profiles of the receptor bindings of a variety type of opioid by DTNB analogs are shown in fig.1.

Positively charged analog of DTNB, 5-nitro-2-PDS, and neutral analog, DTNHEB, inhibited the receptor bindings of all types of opioid ligands except phencyclidine on a concentration dependent manner. The effect of 5-nitro-2-PDS was more potent than that of DTNHEB. DTNB inhibited agonist bindings of mu and delta ligands. and showed no effect on the binding of naloxone. In the case of EKC and dynorphin-A(1-9) bindings, the inhibitory effects of DTNB were incomplete, resulting in only 50% at the maximum inhibition. When DTNHEB was applied to the DTNB-treated membrane which showed the maximum inhibition on the binding of EKC, DTNHEB exerted an additional inhibitory effects on EKC and dynorphin bindings (Niwa et al. 1983).

These suggest that a state of SH-group at kappa receptor, being sensitive to DTNB, might be different from that of mu or delta receptor. No influences of DTNB and its analogs were observed at the binding of phencyclidine.

After preincubating the membrane with 50 nM DADLE for 20 minutes, 500 μM DTNB or 10 μM DTNHEB was added. At appropriate time, the membrane was washed and DADLE binding was evaluated. The inhibitory effect of DTNB on DADLE binding to the membrane was not modified by the preincubation with DADLE. However, the effect of DTNHEB decreased at the DADLE-pretreated membrane.

DTNB and its analogs potentiated sodium effects on opioid agonist binding. There is no difference in the potentiation effects between DTNB- and DTNHEB-treatment.

Guanine nucleotide inhibits opioid agonist binding to the receptor. When the membrane was pretreated with DTNB, the inhibitory effect of guanine nucleotide completely disappeared, but DTNHEB showed no effect (fig. 2). Preteating the membrane with guanine nucleotide diminished the effect of DTNB, but not DTNHEB. A SH-group, blocked by DTNB, may be different on a role from a SH-group, blocked by DTNHEB.

These results suggest that DTNB-sensitive SH-group exists in a binding site of guanine nucleotide in GTP binding protein and DTNHEB-sensitive SH-group is a functional group in the center of the receptor binding site.

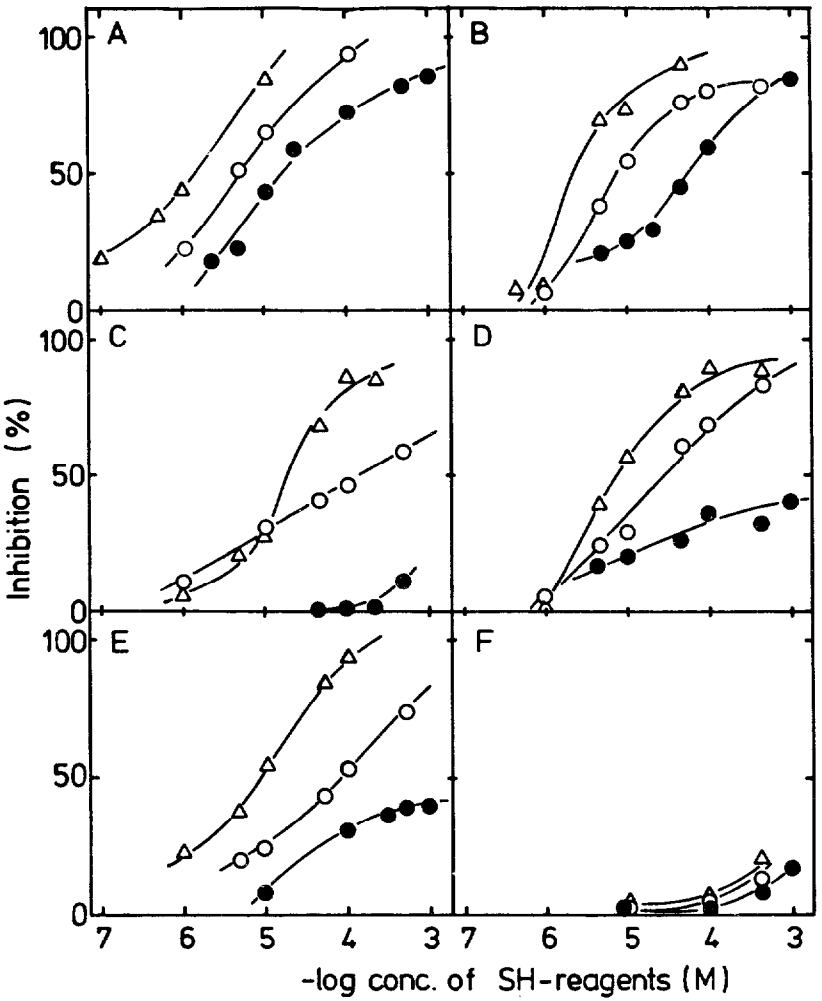

FIGURE 1

Inhibitory effects of DTNB analogs on the receptor binding of a variety type of opioid; A, dihydromorphine: B, DADLE; C, naloxone; D, ethylketocyclazocine; E, dynorphin-A(1-9) and F, phencyclidine.
DTNB, (●); DTNHEB, (○) and 5-nitro-2-PDS, (△).

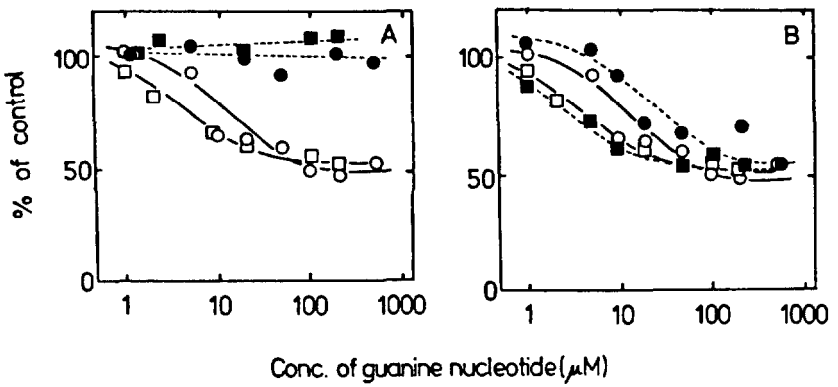

FIGURE 2

Effects of guanine nucleotides on ^{3}H-DADLE specific bindings to (A) 500 μM DTNB- and (B) 10 μM DTNHEB-treated membranes. Open symboles represent results at non-treated membrane (control) and closed symbols represent results at DTNB- and DTNHEB-treated membranes: GTP, (○,●) and GPP(NH)p, (□,■).

Reactivity of a SH-group in protein depends on its environmental factor, such as steric interactions and electrostatic effects. There are little differences in molecular size and general reactivity, except the effect of attracting force of charged group, among DTNB. DTNHEB and 5-nitro-2-PDS.

One SH-group in the opioid receptor may be located in an active center of an anionic binding site for the ligand binding. This SH-group is blocked by DTNHEB and 5-nitro-2-PDS. Another SH-group which is located in GTP-binding protein, is in a positively charged environment and specifically blocked by DTNB. The fact that opioid agonist binding is selectively inhibited by DTNB supports this conclusion.

Finally, since the maximum inhibitory effect of DTNB on kappa agonist binding is approximately 50%, a coupling mechanism at kappa receptor between the ligand binding site and GTP-binding protein may be different from those of mu and delta receptors.

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ENHANCEMENT OF PERFORANT PATH TRANSMISSION BY ENKEPHALIN ANALOGUES; ALTERATION OF GRANULE CELL INHIBITION/POTENTIATION

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ABSTRACT

Enkephalin analogues were electrophoretically applied into the dentate granule cell layer of the hippocampus in vivo to determine opioid effects on granule cell responsiveness to afferent input. The results indicate that opioids, probably acting at μ receptors, enhance dentate responsiveness to perforant path transmission. This enhancement includes decreased inhibition and increased potentiation.

INTRODUCTION

Immunocytochemical studies have shown that the dentate gyrus of the hippocampus of the rat contains endogenous opioid peptides (Gall et al. 1981, McGinty et al. 1984). To begin to understand the roles of these opioids, we have applied opioid peptides locally to the dentate granule cells, *in vivo*, to determine the effects of opioids on responsiveness of these cells to afferent input. In addition, we examined the relative potencies of selective opioid agonists in exerting these effects.

MATERIALS AND METHODS

These studies involved stimulation of the perforant path, which evokes an excitatory response by the granule cells. The excitatory response was recorded as a 'population spike' (PS) within the evoked field potential. Using halothane-anesthetized rats (250-350), a concentric bipolar stimulating electrode was stereotactically positioned within the perforant path (AP -8.1, L 4.4, V dura-3.5), and a glass 5-barrel recording pipet (8-12 m) was positioned within the ipsilateral granule cell layer (AP -4.0, L 2.5, V dura-2.7 to -3.5). The center barrel of the pipet contained Pontamine Sky Blue (2% in 0.5 M sodium acetate), used for marking the recording site. The outside barrels contained opioid peptides (5mM, pH 7-8 in saline) for electrophoresis; one barrel contained 3 M NaCl for current balancing.

Input/output (I/O) curves were constructed by plotting PS amplitude as a function of stimulus intensity. Paired-pulse data were obtained by presenting pairs of identical stimulus pulses (a Conditioning pulse followed by a Test pulse) at varying interpulse intervals; the paired-pulse curves represent the amplitude of the second evoked PS (Test PS) expressed as a percentage of the first evoked PS (Conditioning PS) for each interpulse interval.

RESULTS AND DISCUSSION

To obtain an initial assessment of opioid effects on dentate responsiveness to single or paired-pulse stimulation, analogs of

methionine-enkephalin or leucine-enkephalin were electrophoresed within the granule cell layer. The analogs, [2-D-Ala,5-Met]enkephalinamide (DAMEA) and [2-D-Ala,5-D-Leu]enkephalin exerted similar effects. Figure 1 illustrates the effect of DAMEA (25 and 75 nA) on responsiveness to single-pulse stimulation. DAMEA shifted the I/O curve to the left and increased the maximum PS amplitude, indicating an increased responsiveness to perforant path stimulation. This effect was reversed by intravenous naloxone administration (figure 1) as well as by local electrophoretic naloxone administration (not shown).

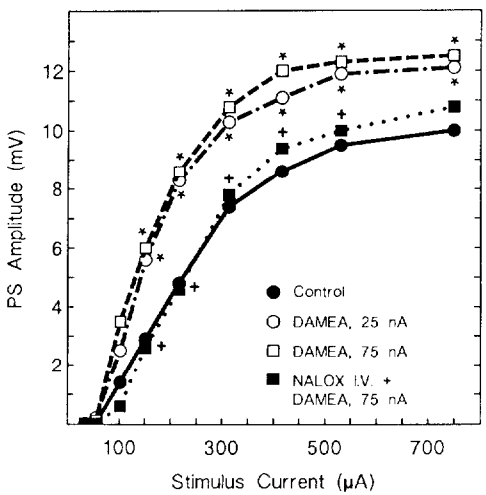

FIGURE 1. Effect of DAMEA (25 and 75 nA) on I/O curves, and blockade by naloxone (5 mg/kg i.v.); N=5.
* p<.05 vs. Control; + p<.05 vs. 75 nA DAMEA [ANOVA].

In order to investigate potential opioid modulation of local hippocampal circuitry characteristic of the dentate gyrus, we have employed a standard paired-pulse paradigm of stimulation. When the stimulus current employed is adjusted to evoke a half-maximal conditioning PS, two local circuitry effects characteristic of the dentate gyrus can be observed (figure 2, Control). One such effect, that of recurrent (or feed-forward) inhibition, is associated with interpulse intervals of less than 45 msec and is marked by an inhibited test response. A second, potentiating effect is observed at interpulse intervals ranging from 50-150 msec.

DAMEA increased the Test PS relative to the Conditioning PS at interpulse intervals ranging from 30 to 120 msec (figure 2). This effect could be antagonized by both intravenous and local naloxone administration. Since the effect of DAMEA is observed over a wide range of interpulse intervals, the increase could be due to a change in the sequential inhibition or in the sequential potentiation. We have found, however, that these two phenomena can be partially dissociated by altering the experimental parameters. When the electrophoretic application of the opioid is restricted to a short time period (eg. less than 11 min.), the relative enhancement of the

test response is restricted to the portion of the curve dominated by the sequential inhibitory effect (Wiesner and Henriksen 1986). This result suggests that DAMEA is able to exert a disinhibitory action in the dentate similar to that seen in the CA subfields (Zieglegansberger et al. 1979). Alternatively, when the stimulus current is adjusted to evoke only a threshold conditioning PS, the effect of sequential inhibition is minimized, essentially isolating the sequential potentiation effect. Under these experimental conditions, DAMEA greatly increased the relative amplitude of the test response over a broad range (not shown). It therefore appears that DAMEA increases sequential potentiation as well as decreasing sequential inhibition.

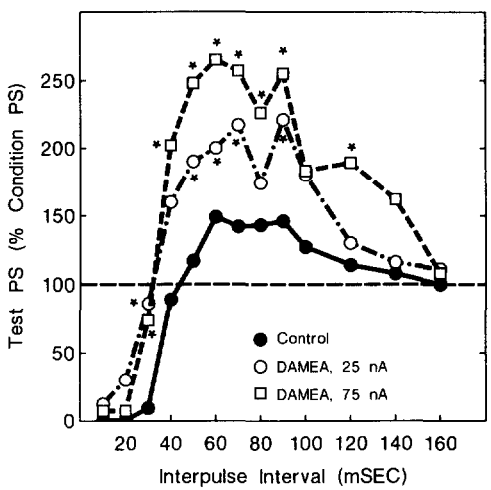

FIGURE 2. Effect of DAMEA (25 and 75 nA) on paired-pulse curves (see MATERIALS AND METHODS); N=5. * p<.05

To obtain an initial indication of the specific opioid receptor type(s) which may be involved in these opioid effects on perforant path transmission, we applied various selective opioid agonists to the granule cell layer. Figure 3 shows a comparison of effects on the I/O curve of a few prototypic agonists, along with DAMEA (25 nA for 4-6 min). The most effective of the selective agonists was the μ agonist PL017 morphiceptin. Neither the 6 agonist D-pen,L-pen-enkephalin (DPLPE), nor the κ agonist U-50488H were effective at this dose level. Similar differential effects were observed using paired pulse paradigm. These results are suggestive that the opioid effects may be mediated at μ receptors.

We conclude from these results that opioid peptides,by acting at opioid (possibly μ) receptors, can enhance dentate responsiveness to perforant path input. Components of this enhanced responsiveness are: (1) increased primary responsiveness to single stimuli; (2) decreased sequential inhibition; and (3) increased sequential potentiation. In excitatory effects of opioids in the CA subfields (Zieglegansberger et al. 1979), it seems likely that the disinhibitory effect indicated in the present studies may in turn be responsible for the increased

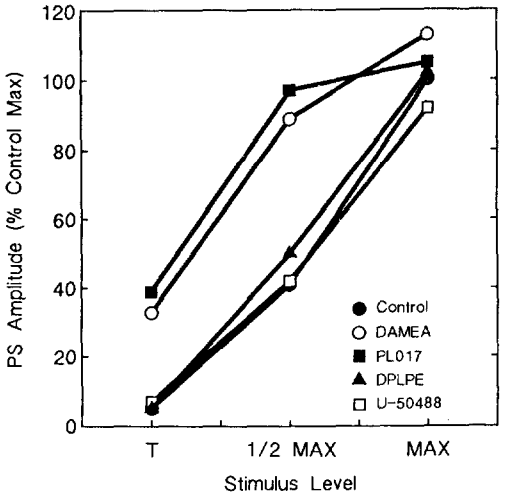

FIGURE 3. Effect on the I/O curve of DAMEA, PL017 (μ agonist), DPLPE (δ agonist), and U-50488H(κ agonist), (25 nA, 4-6 min, N=3-6). Stimulus levels were threshold (T), half-maximal (1/2 MAX), or maximal (MAX) for response levels of each animal; responses were normalized as a percentage of the Control maximum for each test.

primary responsiveness. Finally, our studies of single unit activity have shown that, while exerting these facilitatory effects on perforant path transmission, opioids, in fact, suppress the spontaneous activity of individual granule cells (Wiesner and Henriksen 1986). The facilitating effect reported here, therefore, may be specific to perforant path transmission.

ACKNOWLEDGEMENTS

Sources of peptides were: Dr. N. Ling (DAMEA), Endo Laboratories (naloxone), Peninsula (PL017, DPLPE), Upjohn, Inc. (U-50488H). The work was supported by NIDA grant DA03365.

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MODULATION OF BRAIN NMDA RECEPTORS: COMMON MECHANISM OF σ/PCP RECEPTORS AND THEIR EXOGENOUS AND ENDOGENOUS LIGANDS

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ABSTRACT

Quantitative autoradiographic distribution patterns of σ/PCP and NMDA receptors show striking similarities but not in all brain areas. We have identified an endogenous ligand of brain PCP receptors which noncompetitively antagonizes NMDA-induced neurotransmitter release from brain slices. These data support the concept that the σ/PCP receptor ligands may exert their unique behavioral effects by indirect modulation of NMDA-mediated transmission.

INTRODUCTION

Binding of SKF10,047-like benzomorphans, phencyclidine (PCP) derivatives, dioxolanes, and benz-f-isoquinolines to σ/PCP receptors selectively modulates the excitatory effects of the prototypic excitatory amino acid agonist n-methyl-d-aspartate (NMDA). Such drugs potently and stereoselectively antagonize the excitatory effects of NMDA on spinal neurones noncompetitively (Berry et al. 1984). In addition PCP-like drugs potently inhibit NMDA-stimulated acetylcholine and dopamine release from rat striatal slices (Snell and Johnson 1985). These effects are selective in that these drugs fail to modulate the excitatory responses to quisqualate (q) or kainate (k) type excitatory amino acid receptors. In this study we investigate the neuroanatomical and functional relationships between σ/PCP and NMDA receptors by autoradiographic and biochemical techniques using exogenous and endogenous ligands of the σ/PCP receptor.

MATERIALS AND METHODS

1. Comparative Quantitative Autoradiographic Localizations:

Adult male Sprague-Dawley rats were decapitated and brains were rapidly removed and frozen on powdered dry ice, 6 µ sections were cut at various levels of the brain. Alternate sections were radiolabeled for PCP and NMDA receptors.

a) [^{3}H]TCP Binding to PCP Receptors:

Brain sections were preincubated in 5 mM Tris-HCl at 37°C for 30 min, then incubated with 5nM [^{3}H]TCP (52.9 Ci/mmol, NEN, MA) in 5 mM Tris-HCl ph 7.4 at 4°C for one hr (Sircar and Zukin 1985). Adjacent sections were incubated under the same conditions but in the presence of 10 µm non-radioactive PCP. At the end of incubation,

slides were washed sequentially through 6 rinses (6 sec each) of 5 mM Tris-HCl, pH 7.4 At 4°C. Slides were rapidly dried under a stream of cold air. They were tightly juxtaposed against tritium sensitive film (Ultrofilm, LKB Instruments, Gaithersburg, MD) at room temperature for 4 weeks. 10 μ thick sections were cut from a tritium standard (^{3}H-microscale block, Amersham, IL). These sections were dry-fixed to slides and at least one such ^{3}H-microscale containing slide was placed against each film.

b) [^{3}H]L-Glutamate Binding to NMDA Receptors:

Slides containing brain sections were preincubated at 30°C for 10 min to remove endogenous lutamate. The sections were incubated for 30 min at 4°C in 100 nM [^{3}H]L-glutamate (47.7 Ci/mmol, NEN, MA) in 50 mM Tris-acetate ph 7.2 at 4°C (Monaghan and Cotman 1985). Alternate sections were incubated under the same conditions but in the presence of 100 um NMDA. Following incubation the sections were washed four times in ice-cold 50 mM Tris-acetate buffer for a total time of 30 sec. Sections were dried and tightly juxtaposed against tritium-sensitive film for 1-3 weeks at 4°C. Each film also carried ^{3}H-microscale-containing slides.

c) Development and Quantification of Films:

Following exposure, films were developed in Kodak D19 for 5 min at 20°C, rinsed in water for 20 sec and fixed for 10 min. Autoradiograms were analyzed by an image processor (Quantimet 920, Cambridge Instruments, UK) which produced an electronic image of the autoradiographic section with point-to-point digitization to give optimal resolution and gray-scale separation. The system was then calibrated to convert gray levels into optical density values by using standards of known optical density. Color-coded images of the brain section for receptor density (fmol/mg tissue) were generated.

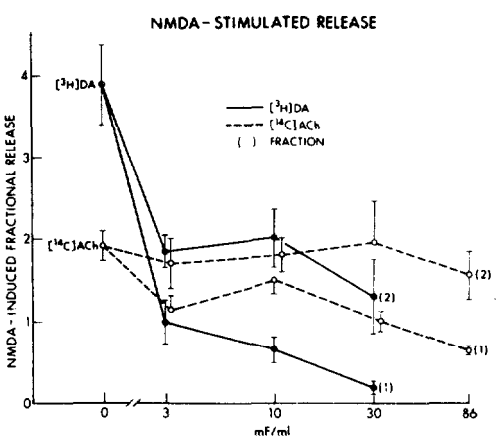

FIGURE 1: Effect of Hippocampal HPLC Fractions 23-38 (1) and 56-66 (2) on NMDA-stimulated [^{3}H]DA and [^{14}C] ACh Release

2. Extraction of Endogenous PCP-like Ligand:

Six hundred bovine hippocampi were freshly dissected from fresh

whole brains, immediately frozen on dry ice and lyophilized. Dried fragments (1 fragment = 1 hippocampus) were acetone-defatted and hot-acid extracted using the method of Vale et al. (1983). Extracts were further purified with 300 g Vydac reverse phase C_{18} sorbent. Prior to assay, aliquots of starting material were again purified by adsorption to hydrophobic derivatized gel using prepacked 6-ml bond elute columns containing 500 mg C_{18} sorbent. The crude starting material showed dose-dependent inhibition of [^{3}H]TCP binding at doses up to 100 millifragments (mf) per assay tube. This material was further purified by HPLC on a Vydac C_{18} preparative cartridge (5 X 30 cm) using a TFA/CH_3CN solvent system consisting of buffer A: 0.1% TFA and buffer B: 40% buffer A and 60% CH_3CN at a flow rate of 75 ml/min (Zukin et al. submitted for publication).

3. Effect of Endogenous Ligand on NMDA-Induced ACh and NE Release:

Male Sprague Dawley rats were decapitated and the corpora striata were removed, sliced and preincubated in a modified Tyrode's buffer adjusted to pH 7.4 for 10 min at 4°C. [^{3}H]choline hydrochloride or [^{3}H]dopamine was added to the buffer at final concentrations of 50 and 100 mM, respectively. Slices were then su erfused with the buffer solution containing 10 µM hemicholinium ([^{3}H]choline release) or 10 µM pargyline ([^{3}H]DA release). The superfusate was then collected and radioactivity was determined. NMDA-stimulated tritium release was calculated by the method of Snell and Johnson (1985). Assays were carried out in the absence of magnesium ions, which inhibit NMDA-induced neurotransmitter release.

RESULTS AND DISCUSSION

TABLE 1: Regional Distributions:A Similarities

REGION	RECEPTOR DENSITY [^{3}H]TCP*	RECEPTOR DENSITY [^{3}H]L-glutamate**
CA_1	98.4%	100.0%
CA_2	100.0%	97.0%
Dentate gyrus	87.7%	93.3%
Medial amygdala	71.9%	55.9%
CA_3	54.3%	62.0%
VMH	53.3%	34.8%
Post. cingulate cortex	38.2%	37.6%
Entorhinal cortex	36.4%	48.0%
Median eminence	34.2%	44.9%
Arcuate nucleus	33.9%	36.3%
Subiculum	28.7%	24.8%
Substantia nigra	25.4%	13.1%
Superior colliculus	17.9%	29.4%
Cerebellum	8.1%	13.7%
Ventral tegmental area	6.6%	20.9%
Pontine ret. Formation	5.1%	13.1%
Globus pallidus	1.3%	10.6%

* data expressed relative to CA_2 (receptor density = 100%)

** data expressed relative to CA_1 (receptor density = 100%)

We have demonstrated neuroanatomical and functional relationships between σ/PCP and NMDA receptors by autoradiographic, biochemical and bioassay techniques. Regional distribution of NMDA and PCP

receptors show striking similarities in certain brain areas (table 1) in agreement with Maragos et al. (1986). Hippocampus had the highest level of both types of receptors with hippocampal fields CA_1, CA_2, and dentate gyrus having higher levels than CA_3. Other regions that showed similar distributions included medial amygdala, VMH, various cortical areas, substantia nigra, cerebellum, ventral tegmental area, arcuate nucleus, median eminence, subiculum, pontine reticular formation and globus pallidus. NMDA and PCP receptor distributions were dissimilar in certain regions, most notably central gray, anterior cingulate cortex, caudate-putamen, nucleus accumbens, dorsal thalamus, septal area and preoptic nucleus (table 2). The endogenous ligand of σ/PCP receptors which we have identified mimics the effects of PCP-like drugs on the NMDA system. Partially purified bovine hippocampal extract was found to have PCP-like inhibitory effects on both NMDA-stimulated DA and ACh release (figure 1).

TABLE 2: Regional Distributions: Dissimilarities

REGION	RECEPTOR DENSITY [^{3}H]TCP*	RECEPTOR DENSITY [^{3}H]L-glutamate**
Dorsomedial hypothalamus	68.9%	44.5%
Central gray	30.5%	11.0%
Ant. cingulate cortex	26.9%	88.1%
Auditory cortex	22.0%	53.1%
Interpeduncular nucleus	21.9%	4.2%
Caudate-putamen	12.4%	60.7%
Preoptic	11.0%	33.5%
Laterodorsal thalamus	6.4%	81.5%
Mediodorsal thalamus	6.3%	36.4%
Nucleus accumbens	5.3%	83.8%
Lateral septum	3.9%	87.4%
Medial septum	1.7%	22.9%

* data expressed relative to CA_2 (receptor density = 100%)

** data expressed relative to CA_1 (receptor density = 100%)

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AN ENDOGENOUS LIGAND FOR PHENCYCLIDINE RECEPTORS: ISOLATED FROM HUMAN BRAINS

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ABSTRACT

Using preparative HPLC, Sephadex G-25 and analytic HPLC, we obtained an active peak inhibiting (^{3}H) PCP receptor binding, with a molecular weight of 3,000 daltons. Another isolation process included acetic acid extraction, Toyopearl HW gel filtration, Sephadex G-10 chromatography and analytic HPLC. An active peak was also observed, which was consistent with that from the first isolation process.

INTRODUCTION

Phencyclidine (1-(1-phenylcyclohexyl) piperidine, PCP) is a psychotomimetic drug. The existence of specific, high-affinity, stereoselective PCP binding site in mammalian brains has been reported by various groups since 1979 (Zukin and Zukin 1979, Vincent et al. 1979). Elucidation of endogenous ligand for PCP receptor may bring important imformation to the studies on mental activities and related diseases. Quirion et al. (1984) reported a fraction of PCP-like peptide isolated from the porcine brains. But the sequence has not been published yet. In our laboratory, an active peak inhibiting (^{3}H) PCP receptor binding was observed from human brains (Lu et al. 1986). I- this paper, we report further isolation and purification of this fraction.

METHODS

First isolation process: Human brains (willed contribution) were processed into acetone powder, extracted by acids and chromatographed on a preparative HPLC (Lu et al. 1986). The fractions containing activity were lyophilized and chromatographed by Sephadex G-25 (total volume 55 ml, superfine), and eluted with 0.5 M acetic acid. Then the active fraction from Sephadex G-25 was further purified by analytic HPLC with μ Bondapak C_{18} column, and eluted with a gradient of acetonitrite.

Second isolation process: Sample was extracted by 0.5 M acetic acid from acetone powder, and chromatographed on Toyopearl HW column (total volume 400 ml) at a flow rate of 40 ml/hr. The active fraction was processed through Sephadex G-10 for desalination and analytic HPLC for further purification.

(^{3}H) PCP receptor binding assay: Same as which described in our privious paper (Lu et al. 1986).

RESULTS

The 10th min fraction eluted by 30% acetonitrile in preparative HPLC

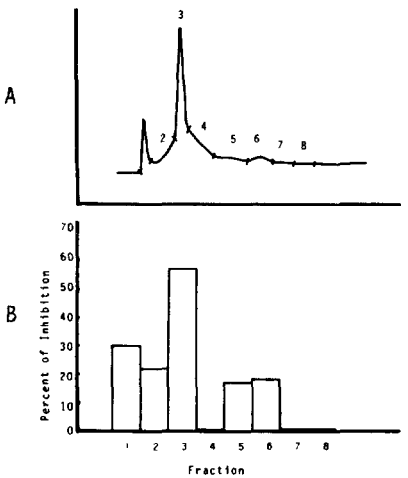

Fig. 1. Active peak (B) eluted from Sephadex G-25 column and peaks monitored by U.V. 254 nm (A); a further purification for active fraction obtained from preparative HPLC. The column was eluted by 0.5 M acetic acid, and collected by O.D. peaks and screened with (^{3}H) PCP binding assay.

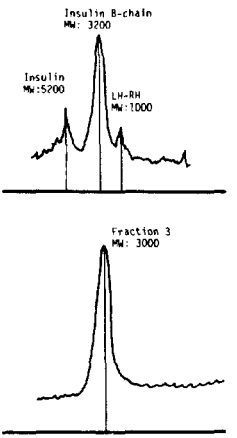

Fig. 2. Molecular weight of endogenous PCP-like substance. MW of fraction 3 from Sephadex G-25 (in fig.1) was determined on Sephadex G-25 column, checked with standards: insulin (MW 5,200), insulin B-chain (MW 3,200) and LH-RH (MW 1,000).

was chromatographed on a Sephadex G-25 column. An active peak (fraction 3) was overlapped with the main peptide peak monitored under U.V. 254 nm (fig.1). The molecular weight of the active substance is around 3,000 daltons (fig.2). The fraction 3 underwent through analytic HPLC; the active peak was located at around 30% acetonitrile elutions and the recovery was about 1 %, so another isolation process was used. As shown in fig. 3, desalted fraction G from Toyopearl HW gel filtration potently inhibited (^{3}H) PCP receptor binding, to non-specific binding level. The fraction G was not degraded by pronase (Sigma) (fig. 4). After the fraction G was chromatographed with analytic HPLC, the activity was decreased about 99 %.

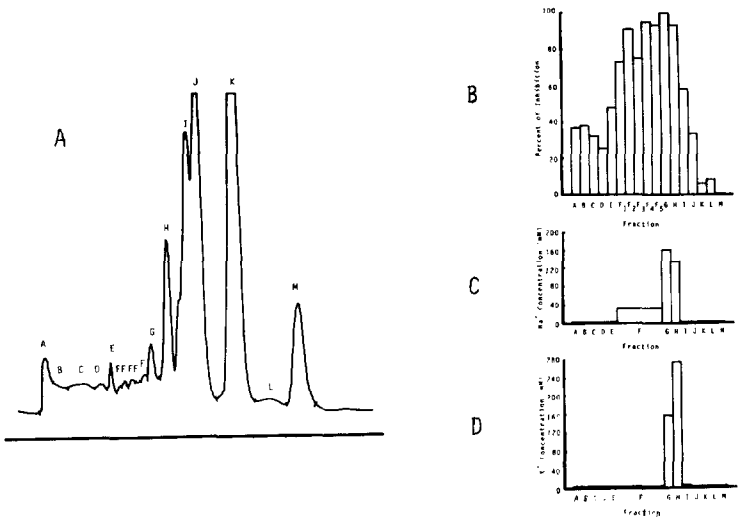

Fig. 3. Chromatogram (A) and peaksdetecting (^{3}H) PCP receptor activity (B), sodium (C) and potassium (D) content of fractions from Toyopearl HW gel filtration. Sample was extracted by 0.5 M acetic acid from acetone powder, and eluted by the same acid. Fractions were collected according to peak of O.D. under 254 nm ultraviolet spectrum. The fraction G and H were processed through Sephadex G-10 for desalination before (^{3}H) PCP receptor binding assay.

DISCUSSION

Many authors reported that known neurotransmitters and neuropeptides could not inhibit (^{3}H) PCP receptor binding (Quirion et al. 1984). In our experiments, the endogenous PCP-like substance was obtained with both isolatIon processes. Other factors, pH and salts, which interfere with (^{3}H) PCP binding, were ruled out in our experiments. So we consider that the active peak obtained in present studies represents the endogenous ligand for PCP receptors.

Quirion et al. (1984) reported that the PCP-like substance was a peptide. Analytic HPLC with reverse phase C_{18} column was usually used for purification of peptides, but in our experiment, the recovery of both preparative HPLC and analytic HPLC were as low as 1 %. Now we are trying new methods for purification of PCP-like substances.

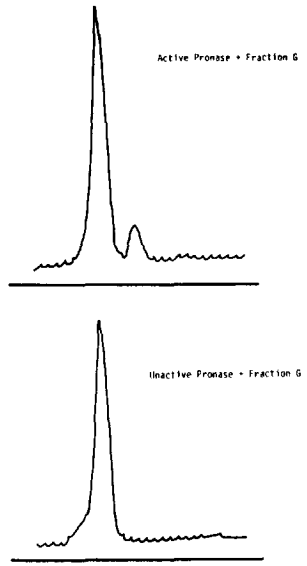

Fig. 4. Effect of pronase on fraction G from Toyopearl HW gel filtration and Sephadex G-10. In control (below), sample of fraction G was incubated with unactive pronase, which pretreated in boiling water for 10 min, in Tris-HCl buffer (pH 7.5) at 37°C for 30 min, then isolated by Sephadex G-25. Only one peak was observed, with receptor binding activity and a MW of around 3,000 daltons. But in incubation with enzyme (upper), the 3,000 daltons peak with (^{3}H) PCP binding activity was remained intact. Beside, there was a smaller peak (without binding activity, MW around 1,000) behind the active peak.

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The authors thank Dr. DK Huang, Mr. WM Xu and SD Lu for brains collection, and Ms. GZ Du for her excellent technician work. This work is sponsored by the Chinese Academy of Sciences and W.H.O.

AN ENDOGENOUS LIGAND FROM HUMAN CSF FOR THE PHENCYCLIDINE RECEPTOR

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ABSTRACT

A fraction which potently and specifically competed-with ^{3}H-PCP binding to rat membranes was isolatedfrom human CSF. This substanceran just before the salts in Sephadex G-25 column and was luted with low concentrations of acetonitrile in reverse phase HPLC predicating that it may be a small hydrophilic compound with a MW. of less than 1,000 daltons.

INTRODUCTION

Phencyclidine(PCP) has received increasing attention recently not only because of its abuse hazards, but also its unique CNS effects. PCP-induced psychosis resembles schizophrenia. Various laboratories described the specific binding sites of PCP in rat and human brains with characteristic regional distribution (Vincent et al.1979;Zukin and Zukin 1979;Sircar and Zukin 1983). Several papers suggested that PCP receptors were well related to its psychotomimetic effects and that endogenous ligands for the receptors might be present in CNS (Quirion et al. 1984). Therefore, a search for this kind of ligand will cast some light on the pathogenesis of schizophrenia. Quirion et al.(1984) reported a peptide ligand in porcine brain. In our laboratory, with preparative HPLC, a fraction which inhibited ^{3}H-PC? binding to rat membrane was found in human brain (Lu et al. 1986). Here, we report a fraction isolated from human CSF with potent 3-PCP binding activity.

MATERIALS AND METHODS

(+)-3H-PCP (48 Ci/mmol; 1 Ci=37 G Bq, New England Nuclear). PCP was synthesized by Dept. of Pharmaceutical Chemistry of this Univ. CSF was collected via lumbar puncture at 8-9 A.M. from patients suffering from peripheral neuritis or myelopathy without any psychotropic medication. It was frozen immediately after the collection, then lyophilized (5 ml in each tube) for use.

The lyophilizsd samples were resuspended in 0.5 M acetic acid and chromatographed on column of Sephadex G-25 fine (1.2x60 cm, 9 ml/hr) eiuted with 0.5 M acetic acid using LKB automatic collector, Aliquots were collected, lyophilized for H-PCP receptor binding assay. The fractions containing ^{3}H-PCP binding activity were pooled and chromatographed in 0.1% heptafluorobutyric acid, They were chromatographed on 3.9x30 cm u Bondapak C_{18} column at a flow rate of 0.6 ml/min. eluted with gradient 0-40% acetonitrile and were moni-

tored under 230 nm ultraviolet wave spectrum. The samples from analytical HPLC were further screened by ^{3}H-PCP binding assay.

Rat brain membranes were prepared according to Quirion et al.(1984) with a few modifications. For ^{3}H-PCP receptor binding assay, duplicate assay tubes contained 8 nM ^{3}H-PCP, membrane preparation of 10 mg tissue and chromatographic fractions (their pH was adjusted to 7.6) or standards(PCP) all in 5 mM Tris-Hcl/50 mM sucrose pH 7.6 at 4°C in a final volume of 0.5 ml. Incubation took 45 min.at 4°C, then the samples were filtered under reduced pressure through GF/C fiters(Whatman) which presoaked for at least two hrs. at 4°C in 0.05% polyethlenimine, then rapidly washed with 2x5 ml aliquots of ice cold 5.0 mM Tris-Hcl buffer. Each filter was dried by infrared lamp, then placed in vials containing 5 ml of liquid (TP 0.3%, POPOP 0.03% in toluene) and counted by liquid scintillation spectrometry. ^{3}H-PCP specific binding to synaptic membranes was defined as the difference in radioactivity bound in the-absence and presence of 20 uM PCP. In a typical experiment, total binding was 2,400 cpm and non-specific binding was 800 cpm.

RESULTS

1. Human CSF went through Sephadex G-25 fine column
Human CSF was applied to a Sephadex G-25 fine column at a flow rate of 9 ml/hr and monitored under UV 254 nm. Aliquots of the 3 ml eluate in each tube were collected and lyophilized. Each fraction was assayed with ^{3}H-PCP receptor binding and salt concentrations measured by Beckmenn system E-4A electrolites analyze.(See fig.1)

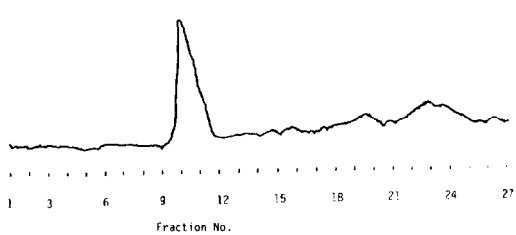

Fig.1 Human CSF went through Sephadex G-25 fine column

2. Active peak and salt peaks
As fig.2 shows, human CSF contains an active fraction No.17-19 and salts peaks No.20-21. The pH of all fractions for binding assay were 7.5. Some were adjusted by 5 mM Tris solution(pH 8.5 at room temperature). The experiment was repeated three times. Since the salt will interfere the ^{3}H-PCP binding, the salt containing fractions (20,21) were discarded. The fractions No.17-19 were combined and lyophilized for analytical HPLC.

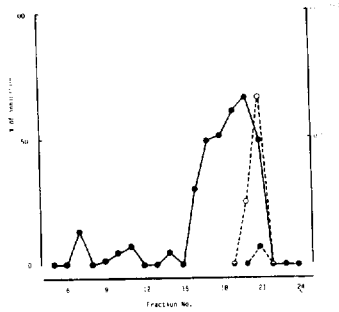

Fig.2. Active peaks and salt peaks screened by ^{3}H-PCP binding assay and salt analyzer respectively

●————● active peaks
O--------O sodium peak
●-------● potassium peak

3. Analytical HPLC elution pattern of fraction No.17-19

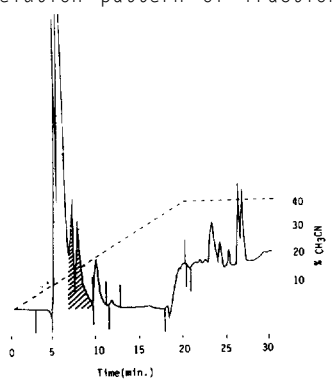

Fig.3 Analytical HPLC elution pattern of fraction No.17-19 from Sephadex G-25 chromatography

Fig.3 showed the peaks within 30 min. elution after injection of fraction No.17-19(see Fig.2). Elutes were collected by eaks monitored with UV 230 nm and lyophilized, then screened by ^{3}H-PCP binding assay. The bared peaks possessed activity for inhibiting ^{3}H-PCP binding.

DISCUSSION

According to work of many laboratories, including our past work, ^{3}H-PCP binding assay was specific, and none of the known neurotransmitters and neuropeptides could displace its binding in reasonable concentrations(Quirion et al.1984;Lu et al.1986). In this experiment, we excluded the factors which might interfere with binding, such as pH

and salts. Therefore, we consider the endogenous ligand in human CSF is conceivable. It may be released directly from brain or as active metabolite of some authentic forms of PCP-like substance. Unlike the neuropeptide reported by Quirion et al., our fraction may be small hydrophilic compound, since it ran just before the salts in Sephadex G-25 column and was rapidly eluted with low concentrations of acetonitrile in reverse phase HPLC. Its molecular weight may be less than 1,000 daltons. The biochemical properties of the ligand are to be determined.

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DEXTRORPHAN: AN ANTAGQNIST FOR PHENCYCLIDINE RECEPTOR

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ABSTRACT

Radio-binding assay, bioassay and HPLC detection were used to observe the antagonistic effects of dextrorphan on PCP's actions. Dextrorphan displayed high affinity to PCP receptors and it had weak PCP-like bioactivity, but could antagonize PCP's action dose-dependetly in vitro and shift the dose-response curve to the right. PCP increased the contents of norepinephrine in bath medium, which was reversed by dextrorphan. Thus, the results suggest that dextrorphan is a partial antagonist for PCP receptors.

INTRODUCTION

Using radio-binding assay, we have demonstrated phencycliding(PCP) receptors in the blood vessel and in vitro bioassay showed that PCP markedly enhanced the electrical field stimulated construction of rabbit central ear artery(ESC)(Zhu et al.1986). Dextrorphan, a d-isomer of levorphanol, could displace PCP binding to the membrane preparation of blood vessels and it could decrease the excitatory effects of PCP on ESC. In this paper, we used three different ways to demonstrate dextrorphan being a partial antagonist for PCP receptors.

METHODS AND MATERIALS

Animal: New Zealand rabbit weighting 2-2.5 Kg.

Materials: ^{3}H-PCP(38Ci/mmol) and PCP were made by Drs. Gong JL and Sun OF in the school of pharmacy of this university. Dextrorphan and levorphanol were generous gifts of Dr. Avram Goldstein, Addition Research Foundation, U.S.A. Phentolamine was made by the pharmaceutic factory of this university, and norepinephrine bitartratis, by Shanghai Tenth Pharmaceutic Factory.

Radio-binding assay: Rabbit mesenteric blood vessels were homogenized in ice-cold 5mM Tris-HCl, pH7.4. The homogenate was centrifuged at 500xg for 10 min and at 16,500xg for 30 min(4°C). The pellet (2.4mg ptotein) and ^{3}H-PCP(5.2nM) were incubated with various ligands with a final volume of 0.5ml at 4°C for 60 min and terminated by rapid filtration(GF/C or GF/B filters preincubated in 0.05& polyethylenimine). Non-specific binding and specific binding were observed in the presence or absence of unlabelled PCP (20μM). Filters were counted by liquid scintillation spectrometry.

Bioassay: The central ear artery of rabbit was prepared in a length of 4-5mm. The preparation was suspended under 0.5g of tension and incubated in 4ml bath organ containing Krebs solution at 37°C through a mixture of 95% O_2 and 5% CO_2 and equilibrated for 4-5hrs prior to

receiving electrical field stimulation(25v, 5-6Hz, trains of 5 pulses, 1ms per pulse, 2.5 min of interval between trains). Dextrorphan and levorphanol were given 10 min before administration of PCP. Dose-response curves of PCP were constructed by increasing bath medium concentration of PCP cumulatively.

High performance liquid chromatography(HPLC): The amine was separated on a GYT-C_{18} column (20x0.5cm I.D., 10μM partical size) and 0.15M chloracetic acide-sodium hydroxide buffer with 0.83 nM EDTA, 9 nM D-Campher sulfonic acide, pH 4.2 and methanol (92:8) at a flow rate of 1.5ml/min and detected by a LC-4B/17 electro-chemical detector.

RESULTS

Radio-binding assay showed that ^{3}H-PCP could bind with high specificity to rabbit blood vessel membranes with Kd=31.9±3.0nM, Bmax=4.96±0.29pmol/mg protein. Dextrorphan and levorphanol could displace ^{3}H-PCP binding to rabbit blood vessel with IC_{50}=3.01±0.19 and 18.2±1.2 μM, respectively (fig.1).

In bioassay, the ESC was enhanced by PCP in a dose-dependent manner. Dextrorphan reduced, even abolished the effect of PCP on ESC and shifted its dose-response curve to the right (fig.2 and fig.3). Dextrorphanand levorphanol decreased the maximum effect of PCP, the pD2' for them were 6.8463 and 5.5862, respectively.

The contents of norepinephrine in bath medium were 16.53±0.37ng/ml after 20 min electrical field stimulation only. The contents were increased to 20.36±1.13 nq/ml after administration of 10^{-5}M of PCP, and decreased to 17.04±0.698 ng/ml after using 10^{-6}M of dextrorphan (fig. 4).

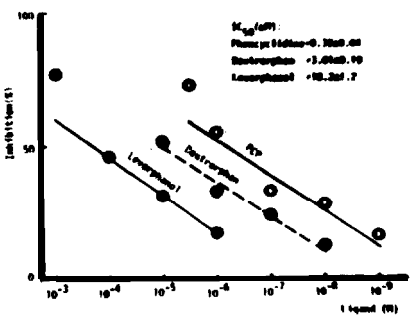

Fig.1 Displacement curves of ligands inhibiting ^{3}H-PCP binding to the homogenate of mensenteric blood vessel.

The ordinate represents persentage inhibition of specific binding of ^{3}H-PCP (5.2nM) to the homogenate(2.4mg protein per assay tube). IC_{50}s represent cone centrations of ligands that displacing 50% of specific bound. The experiment has been replicated three times.

DISCUSSION

Using the binding assay, bioassay and HPLC detection, we have demonstrated that dextrorphan is a specific antagonist for PCP receptors.

In radio-binding assay, we have found that specific, saturable and stereoselective PCP binding sites in the blood vessels(Zhu et al. 1986). The binding sites could be displaced by PCS analogs and some benzomorphan derivatives. Here, we observed that H-PCP binding to

the blood vessel could be displaced by dextrorphan dose-dependently and sereospecifically, thus demonstrating that dextrorphanhad high affinity to PCP receptor.

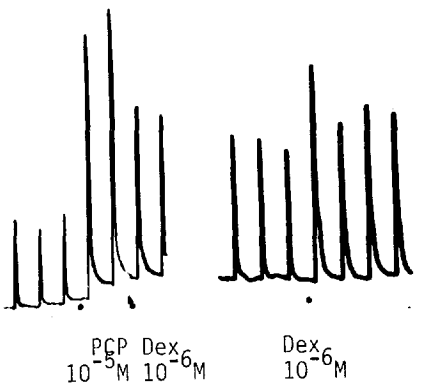

Fig.2 The effects of dextrorphan on ESC and the reversal effects of it on PCP's action.

The vasoconsitriction was induced by electric-field stimulation. PCP enhanced such vasoconstriction and dextrorphan reversed the effect of PCP(left). Dextrorphan alone has a slight excitatory effect on the vasoconstriction(right).

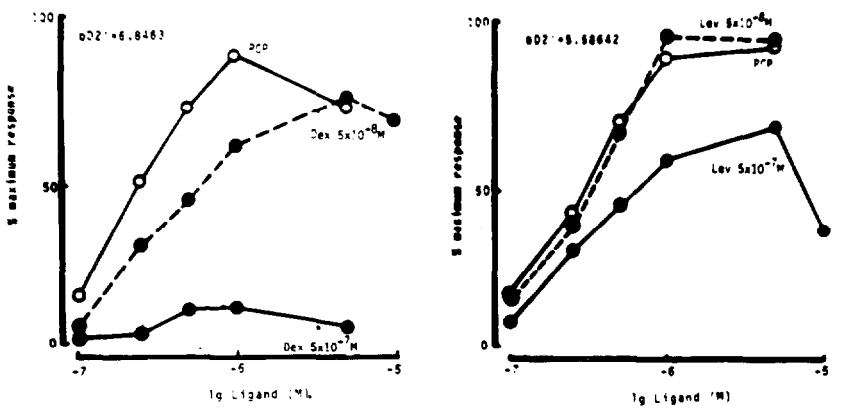

Fig.3 Right-shited dose-response curves of PCP by dextrorphan (left) and levorphanol(right).

The ordinates showed vasoconstrictive responses of ligands compared with the maximum effect of PCP. Dextrorphan or levorphanol was added in the medium 10 min before administration of PCP. Dextrorphan(10^{-8}M) significantly shifted the dose-response curves to the right with a pD2'=6.8463, while levorphanol showed an effect about 10 times less potent(pD2'=5.5864).

Bioassay showed that PCP could markedly and dose-dependently enhance the ESC. The concentrations for half maximum effect of PCP(pD2) were 6.5928 to 6.6665. Dextrorphan showed very weak PCP-like bioactivity (fig.2), but it could antagonize the enhancing ESC by PCP and shifte the dose-response curves of PCP to the right. Levorphanol, a l-enantimer, also had such antagonizing effect, but with lower pontency(fig.

33). The bioactivity of d- and l-isomers in antagonizing PCP's action on ESC was consistent with the affinity of those ligands to PCP receptor, which also was the same as that obtained from radio-binding assay in the rat brain(Tam 1983). In both binding assay and bioassay, dextrorphan was about 10 times more potent than that of levorphanol, which is another evidence for dextrorphan being an antagonist for PCP receptors.

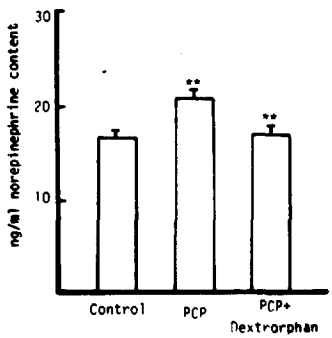

Fig.4 The norepinephrine contents in the medium after administration of PCP or PCP and dextrorphan.

The bath medium was collected after 20 min of electric-field stimulated without any drugs or with PCP(10^{-5}M or/and dextrorphan(10 M). The norepinephrine contents in the bath medium were measured by PHLC.

The effects of PCP on ESC could be abolished by phentolamine and the norcpinephrine contents in bath medium detected by HPLC was markedly increased after administration of PCP, suggesting that the vasoconstriction induced by PCP may be produced by increasing the release of norepinephrine from the nerve terminals. On the contrary, dynorphin could inhibit the ESC and its action may produced by inhibiting the release of norepinephrine(Sun et al 1983 and Illes et al 1983). In this paper, we showed that dextrorphan could antagonize the increment of norepinephrine content in bath medium by PCP. This result have further supplied the evidence that dextrorphan is a PCP antagonist.

Since dextrorphan per se showed weak PCP-like effect and it decreased the maximum effect of PCP, we considered it a partial antagonist for PCP receptor.

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ACKNOWLEGEMENT

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HIGH AND LOW AFFINITY PSYCHOTOMIMETIC OPIOID BINDING SITES: CHARACTERIZATION BY A NOVEL ^{3}H-PCP-ANALOG

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ABSTRACT

1-[1-(3-Hydroxyphenyl)cyclohexyl]piperidine (PCP-3-OH) is one of the most potent analogs of phencyclidine (PCP). In the present study we describe the binding properties of ^{3}H-PCP-3-OH to guinea pig brain membranes. Scatchard analysis of saturation binding studies revealed the existence of high (0.44nM) and low (17nM) affinity binding components. High affinity binding sites were completely blocked in the presence of (+)SKF 10047 (50nM). In competition studies PCP analogs compete for ^{3}H-PCP-3-OH specific binding in a monophasic manner whereas psychotomimetic opioid ligands compete for this binding in a biphasic manner. Results from both saturation and competition experiments suggest the existence of a common high affinity binding site for psychotomimetic opioid ligands and PCP analogs and a low affinity binding component primarily for phencyclidines.

INTRODUCTION

On the basis of behavioral experiments (Shanon 1981; Brady et al. 1982) and binding studies (Zukin and Zukin 1979; Vincent et al. 1979; Quirion et al. 1981; Zukin and Zukin 1981; Itzhak et al. 1981a) it has been suggested that PCP and psychotomimetic opioids, such as SKF-10047, may share a common binding site in CNS. Sigma opioid receptors, originally proposed for SKF 10047 and related benzomorphans (Martin et al. 1976), are now termed "sigma opioid/PCP" receptors. The biochemical characterization of these binding sites is somewhat hampered, compared to mu, delta and kappa opioid receptors, primarily by the lack of highly potent and/or selective "sigma" ligands. The high dissociation constant of ^{3}H-PCP (ca. 200nM) (Zukin and Zukin 1979; Vincent et al. 1979) may give rise to difficulties in receptor binding studies involving such a ligand. However, recently by utilizing ^{3}H-(+)SKF 10047 we reported the existence of common high affinity (Kd=20nM) dextrorotatory-opioid binding sites for psychotomimetic opioids and phencyclidines in rat CNS (Itzhak et al. 1985). The aim of this study was to determine whether "sigma opioid/PCP" binding sites represent a single or multiple binding components. Utilizing PCP-3-OH, which produces strong psychotomimetic PCP-like effects (Kalir et al. 1978) in its radiolabeled form (^{3}H-PCP-3-OH), we present evidence for the existence of multiple binding components for psychotomimetic opioids and phencyclidines in guinea pig brain.

MATERIALS AND METHODS

^{3}H-PCP-3-OH (9.8 Ci/mmole) was purchased from Israel Nuclear Research Center-Negev, Israel. The purity of the drug as determined by thin layer chromatography and U.V. spectrophotometer was > 99%. PCP and its derivatives: PCP-3-OH, 1-[1-(3-aminophenyl)cyclohexyl]piperidine

(PCP-3-NH_2), 1-phenylcyclohexylamine (PCNH2), N-ethyl-phenylcyclohexylamine (PCNHEt), and 1-(1-phenylcyclohexyl)-4-piperidinol (PCP-4'-OH), were synthesized as previously described (Kalir et al. 1978; Itzhak et al. 1981b). The two stereoisomers of SKF 10047 were generously supplied by Dr. R. Hawks National Institute on Drug Abuse. Other drugs were purchased from commercial sources. Crude guinea pig brain (minus cerebellum) membranes were prepared as previously described (Simon et al. 1975). Binding assays were carried out in HEPES-KOH buffer 10mM (pH 7.7; 25°C) as previously described (Itzhak et al. 1985). Specific binding of ^{3}H-PCP-3-OH was assessed in the absence and presence of 1µM unlabeled PCP-3-OH or PCP, which resulted in similar levels of non specific binding.

RESULTS AND DISCUSSION

In typical experiments specific binding of ^{3}H-PCP-3-OH (3nM) to guinea pig brain membranes represented 80±3% of total binding. Radioactivity absorbed to GF/B filter blanks was usually less than 5% of the total binding. Specific binding increased linearly within protein concentration of 0.2-1.7mg/ml and was reduced in the presence of trypsin (20µg/ml) or N-ethylmaleimide (0.5mM) by 66 and 54%, respectively, from control binding. Preincubation of membranes at 60°C/20 min resulted in 90±5% decrease in specific binding. These findings are consistent with the requirements for drug-receptor interaction and indicate the protein nature of the binding sites. Saturation binding experiments of ^{3}H-PCP-3-OH were carried out at concentrations of 0.2-40nM and revealed two saturable binding components. Scatchard analysis of the specific binding resulted in a biphasic plot (Fig. 1). Apparent Kd values, calculated as described by Munson and Rodbard (1980), are 0.44nM and 17nM for high and low affinity binding components, respectively.

The ability of various PCP-analogs and psychotomimetic opioid ligands to compete for ^{3}H-PCP-3-OH (3nM) specific binding was also studied. The rank order of potency among PCP analogs was as follows: PCP-3-OH> PCP=PCNHEt>PCP-3-NH_2>PCNH_2>PCP-4'-OH. The IC_{50} determined for PCP-3-OH was 7nM, whereas the I?50 for the least potent analog, PCP-4'-OH, was 860nM. This rank order of potency is consistent with that found in behavioral studies (Kalir et al. 19 8; Shanon 1981; Solomon et al. 1982) and competition studies for ^{3}H-(+)SKF 10047 binding sites (Itzhak et al. 1985). All PCP analogs competed for ^{3}H-PCP-3-OH specific binding in a monophasic manner yielding Hill coefficient values near 1.

Psychotomimetic opioids, such as (+)SKF 10047, ketocyclazocine, pentazocine, nalorphine and dextrallorphan competed for ^{3}H-PCP-3-OH specific binding in a biphasic manner (Fig. 2). About 30% of the binding was readily inhibited by low concentrations (1-25nM) of these opioids, whereas the residual binding of ^{3}H-PCP-3-OH was far less sensitive (Fig. 2). The levorotatory isomer of SKF 10047 (Fig. 2), levorphanol and levallorphan inhibited ^{3}H-PCP-3-OH specific binding only at high concentrations and in a monophasic manner, yielding IC values of 350-800nM. However, morphine (u-agonist) naloxone, DADL (δ-prefering ligand) and tifluadom (K-agonist) as well as non-opioid ligands, such as haloperidol and atropine (each at 1nM-10µM) did not affect the binding of ^{3}H-PCP-3-OH.

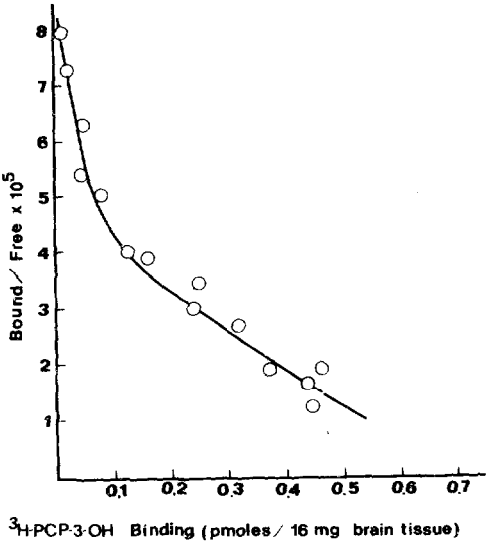

FIGURE 1. Scatchard plot of ^{3}H-PCP-3-OH specific binding to guinea pig brain membranes. Binding of tritiated ligand (0.2-40nM) was carried out at 25°C for 40 min. Specific binding was assessed in the absence and presence of PCP-3-OH (1μM). Points represent a typical experiment which was repeated three times with similar results.

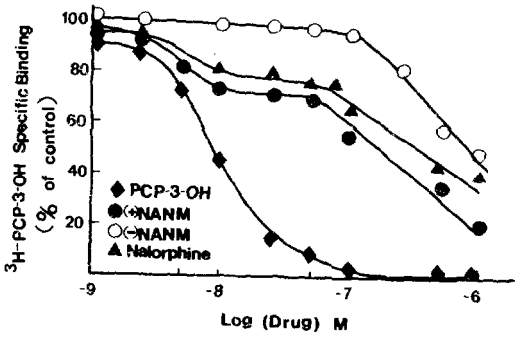

FIGURE 2. Inhibition of ^{3}H-PCP-3-OH (3nM) specific binding to guinea pig brain membranes by unlabeled PCP-3-OH and selected opioids. Assays were carried out at 25°C for 40 min. Control specific binding was 1630±80 cpm. Results represent the mean of three separate determinations (SEM < 10%).
NANM = N-allylnormetazocine (SKF 10047)

The ability of (+)SKF 10047 and the racemic mixture of other psychotomimetic opioids, at rather low concentrations, to inhibit a portion of ^{3}H-PCP-3-OH specific binding, and the inability of (-)SKF 10047, levorphanol and levallorphan to inhibit this binding, at low concentrations, suggests that the high affinity binding component of ^{3}H-PCP-3-OH displays dextrorotatory stereospecificity. Moreover, saturation binding studies of ^{3}H-PCP-3-OH in the presence of (+)SKF 10047 (50nM) yielded a linear Scatchard plot (Kd=21nM). These results indicate that high affinity binding sites for ^{3}H-PCP-3-OH were completely blocked in the presence of (+)SKF 10047, whereas the low affinity binding component was practically unaffected.

We have previously demonstrated that ^{3}H-(+)SKF 10047 binds to a single high affinity binding component and both psychotomimetic opioids and PCP analogs competed for ^{3}H-(+)SKF 10047 binding sites in a monophasic manner (Itzhak et al. 1985). These findings together with the present results strongly support the view that the high affinity binding component, labeled by ^{3}H-PCP-3-DH, corresponds to a common high affinity binding site for both phencyclidines and psychotomimetic opioids. The low affinity binding component may correspond to subpopulation of the sigma binding sites, which interact primarily with PCP analogs. Thus, it is proposed that "sigma-opioid/PCP receptor" may represent two distinct subtypes of binding sites.

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NEW δ-RECEPTOR ANTAGONISTS

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ABSTRACT

A new δ— selective opiate antagonist has been synthesised in which the two glycine residues of diallyl leucine enkephalin have been replaced by 4-aminobenzoic acid. The compound has a different conformation to that of ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu).

INTRODUCTION

Diallyl enkephalin analogues have been reported by Shaw et al. (1982) and by Cotton et al. (1984) with selective antagonist activity at the δ-opioid receptor in which the amidic link between Gly^3 and Phe^4 has been replaced by a methylenethio link (ICI 154.129, structure 1 in the results table) or in which the two glycine residues have been replaced by two aminoisobutyric acid residues (ICI 174,864, structure 2 in the results table). We now report the synthesis of the series of diallyl enkephalin analogues in which the two glycine residues have been replaced by a single 4-aminobenzoic acid residue or one of its close relatives. The objectives of the study were to examine the structure activity relationships of the compounds as opiate antagonists and to compare the conformation of the most potent and selective agents with that of the selective δ-receptor antagonist, ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu).

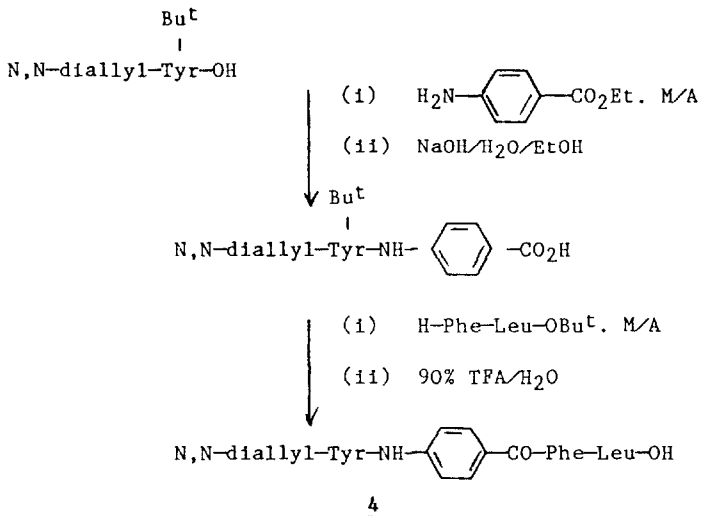
MATERIALS AND METHODS

Synthetic methods

The novel compounds indicated in the results table were prepared by standard chemical methods. The route to the 4-aminobenzoic acid analogue, 4, is indicated in figure 1. The 4-methylaminobenzoic acid analogue, 6, was prepared by N-methylation (NaH/DMF/MeI) of the amide formed by the mixed anhydride coupling of 4-aminobenzoic acid ethyl ester with N,N-diallyl-Tyr(Bu^t)-OH. The 3-aminobenzoic acid derivative, 7, the 4-aminophenylacetic acid derivative, 8, and the 4-carboxybenzylamine derivative, 9. were made by initial chain extension at the carboxy-terminus (H-Phe-Leu-OBu^t, mixed anhydride) of the starting benzyloxycarbonyl protected amino-acid and subsequent N-acylation with N,N-diallyl-Tyr(Bu^t)-OH. For compound 10, the coupling of 4-benzyloxycarbonylaminobenzoic acid with N—methylphenylalanylleucine methyl ester was accomplished with dicyclohexylcarbodiimide and hydroxybenztriazole. The 4-amino-2-methylbenzoic acid analogue was prepared starting from 2-methyl-4-nitrobenzoic acid which was used to acylate the dipeptide ester, H-Phe-Leu-OBu^t, by mixed anhydride coupling. Catalylic hydrogenation (Pd/H_2) then reduced the nitro-group to an amino-group for subsequent

acylation with N,N-diallyl-Tyr(Bu^t)-OH. Mixed anhydride (M/A) couplings were carried out using ethyl chloroformate and triethylamine and t-butyl ethers and esters were cleaved by 90% trifluoroacetic acid.

FIGURE 1. Synthetic route



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Biological methods

The compounds were tested as opioid antagonists on the field stimulated mouse vas deferens against the selective δ--agonist, [Leu^5] enkephalin and the μ-agonist, normorphine, as previously described by Miller et al. (1986)

RESULTS

The results in the table indicate that the 4-aminobenzoic acid derivative, 4, is the most potent δ--receptor antagonist with a selectivity ratio of about 100 between the δ-- and μ-receptors. The presence of methyl substituents on the aromatic ring (11) on the amino-group (6) or on the nitrogen of the adjacent phenylalanine (10) all led to loss of potency at the δ-receptor. Similarly the inclusion of an extra methylene group as in compounds 8 and 2 caused loss of activity. These observations were of value in studying the conformation of the most potent δ-receptor antagonist, the 4-aminobenzoic acid derivative, 4, (ICI,167,853).

TABLE 1. Assay for opiate antagonist activity on field stimulated mouse vas deferens

N,N-Diallyl-Tyr-X-Y-Leu-OH

Compound	X	Y	Ke in nM [Leu^5]-enkephalin	Ke in nM normorphine
1#	Gly-NH-CH_2-CH_2-S-CH(CH_2-Ph)-CO		254 ± 27	7,400 ± 950
2#	Aib-Aib	Phe	33 ± 4.7	>10,000
3 ≠	Gly-Gly	Phe	204	14,000
4	4-NH-ø-CO	Phe	202 ± 16	>20,000
6	4-NMe-ø-CO	Phe	>20,000	>20,000
7	3-NH-ø-CO	Phe	2,600 ± 940	>20,000
8	4-NH-ø-CH_2-CO	Phe	800 ± 115	>15,000
9	4-NH-CH_2-ø-CO	Phe	1,900 ± 200	13,900 ± 6,000
10*	4-NH-ø-CO	MePhe	1,370 ± 270	2,800 ± 900
11	4-NH-2-Me-ø-CO	Phe	922 ± 140	7,500 ± 3,200

Aib — Aminoisobutyric acid, Cotton et al. (1984). ø is phenyl
≠ n=3 for all results except this where n=1, # Shaw et al. (1982)
* Tested as the methyl ester

Conformational Studies

A major source of flexibility within the diallyl-[Leu^5]-enkephalin molecule is the central Gly^2-Gly^3 dipeptide unit which can adopt more than 100 conformations. Energy calculations using ECEPP (Momany et al. 1975) identified 18 distinct, accessible conformers. Following earlier studies with L- and D-alanine at the 2 and 3 positions, where it was shown that only the D-Ala^3 analogue was as potent as the parent compound, two conformers were selected for further study where the conformation of residue 2 was the same (C7 ax: phi = 80, psi = -80). The other residue of conformer 1 was relatively extended (phi = 65, psi = -175), whereas in conformer 2 the right-handed alpha helical conformation (AR: phi = -54, psi = -60) was postulated. Substitution of a-aminoisobutyric acid (Aib) for glycine at the 2 and 3 positions led to a 6 fold increase in δ-antagonist potency. Further energy calculations on N,N-diallyl-Tyr-Aib-Aib-Phe-Leu suggested that the conformation adopted by the central dipeptide was helical but could be either right- or left-handed. Indeed the right-handed form could be related to conformer 2, in that the tyramine and phenylalanine entities could be superposed with an RMS deviation of about 1.5Å, however the relative orientations of the leucine side chains were different.

In the current work, aminobenzoic acids and homologues were incorporated as replacements for the two glycine residues. These "dipeptide mimics" are conformationally restricted ie. the conformation of the p-aminobenzoic acid (PABA) analogue can be defined in terms of two torsions corresponding to rotation about the N-C bond (phi2) and the C-CO bond (psi3). The energetically accessible values for these torsions are:

phi2	+45,-45,+135,-135
psi3	+50,-50,+130,-130

There are 16 possible combinations of these angles, however, because of the symmetry and planarity of the phenyl ring, there are only 4 distinct conformations. In addition to the considerable reduction in flexibility when compared with the Gly^2-Gly^3 unit (>100 conformations), the PABA moiety cannot mimic the spatial characteristics of a helical dipeptide such as that suggested for the Aib^2-Aib^3 analogue. It was thus intriguing to discover that the PABA derivative was equipotent with diallyl-[Leu^5]-enkephalin, whilst the meta counterpart (MABA) was an order of magnitude poorer.

CONCLUSIONS

Although energy calculations have suggested a low energy conformation of the PABA analogue which can be related to the originally identified conformer 2. the spatial relationships between the PABA and Aib^2-Aib^3 analogues are, at best, unclear.

It would appear that the two types of conformational restriction have both generated potent delta-receptor antagonists, but the restricted conformations adopted differ in the two cases. This conclusion is unusual if not unique and was certainly unexpected.

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DIMERIC OPIOID ANTAGONISTS

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ABSTRACT

Symmetrical and unsymmetrical dimeric pentapeptide opioid antagonists have been prepared and studied on the mouse vas deferens preparation. The findings do not support the hypothesis that such agents bind to dimeric δ-opioid receptors.

INTRODUCTION

Dimeric opioid peptides have been reported which have enhanced selectivity for the opioid b-receptor compared to their monomeric analogues. Those dimers, prepared by linking two tetrapeptides as amides of α,ω-alkylenediamines, have receptor binding affinities at the δ -receptor similar to those of pentapeptide monomers but some achieve high &selectivity through loss of affinity for the μ-receptor. However, where two molecules of the pentapeptide Tyr-D-Ala-Gly-Phe-Leu. were linked as amides of ethylenediamine, a compound was obtained with a ten-fold enhanced affinity for δ -receptors and similar affinity for p-receptors as Tyr-D-AlaXly-Phe-Leu-NH_2, as measured by binding studies. (Shimohigashi et al.1982).

The hypothesis has been advanced that the δ-selectivity observed is as a consequence of the dimeric ligands interacting with two, closely associated, δ— receptors. The objective of this study was to explore the hypothesis that dimeric peptides have enhanced b-selectivity because of interaction with a dimeric δ-receptor. Antagonists rather than agonists were chosen for investigation so that Ke values obtained from isolated tissue assays would be a reflection of affinity rather than affinity and intrinsic activity. The key experiment was the comparison of symmetrical and unsymmetrical dimeric pentapeptides as antagonists of [Leu^5] enkephalin and normorphine in the electrically stimulate mouse vas deferens preparation.

Theoretical Considerations

For the binding of a ligand to a receptor, the free energy of interaction, ΔG, is related to the affinity constant by the expression :-

$$\Delta G = \Delta H - T\Delta S = -1.4 \log K$$

where AH is the change in enthalpy, AS the change in entropy and T the absolute temperature. Thus, if it were possible to dimerise a ligand and to allow it to associate with a dimeric receptor such that the value of ΔG were to be doubled, then the doubling of log K would be reflected in a squaring of the value of K. Consequently, one

might see a change of affinity from say 10^{-7}M to 10^{-14}M.

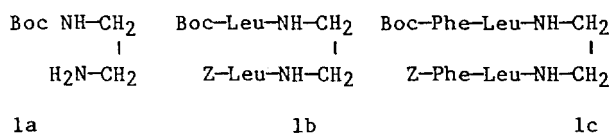
Such enhancements in potency have not been observed for opioid peptide dimers. On the dimeric receptor theory this could be explained by entropic factors involved in the association of two receptors into a dimer, by entropic factors associated with the flexible linkage in the dimer between the two pharmacophores, or by unfavourable interactions between the receptor and the polymethylene chain linking the two monomeric pentapeptides.

MATERIALS AND METHOD

Synthetic methods

Peptide dimers were prepared in which the carboxyl termini of the two peptide chains were held together as amides of ethylenediamine. The synthesis used an orthoganol protection strategy in which one arm of the dimer was elaborated using t-butyloxycarbonyl nitrogen protection (deprotection with acid) and the other arm was elaborated using benzyloxycarbonyl nitrogen protection (deprotection with catalytic hydrogenation). Standard peptide methodology was used throughout the synthesis.

Thus t-butyloxycarbonyl ethylenediamine, 1a, was elaborated first to the unsymmetrically protected leucine dimer 1b, and thence to the phenylalanylleucine dimer, 1c. Peptide chain extension was accomplished either by the use of protected amino-acid active esters (4-nitrophenol or 3, 4, 5 - trichlorophenol esters) or by the mixed anhydride method employing ethyl chloroformate and triethylamine. The intermediate 1c, was extended to give 2 by a fragment coupling employing the t-butyl ether of N,N-diallyltyrosinylglycylglycine and mixed anhydride activation. Similar extension on the second phenylalanine gave the symmetrical dimer. 3, the unsymmetrical dimer, 4, and the truncated acetyl derivative, 5. The fragment couplings to convert 2 to 3 and 4 were accomplished using dicyclohexylcarbodiimide and hydroxybenztriazole activation.



1a 1b 1c

Biological methods

The compounds were tested as opioid antagonists on the field stimulated mouse vas deferens against the selective δ-agonist, [Leu^5] enkephalin and the p-agonist. normorphine, as previously described by Miller et al. (1986).

RESULTS AND DISCUSSION

FIGURE 1. Assay for opioid antagonist activity on the field stimulated mouse vas deferens

Compound	Ke in nM versus [Leu^5] -enkephalin	Ke in nM versus normorphine
MONOMERIC ANTAGONISTS		
N,N-diallyl-Tyr-Aib-Aib-Phe-Leu#	32.9±4.7	>10,000
N,N-diallyl-Tyr-Gly-Gly-Phe-Leu-NHEt	133±61	1,010 ± 504
DIMERIC ANTAGONISTS		
N,N-diallyl-Tyr-Gly-Gly-Phe-Leu-NH-CH_2 X-Phe-Leu-NH-CH_2		
(2) X = H	460±189	2,200 ± 770
(3) X = N,N-diallyl-Tyr-Gly-Gly	14.3±2.0	1,340 ± 362
(4) X = N,N-diallyl-Phe-Gly-Gly	1,200±140	17,100 ±4100
(5) X = Acetyl	65±20	2,800 ±1300

Key : Aib – Aminoisobutyric acid. # Cotton et al. (1984)

* All compounds were tested in triplicate

The symmetrical dimer (3) is a more potent δ-antagonist than the monomeric ethylamide but is only comparable in potency to and less selective as a δ-antagonist than the monomeric pentapeptide ICI 174,864 (diallyl-Tyr-Aib-Aib-Phe-Leu). The unsymmetrical dimer (4) is 80 times less potent than 3 but retains some δ-selectivity. The most surprising finding is that the truncated dimer (5) is almost as potent as the symmetrical dimer (3) and has a high δ-selectivity.

The increase in potency at the δ-receptor caused by dimerisation is about nine-foid in this series and is comparable to that obtained in other compounds with a single tyrosine moiety such as ICI 194,864. The 80-fold loss of potency at the δ-receptor caused by the removal of one hydroxyl moiety from 3 to give 4 is of interest. If the two peptide chains do not interact with each other, then such a compound would be expected to be two-fold less potent for a monomeric receptor system. However, the 80-fold loss of potency going from compound 3 to 4 and the nine-fold difference between the monomeric and dimeric amide antagonists are still small compared to the orders of magnitude which might be expected. The high potency and selectivity of the truncated dimer (5) suggests that the δ-receptor, but not the μ-receptor, can tolerate lipophilic residues beyond the normal binding site of the pentapeptide carboxy-terminus.

Thus part, if not all, of the potency and selectivity of dimeric enkephalins can be explained without invoking a dimeric δ--receptor system. The differences in potency between dimers and monomers and between symmetrical and unsymmetrical dimers could be a consequence of conformational factors whereby a dimer folds as a single large molecule rather than as a linked pair of monomers each with the tertiary structure it has in the monomeric state.

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MELPHALAN-CONTAINING N,N-DIALKYLENREPHALIN ANALOGS AS POTENTIAL IRREVERSIBLE ANTAGONISTS OF THE δ OPIOID RECEPTOR

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ABSTRACT

N,N-Dialkylated leucine enkephalin analogs containing melphalan in place of Phe[4] were synthesized as potentially irreversible antagonists of the δ opioid receptor. These compounds, along with the corresponding Phe[4] pe tides, were tested for both agonist and antagonist activity in the GPI and MVD smooth muscle preparations. All but one of the compounds showed antagonist activity at 10^{-6}M against [D-Ala[2],D-Leu[5]]-enkephalin in the MVD when tested under reversible conditions; in all cases the Mel[4] peptide had lower activity against DADLE than did the corresponding Phe[4] peptide. At high concentrations (10^{-5}M) the active Mel[4] analogs, $(benzyl)_2$Tyr-Gly-Gly-Mel-Leu and $(allyl)_2$Tyr-Aib-Aib-Mel-Leu, both showed weak irreversible antagonism at the δ receptor.

INTRODUCTION

Selective irreversible opioid receptor antagonists can be valuable pharmacological tools, as evidenced by the use in a variety of studies of β-funaltrexamine (β-FNA) (Portoghese et al. 1980, Takemori and Portoghese 1985), an irreversible antagonist of the μ receptor. N,N-Diallyl leucine enkephalin analogs are selective reversible antagonists of the δ opioid receptor (Shaw et al. 1982, Cotton et al. 1984) which have been useful in studying the role of the δ receptor in various physiological processes. In a combination of these two approaches, several enkephalin analogs with the eneral structure R_2Tyr-Y-Y-X-Leu, where R = allyl or benzyl, X = Gly or Aib (α-aminoisobutync acid) and X = Mel (melphalan), the nitrogen mustard-containing analog of phenylalanine, were synthesized and tested as potential irreversible antagonists of the δ opioid receptor. Here we present the results of the biological evaluations for this series on the guinea pig ileum and the mouse vas deferens preparations.

MATERIALS AND METHODS

Synthesis

Melphalan-containing peptides were synthesized in solution by mixed anhydride couplings of R_2Tyr(OtBu)-X-X with Mel-LeuOtBu, followed by deprotection with trifluoroacetic acid. Except for compound 2b (ICI 174,864), which was a gift from ICI Pharmaceuticals Division, Macclesfield, Cheshire, England, the corresponding phenylalanine-

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containing peptides were synthesized in a similar manner, or were prepared by dialkylation of a protected pentapeptide followed by deprotection. Details of these syntheses will be published elsewhere.

Biological Evaluation

Compounds were evaluated in the electrically stimulated longitudinal muscle of the guinea pig ileum (GPI) (Rang 1965) and in the mouse vas deferens (Henderson et al. 1972). The melphalan-containing compounds were added to the tissues as a solution in methanol; in the concentrations used methanol had no effect on the tissues. Agonist activity is expressed as the percent inhibition of the twitch after a 10 minute incubation of the tissue with the test compound. The reversible antagonist potency was determined against morphine, ethylketazocine (EK), or [D-Ala^2,D-Leu^5]enkephalin (DADLE), and is expressed as the ratio of the IC_{50} of the agonist in the presence of the test compound to the IC_{50} in the absence of the test compound in the same preparation. Irreversible antagonism was evaluated by incubating the MVD with the antagonist for 10 minutes, washing the tissue 30 times, and then determining the IC_{50} of DADLE; the activity is expressed as the ratio of the IC of DADLE after exposure to the antagonist to that of the same tissue before exposure.

RESULTS AND DISCUSSION

All but one of the compounds were reversible antagonists in the MVD when tested against DADLE, a δ receptor agonist (table 1). Of the Phe^4 peptides, all of which were active, 2b (ICI 174,864) was significantly more potent than the corresponding dibenzyl analog 3b. The N,N-dibenzyl modification was chosen for further examination because for the Gly-containing peptides the reverse was true; 1b was 2.5-fold more potent than N,N-diallyl leucine enkephalin (IC_{50} ratio = 2.7±0.6, n = 3). Possible contributing factors to this reversal are conformational changes in the peptide chain imposed by the Aib residues which alter the position of the alkyl groups attached to the amine terminus and the greater lipophilicity of peptides containing both the benzyl groups and the Aib residues. The two dibenzyl peptides 1b and 3b showed similar antagonist activities against DADLE.

Replacement of phenylalanine by melphalan, the para-substituted nitrogen mustard analog of phenylalanine, resulted in decreased affinity for the δ receptor. The decrease in activity was only 2.2-fold in the case of the dibenzyl leucine enkephalin analog 1a, but was larger for the Aib-containing peptides. The 4.7-fold decrease in activity for 3a resulted in an inactive compound at 10^{-6}M. The decrease for 2a was approximately 3.1-fold, but the resulting compound was still more potent than 1a.

Most of the compounds showed little or no activity in the remaining tests. Only one compound, 2a, had an IC_{50} ratio against morphine in the MVD which was greater than one. The compounds did show some agonism in the MVD at 10^{-6}M, but the activity was weak (7.4% to 25.7% inhibition of the twitch, see table 1). One compound, 3b, showed significant agonist activity in the GPI (68.7±6.7% inhibition of the twitch at 10^{-6}M (n = 3), IC_{50} = 2.7 x 10^{-7}±1.4 x 10^{-7}M (n = 4)); the remaining compounds at 10^{-6}M showed some agonism in the GPI, but the activity

was much weaker (less than 10% inhibition of the twitch for compounds 1a, 1b, and 3a, 11.4±3.3% inhibition (n = 2) for 2a, and 27.0±10.8% inhibition (n = 2) for 2b). These compounds were inactive as antagonists at 10^{-6}M in the GPI against both morphine and EK (data not shown).

TABLE 1. Reversible Agonism and Antagonism in the MVD of N,N-Dialkyl Leucine Enkephalin Analogs, R_2Tyr-Y-Y-X-Leu.

Cmpd.	R	X	Y	Agonism: Percent Inhibition[1]	Antagonism: IC_{50} Ratio[1] DADLE	Morphine
1a	benzyl	Gly	Mel	16.2 ± 1.7%[2]	3.0 ± 0.5[2]	1.0 ± 0.4
1b	"	"	Phe	11.4 ± 5.9%	6.6 ± 1.4[2]	0.6 ± 0.2
2a	allyl	Aib	Mel	20.6 ± 6.5%	5.0 ± 0.9	2.2 ± 0.72
2b	"	"	Phe	7.4 ± 7.4%	15.6 ± 2.4	0.8 ± 0.1
3a	benzyl	"	Mel	22.5 ± 5.2%	1.2 ± 0.2	1.0 ± 0.3
3b	"	"	Phe	25.7 ± 4.9%	5.6 ± 1.7	1.0 ± 0.1

[1]Compounds were tested at 10^{-6}M. Results are ± standard error of the mean (S.E.M); n = 3 unless otherwise indicated.
[2]n = 4.

The two melphalan-containing peptides which showed reversible δ antagonist activity were tested for irreversibility against DADLE in the MVD. At 10^{-6}M no irreversible antagonism was observed (table 2), but at 10^{-5}M both compounds did show weak irreversible antagonist activity. The irreversible antagonism was similar for the two compounds in spite of the 2.5-fold higher reversible antagonism of 2a. Although extremely high concentrations were required to demonstrate any irreversibility, these results do demonstrate the potential feasibility of developing irreversible antagonists based on the enkephalins. Other less bulky and

TABLE 2. Reversible and Irreversible antagonism of 1a and 2a against DADLE in the MVD.

	DADLE IC_{50} Ratio[1]			
	10^{-6}M		10^{-5}M	
Cmpd	Reversible	Irreversible	Reversible[2]	Irreversible
1a	3.0 ± 0.5(4)	1.2 ± 0.2 (4)[3]	7.7 ± 3.3(4)	2.4 ± 0.8 (5)
2a	5.0 ± 0.9 (3)	1.1 ± 0.2 (3)	19.4 ± 4.7 (4)	2.9 ± 0.9 (5)

[1] ± S.E.M. (n).
[2]At 10^{-5}M both 1a and 2a showed significant reversible agonism (47.8±5.7% and 42.9±7.0% inhibition of the twitch, respectively).
[3]After 30 minutes incubation.

more reactive functional groups at the para position of the phenylalanine ring may result in increased affinity of the peptide for the receptor and greater irreversible antagonism at lower concentrations.

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DIHYDROMORPHINE-PEPTIDE HYBRIDS HAVE MU RECEPTOR ANTAGONISTIC AND DELTA RECEPTOR AGONISTIC ACTIVITY ON THE MOUSE VAS DEFERENS AND BIND WITH HIGH AFFINITY TO OPIOID RECEPTORS IN RAT BRAIN

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ABSTRACT

The actions of three morphine derivatives with short peptide side chains were evaluated upon the contraction of the isolated, electrically stimulated mouse vas deferens preparation and upon displacement of specifically bound ^{3}H-etorphine in rat brain membranes. NIH-9834 (N-[6, 14-endoetheno-7, 8-dihydromorphine-7-alpha-carbonyl]-L-phenylalanyl-L-leucinol) and its ethyl ester, NIH-9833, were potent agonists upon the vas deferens. ICI-174864, 100 nM, markedly antagonized the actions of both NIH-9833 and NIH-9834 which indicates that these are delta receptor agonists. NIH-9835 (N-[6, 14-endoetheno-7, 8-dihydromorphine-7-alpha-carbonyl]-L-glycyl-L-phenylalanyl-L-leucine ethyl ester HCl) differs from NIH-9833 and NIH-9834 by the presence of a single amino acid residue. Although this drug had no agonistic activity on the vas deferens, it was a potent antagonist of mu agonists. All three hybrids were potent displacers of ^{3}H-etorphine in rat cerebral membranes. The observation that addition of a single glycyl residue changes dihydromorphine-peptide analogs from potent delta receptor agonists to equally potent mu receptor antagonists suggests that the two receptor sites might be structurally quite similar.

INTRODUCTION

Morphine is a mu receptor agonist on the mouse vas deferens. In contrast, the enkephalins are most potent as delta receptor agonists in that preparation (Lord et al. 1977). The purpose of the present study was to evaluate the effects of several dihydromorphine-peptide analogs upon the mouse vas deferens preparation and examine their binding to opioid receptor in rat brain membranes. These analogs incorporate peptide fragments of the enkephalins into the dihydrcmorphine molecule.

METHODS

In the binding assay, the competition of the investigated compounds with ^{3}H-etorphine for opioid receptor sites in a membrane preparation from rat brain cerebrum was determined (Medzihradsky et al. 1984). After incubation at 25°C to reach binding equilibrium, the suspension of brain membranes in the assay medium was quickly filtered and the bound radioactivity determined. The binding affinity of the tested compounds is expressed as their EC 50 (from log-probit plots) in displacing specifically bound ^{3}H-etorphine.

For the vas deferens experiments, male, albino ICR mice, weighing between 25 and 30 g, were used. The mice were decapitated, the vasa deferentia removed, and 1.5 cm segments were suspended in or-

gan baths which contained 30 ml of a modified Kreb's physiological buffer. The buffer contained the following (mM): NaCl, 118; KCl, 4.75; $CaCl_2$ 2.54; $MgSO_4$, 1.19; KH_2PO_4, 1.19; glucose, 11; $NaHCO_3$, 25; pargyline HCl, 0.3, tyrosine, 0.2; ascorbic acid, 0.1; and Na_2EDTA, 0.03. The buffer was saturated with 95% 02 - 5% CO_2 and kept at 37 C. The segments were attached to strain gauge transducers and suspended between two platinum electrodes. After a 30-min equilibration period, the segments were stimulated once every 10 sec with pairs of pulses of 2 msec duration, 1 msec apart and at supramaximal voltage. Naltrexone, ICI-174864 or NIH-9835 were added to the organ baths 15 minutes before the determination of cumulative concentration-effect relationships for the various agonists. beta-FNA was added to the organ baths after the initial equilibration period. Thirty min later the beta-FNA was removed from the organ baths by repeated washings with fresh buffer. The tissues were washed three times every 5 min for 15 min. Cumulative concentration-effect relationships for the various agonists were then determined 20 min after the last wash (i.e. 30 min after the beta-FNA was removed from the organ baths). The EC 50's were calculated by probit analysis. All values are the means of at least 6 determinations ± the standard error of the mean. The following drugs were studied: ethylketazocine, etorphine, DSLET [Tyr-D-Ser-Gly-Phe-Leu-Thr], NIH-9824, NIH-9833, the ethyl ester of NIH-9834, NIH-9835, naltrexone HCl, ICI-174864 [N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH], morphine sulfate, U50, 488H [trans-3, 4-dichloro-N-methyl-N (2- (1-pyrrolidinyl) cyclohexyl) benzeneacetamide methanesulfonate hydrate] and beta-funaltrexamine (beta-FNA).

RESULTS AND DISCUSSION

NIH-9833 was an extremely potent agonist upon the mouse vas deferens preparation with an EC 50 of 0.85 + 0.53 nM (n=8, FIGURE 1). Naltrexone, 100 nM, beta-FNA, 1 µM, and ICI-174864, 100 nM, antagonized the actions of NIH-9833. EC 50's were 1.7 ± 0.3 nM in the presence of naltrexone and 2.4 ± 0.5 nM in the presence of beta-FNA. Naltrexone competitively blocks mu, kappa and delta receptors, and beta-FNA blocks mu and delta, but not kappa receptors in the mouse vas deferens preparation (Smith in press). In the presence of ICI-174864 the EC 50 for NIH-9833 was 1.9 nM ± 0.5. ICI-174864 is a highly selective delta receptor antagonist (Cotton et al. 1984, Smith et al. 1984), and blockade by ICI-174864 suggests that NIH-9833 acts primarily upon delta opioid receptors. Similar results were obtained with NIH-9834.

NIH-9835 was a potent mu receptor antagonist upon the mouse vas deferens preparation. It caused a parallel shift (12.3-fold shift) to the right in the morphine concentration-effect curve. In control experiments the EC 50 for morphine was 350 nM ± 117 (n=3), and in the presence of NIH-9835, 100 nM, the EC 50 for morphine was 4.3 µM ± 1.48. NIH-9835 caused a slight shift (3-fold) to the right in the DSLET concentration-effect curve. In control experiments, the EC 50 for DSLET was 0.49 nM ± 0.08 (n=4), and in the presence of NIH-9835, 100 nM, was 1.6 nM ± 0.2. NIH-9835 did not significantly shift the U50,488H concentration-effect curve. In control experiments, the EC 50 for U50,488H was 473 nM ± 145, and in the presence of NIH-9835, 100 nM, the EC 50 was 473 nM ± 125. Thus,

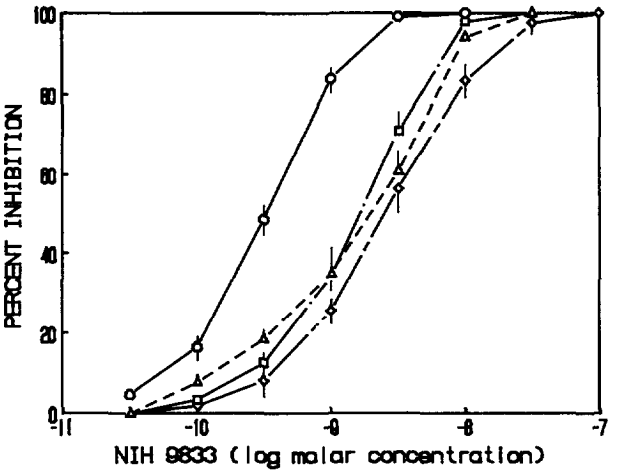

FIGURE 1. Antagonism of the effects of NIH-9833 upon the electrically stimulated mouse vas deferens. Abscissa: concentration of NIH-9833; Ordinate: percent inhibition of the twitch. Each point represents the mean of 6-9 preparations in the absence of antagonist (circles) or in the presence of 100 nM naltrexone (triangles), 100 nM ICI-174864 (squares), or after pretreatment with 1 µM beta-FNA (diamonds). Vertical bars, S.E.M.

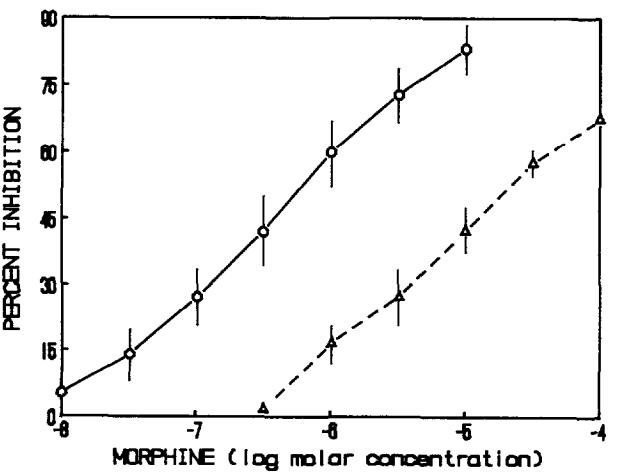

FIGURE 2. Antagonism by NIH-9835 of the effects of morphine upon the mouse vas deferens preparation. Abscissa: concentration of morphine; ordinate: percent inhibition of the twitch. Circles, controls; triangles, observations in the presence of 100 nM NIH-9835. Each value is the mean of 6-9 determination. Vertical bars, S.E.M.

NIH-9835 is a potent opioid antagonist which appears to act primarily upon mu receptors, although it is slightly active upon delta receptors. Its activity is different than that of naltrexone which blocks mu, kappa and delta receptors and which is more potent as an antagonist at kappa receptors than at delta receptors.

All three hybrids displaced bound ^{3}H-etorphine in rat cerebral membranes. The antagonist, NIH-9835, was less potent than naltrexone as a displacer (TABLE 1). The two agonists, NIH-9833 and NIH-9834 were extremely potent displacers of ^{3}H-etorphine with EC 50's in the range of etorphine.

TABLE 1. EC 50's of NIH-9833, NIH-9834 and NIH-9835 compared to those of representative opiate agonists and antagonists in displacing 0.5 nM ^{3}H-etorpine in a membrane preparation from rat cerebrum (in the presence of Na^{+}).

Compound	EC 50 (nM)
Morphine	23.60
Ethylketazocine	6.60
NIH-9835	1.70
Naltrexone	0.87
NIH-9833	0.58
Etorphine	0.37
NIH-9834	0.34

The observation that the addition of a single glycyl residue changes a dihydromorphine-peptide analog from a potent delta receptor agonists to an equally potent and quite specific mu receptor antagonists suggests that the two receptor sites on noradrenergic nerve terminals in the mouse vas deferens might be structurally quite similar.

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CHARACTERIZATION OF D-ALA^2,LEU^5,CYS^6-ENKEPHALIN: A NOVEL SYNTHETIC OPIOID PEPTIDE WITH SLOWED DISSOCIATION FROM DELTA RECEPTORS

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ABSTRACT

D-Ala^2,Leu^5,Cys^6-enkephalin (DALCE) is a synthetic enkephalin analog which contains a reduced sulfhydryl group. It exhibited moderate delta selectivity (mu/delta IC_{50} ratio 13), but was not as selective as the disulfide-containing peptide, D-Pen^{2,5}-enkephalin (DPDPE) (mu/delta ratio 1121). However, unlike other delta-selective peptides, DALCE exhibited a markedly slowed dissociation from receptors after pretreatment of membranes with micromolar concentrations. Pretreatment of membranes with 10 uM DALCE, followed by extensive washing produced an 85-90% loss of ^{3}H-DPDPE binding sites. D-Ala^2,D-Leu^5-enkephalin (DADLE), D-Ser^2,Leu^5,Thr^6-enkephalin (DSTLE) and DPDPE produced losses of 59%, 70%, and 19%, respectively. The effect of DALCE was not reversed by a 60 min post-incubation in buffer containing 250 mM NaCl + 100 uM GMPPNP, a condition which produced nearly complete reversal of loss of sites by DADLE and DSTLE. DPDPE could be dissociated merely by post-incubation in TRIS-buffer alone for 15 min. The order for ease of dissociation after preincubation was DPDPE > DADLE > DSTLE >>> DALCE. The effect of DALCE was selective for delta sites, although higher concentrations of DALCE produced loss of mu sites. DALCE pretreatment had no effect on recovery of kappa sites. These results indicate that DALCE binds essentially irreversibly to delta receptors.

INTRODUCTION

In the search for more selective and stable delta receptor ligands, several analogs of enkephalins have been synthesized. Examples are DADLE and DSTLE (Kosterlitz et al. 1980, Gacel et al. 1980) which make use of the incorporation of D-amino acids for proteolytic stability. Recently, conformationally restricted enkephalin analogs have been produced by disulfide bridge cyclization of the peptide chain. This is accomplished by introduction of cysteine or penicillamine at positions 2 and 5, followed by oxidation to form the disulfide bond (Schiller et al. 1981, Mosberg et al. 1983). D-Pen^{2,5}-enkephalin is the most delta selective enkephalin analog to date (Mosberg et al. 1983). Although these disulfide-containing peptides have been thoroughly characterized in binding assays and bioassays, the effect of introducing a reduced thiol group has not yet been studied. We report here the synthesis and characterization of D-Ala^2,Leu^5,Cys^6-enkephalin (DALCE). This peptide makes use of the D-Ala^2 residue for stability, but is unique in that it contains a sulfhydryl group at the C-terminus. Although this analog is not

as delta-selective as the disulfide-containing peptides, the free sulfhydryl group endows this peptide with a property lacking in other enkephalin analogs. DALCE has a markedly slowed rate of dissociation from delta and mu receptors. This is quite interesting in view of the fact that delta and mu opiate receptors contain an essential sulfhydryl group (Smith and Simon 1980). DALCE may bind covalently by formation of a disulfide bond to receptor.

METHODS

Tyr-D-Ala-Gly-Phe-Leu-Cys was custom synthesized by Peninsula Labs using solid phase techniques. Purity was established by TLC, paper electrophoresis, and reversed phase HPLC. The peptide tended to dimerize during storage due to sulfhydryl oxidation. This was prevented by storage in presence of 20 mM beta-mercaptoethanol in acidic solution at -20°C.

Lysed P_2 membrane preparations were prepared from brains (minus cerebellum) of male Sprague-Dawley rats (150-200 g). Membranes (650 ug protein/ml) were incubated with peptides for 60 min at 25° in 50 mM TRIS-HCl pH 7.4 containing 100 mM NaCl, 3 mM Mn acetate, 2 uM guanosine 5'-triphosphate (GTP), and the following protease inhibitors to protect susceptible peptides: bacitracin (100 ug/ml), leupeptin (4 ug/ml) chymostatin (2 ug/ml), bestatin (10 ug/ml). Following pelleting and resuspension in an equal volume of the buffers specified in table 1 and figure 1, membranes were incubated for the specified times to effect dissociation of the bound peptide. After this dissociation period (post-incubation), the membranes were washed and incubated with ^{3}H-ligand to assess recovery of binding sites compared to controls which were incubated with no peptide. Except where indicated otherwise, incubations with ^{3}H-ligand were oarried out for 90 min at 25°C in a total volume of 500 ul containing 10 mM TRIS-HCl pH 7.4 325 ug of membrane protein, and the components specified below. ^{3}H-DPDPE. 100 mM NaCl, 3 mM Mn acetate, 2 uM GTP; ^{3}H-DAGO (D-Ala^2,NMe-Phe^4,Gly-ol^5-enkephalin): 3 mM Mn acetate; H-BREM (bremazocine): 100 nM unlabeled DSTLE and DAGO to suppress mu and delta binding. ^{3}H-Ligand concentration was 2 nM. Non-specific binding was determined in presence of 10 uM unlabeled levallorphan. The incubation was terminated by filtration of inoubation mixture through glass fiber filters under reduced pressure and washing with ice-cold buffer.

RESULTS AND DISCUSSION

The receptor selectivity of DALCE was compared to other enkephalin analogs by competition binding assays using ^{3}H-DPDPE, ^{3}H-DAGO, and ^{3}H-BREM to label delta, mu, and kappa receptors, respectively (10 mM TRIS-HCl pH 7.4, 90 min, 25°C). Delta, mu, and kappa IC_{50} values for the following unlabeled peptides were: DALCE - 4.1, 55; >10,000 nM; DADLE - 3.4, 151, >10,000 nM; DSTLE - 4.0, 250, kappa not determined; DPDPE - 5.5, 6167, >10,000 nM. Therefore, DALCE exhibited moderate delta selectivity with roughly a 10-fold selectivity for delta sites over mu sites. Like the other enkephalin derivatives, DALCE had very low affinity for kappa receptors. As reported by others (Mosberg et al. 1983), the disulfide-containing peptide DPDPE exhibited the highest delta

selectivity. All the analogs had similar IC_{50} values at the delta site.

Table 1 shows the effect of various dissociation conditions on occupation of delta sites after preincubation with various delta-selective peptides at a concentration of 10 uM. After a 15 min dissociation period in 50 mM TRIS-HCl, all peptides produced a residual blockade of sites. However, DALCE produced the greatest loss of sites while DPDPE produced the least. Increasing the dissociation time in this buffer to 60 min caused moderate increases in recovery of sites after preincubation with DADLE and DSTLE. Recovery of sites with DALCE was unaffected and remained low. Sodium ion and guanine nucleotides have been shown to lower the affinity of opiate receptors for ligand, therefore inhibiting binding or promoting dissociation (Simon et al. 1975, Childers and Snyder 1980, Zukin and Gintzler 1980). Dissociation in buffer containing 250 mM NaCl and 100 uM GMPPNP resulted in complete and nearly complete recovery of ^{3}H-DPDPE binding sites with DADLE and DSTLE, respectively. However, the marked loss of sites produced by DALCE was unaffected by post-incubation in this buffer. Therefore, DADLE, DSTLE, and DPDPE can readily dissociate from delta receptors, while DALCE remains tightly bound. This effect required both opiate receptor recognition as well as a thiol group since preincubation with 25 uM beta-mercaptoethanol or dithiothreitol had no effect on recovery of sites (not shown).

TABLE 1. Pretreatment of membranes with peptides and effect of various dissociation conditions on recovery of delta binding sites

	Dissociation Condition and % Recovery of ^{3}H-DPDPE Binding		
Peptide Pretreatment (10 uM)	15 min	60 min	60 min, NaCl/GMPPNP
DADLE	41	50	100
DSTLE	30	50	80
DPDPE	81	--	--
DALCE	11	17	14

Figure 1 shows the receptor selectivity of the DALCE effect. Membranes were preincubated with various concentrations of DALCE, post-incubated in buffer containing 250 mM NaCl and 100 uM GMPPNP for 60 min, and recovery of mu, delta, and kappa sites was assessed by binding of ^{3}H-DAGO, ^{3}H-DPDPE, and ^{3}H-BREM, respectively. Delta sites were most sensitive to DALCE. Half maximal loss of sites occurred at 2-3 uM DALCE. Mu sites were not affected at 2 uM and only reached 50% loss at 20 uM DALCE. Kappa sites were not affected by DALCE pretreatment at concentrations up to 20 uM. These results are consistent with the receptor selectivity of DALCE (delta > mu >> kappa).

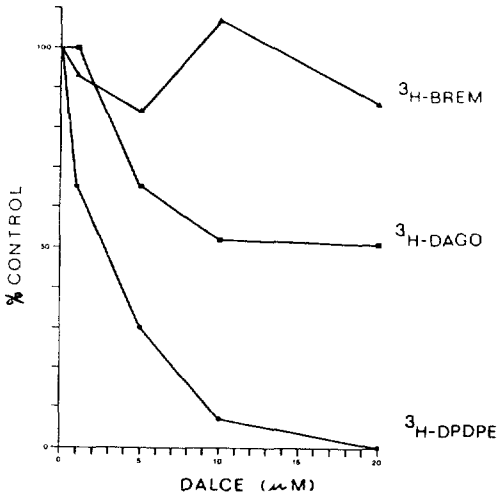

FIGURE 1. Receptor selectivity of the DALCE effect.

In conclusion, DALCE is a sulfhydryl-containing enkephalin analog with preference for delta receptors. It exhibits a markedly slowed rate of dissociation from delta and mu receptors compared to other delta receptor peptides. Taken together with the presence of a critical sulfhydryl group at the ligand binding site of mu and delta receptors (Smith and Simon 1980), it is possible that DALCE binds covalently by forming a disulfide bond with receptor. This is supported by preliminary results suggesting that the effects of DALCE are attenuated by post-incubation with a reducing agent. It would be interesting to ascertain whether incorporation of free sulfhydryl groups into other opioid peptides results in slowed dissociation from receptors.

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[^{3}H]DSTBULET, A NEW LINEAR HEXAPEPTIDE WITH BOTH AN IMPROVED SELECTIVITY AND A HIGH AFFINITY FOR δ-OPIOID RECEPTORS.

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ABSTRACT

The binding properties of the new agonist Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr, DSTBULET, in rat brain tissue shows that insertion of a bulky t.butyl group into the sequence of DSLET leads to a conformationally-induced large increase in selectivity for δ opioid receptors ($K_I(\delta)/K_I(\mu)$ = 0.012). In addition to its similar selectivity to that of the cyclic enkephalins DPDPE and DPLPE. the affinity of [^{3}H]DSTBULET for δ sites (K_D = 2.9 nM) is significantly better than that of [^{3}H]DPDPE (K_D~10.5 nM) or DPLPE ($K_I(\delta)$ ~ 19 nM). DSTBULET *is* therefore the most appropriate probe for both binding studies and pharmacological investigations of δ-receptors as illustrated by a comparison of the analgesic properties of DAGO, DSTBULET and DPLPE.

INTRODUCTION

A clear distinction between the pharmacological responses induced by stimulation of opioid μ or δ-receptors requires highly potent and selective ligands. In the case of δ receptors, linear hexapeptides (DSLET and DTLET) were the first really δ-selective ligands (Fournié-Zaluski et al. 1981; Gacel et al. 1980; Zajac et al. 1983). Recently, two cyclic enkephalins DPDPE and DPLPE have been shown to exhibit even better δ-selectivity than the linear peptides (Mosberg et al. 1983). Unfortunatly this is associated with a large decrease in affinity, preventing the use of [^{3}H]DPDPE for competition studies or for acute quantitative determinations of δ-sites in brain after biochemical lesions or pharmacological manipulations (Delay-Goyet et al. 1985). We report here the binding properties in rat brain tissue, of a new δ-ligand [^{3}H]DSTBULET which shows both a high selectivity and a high affinity.

MATERIALS AND METHODS

The linear hexapeptide Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr, DSTBULET, was prepared by the liquid phase method as described for DSLET and DTLET (Gacel et al. 1981). [^{3}H]DSTBULET (70 Ci/mmol) was obtained from the 2,5-dibromotyrosyl precursor. [^{3}H]DPDPE was from Amersham and [^{3}H]DTLET and [^{3}H]DAG0 from CEA (France). Binding assays (in triplicate) were carried out at 37°C using a crude rat brain membrane preparation as described (Zajac and Roques 1985). K_D and K_I values were obtained from computerized linear regression analysis. Inhibition of electrically evoked contractions of GPI or MVD was performed as described. Analgesic potency of the various compounds was evaluated using the hot-plate test in mice.

RESULTS AND DISCUSSION

Pharmacological profile of DSTBULET v DPDPE. The selectivity of DSTBULET was compared to that of DSLET, DTLET and DPDPE using : i) bioassays on peripheral organs (GPI enriched in μ sites and MVD in δ sites), ii) inhibition of the binding to rat brain tissue of [^{3}H]DAGO (μ selective ligand) and [^{3}H]DTLET (nearly δ-selective ligand). As shown in table 1, the introduction of the bulky O.tBu. group on DSLET leads to a large improved δ -selectivity, as occurs with the cyclic enkephalins. At the level of the brain receptors, DSTBULET was at least as selective as DPDPE but its apparent affinity was significantly better. Besides, DSTBULET is unable (IC_{50}>10,000 nM) to interact with κ receptors labelled with [^{3}H]EKC.

Binding characteristics of [^{3}H]DSTBULET on rat brain. Binding equilibrium of [^{3}H]DSTBULET (5 nM) was reached in 30 min at 35°C. Saturation experiments were performed under these conditions and binding parameters (K_D and B_{max}) calculated from linear regression analysis of the saturation isotherms. As shown in fig.1, [^{3}H]DSTBULET interacts with a single class of site (0.5-15 nM) (equilibrium dissociation constant K_D = 2.90 $\pm$ 0.33 nM and B_{max} = 98.8 $\pm$ 9.4 femtomoles/mg. In addition to its better δ-affinity than [^{3}H)DPDPE (K_D = 10.5 nM) it is very important to observe that [^{3}H]DSTBULET displayed 55% specific binding at 63% saturation against 26% at 67% saturation for [^{3}H]DPDPE. Moreover, as expected from its high selectivity, the B_{max} of [^{3}H]DSTBULET was not affected by blocking of u-sites with high concentration of DAGO (300 nM) and as a consequence of its higher affinity, the binding parameters can be determined with greater accuracy.

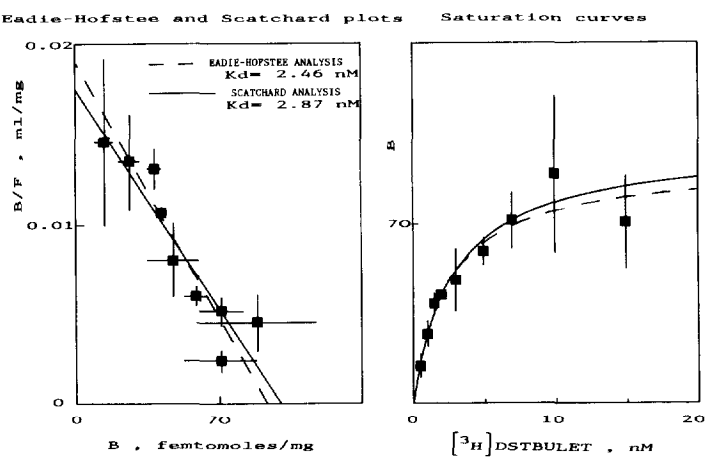
TABLE 1. Pharmacological profile of linear and cyclic enkephalins on peripheral organs and rat brain tissue.

Pharmacological assays (IC_{50} nM ± S.E.M.)		
	GPI	**MVD**
Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET)	406±46 (5)	0.40±0.04 (5)
Tyr-D-Ser-Gly-Phe-Leu-Thr (DSTBULET) OtBu	1800±360 (6)	1.07±0.04 (7)
Tyr-D-Pen-Gly-Phe-L-Pen (DPLPE)	10,000±200 (5)	2.50±0.12 (5)

Binding assays on rat brain membranes (K_I, nM ± S.E.M.)		
	[^{3}H]DAGO, 1nM	**[^{3}H]DSTLET, 2nM**
Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET)	31±5 (4)	4.80±0.80 (4)
Tyr-D-Ser-Gly-Phe-Leu-Thr (DSTBULET) OtBu	404±50 (6)	6.14±0.73 (5)
Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE)	993±151 (3)	19.20±1.40 (4)
Tyr-D-Pen-Gly-Phe-L-Pen (DPLPE)	873±210 (5)	10.90±1.2 (5)

The number of observations is given in parentheses.

FIGURE 1. Eadie-Hofstee and Scatchard analyses of the specific binding of [^{3}H]DSTBULET to rat brain membranes.



Correlation between supraspinal analgesia and affinity for opioid μ sites. As expected, the pharmacological properties of DSTBULET are in agreement with its binding characteristics to brain tissue. This is clearly demonstrated by its 130 fold lower potency than DAGO, a typical μ agonist, to inhibit nociceptive stimuli induced in mice through the hot plate test after i.c.v. administration. The low analgesic potency of DSTBULET and DPLPE, contrasting with their affinity for δ-sites, reinforces the suggested preferential involvement of μ-receptors in the supraspinal control of pain (Chaillet et al. 1984).

CONCLUSION

The new linear hexapeptide DSTBULET possesses the main requirements for both accurate biochemical and pharmacological investigations on delta opioid receptors : - a high affinity with a low non specific binding ; - a good selectivity for the delta sites ; - a satisfactory resistance to peptidases ; - a favourable hydrophobic-hydrophilic balance. Finally DSTBULET belongs to a large series of linear analogues with δ/μ selectivities ranging between 1 and 120. Taken together [^{3}H]DSTBULET and its analogues behave as useful compounds for a complete study of the physiological relevance of delta opioid receptors.

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ANOMALOUS BINDING OF DPDPE AS A RESULT OF BATCH VARIABILITY

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ABSTRACT

The binding of the delta selective agonist [^{3}H]DPDPE to diencephalic rat brain tissue was examined. Although most material examined gave rise to a single receptor model, one commercial preparation (Amersham, batch 8) suggested a two receptor model. Purification by HPLC removed a minor component and restored a single receptor model to the major component.

INTRODUCTION

The endogenous opioid peptides are known to interact with several classes of opioid receptors. The synthetic peptide (2-D-penicillamine, 5-D-penicillamine) enkephalin (DPDPE) is a conformationally restricted enkephalin analogue which has been shown to be a selective delta agonist (Mosberg et al. 1980) and is useful in autoradiographic differentiation of delta receptors in rat brain (Mansour et al. 1986). During the course of these studies several different batches of [^{3}H]DPDPE were utilized. One of these, Amersham batch #8, exhibited binding characteristics different from previous and subsequent batches of synthetic [^{3}H]DPDPE.

METHODS AND MATERIALS

Slide-mounted brain sections (25 um) from the diencephalon were incubated with 200 ul of [^{3}H]DPDPE (Amersham, 41.5 Ci/mmol, batch 8) in a 50 mM Tris buffer (pH 7.5 at 25°C). The concentrations of [^{3}H]DPDPE ranged from 0.1 to 14 nM. After a 60 minute incubation, the slides were drained and washed in four consecutive 250 ml 50 mM Tris washes (pH 7.6, 4°C) for 30 seconds. The slides were then dried with a portable hair dryer and the binding was quantified using liquid scintillation counting (Packard, 2000 CA). Non-specific binding was evaluated by treating a parallel set of slides with the same [^{3}H]DPDPE concentrations with a 1 uM final concentration of unlabelled DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr). The data were graphed as Scatchard plots and the K_D and B_{max} values determined with the LIGAND program (Munson and Rodbard 1980). Similar studies were done on other batches of [^{3}H]DPDPE (Amersham, batch 9) and purified [^{3}H]DPDPE from batch 8.

The liquid chromatography was done on an HPLC system comprised of two Altex 110 pumps coupled to an Altex model 420 controller, a Rheodyne 7125 injection valve, a Waters 3.9 mm X 30 cm uC18 reverse phase column and a Pharmacia model 100 fraction collector. The flow rate was 1.5 ml/min; the elution solvent was 10-60% (over 80 minutes) acetonitrile/0.1% TFA. Samples were collected every minute and a 50 ul aliquot was examined for [^{3}H] activity by liquid scintillation counting.

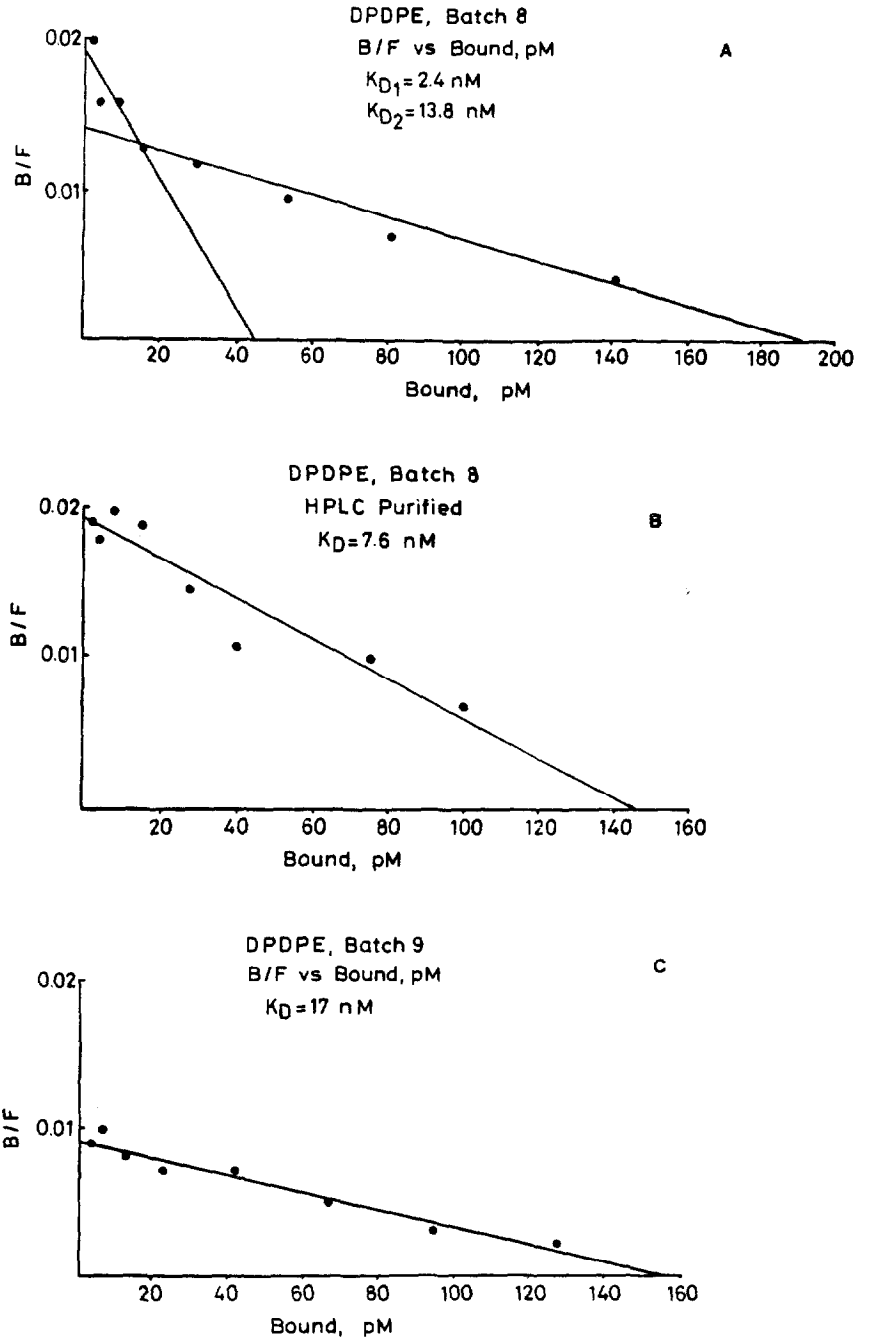

FIGURE 1. Scatchard plots of selected DPDPE batches

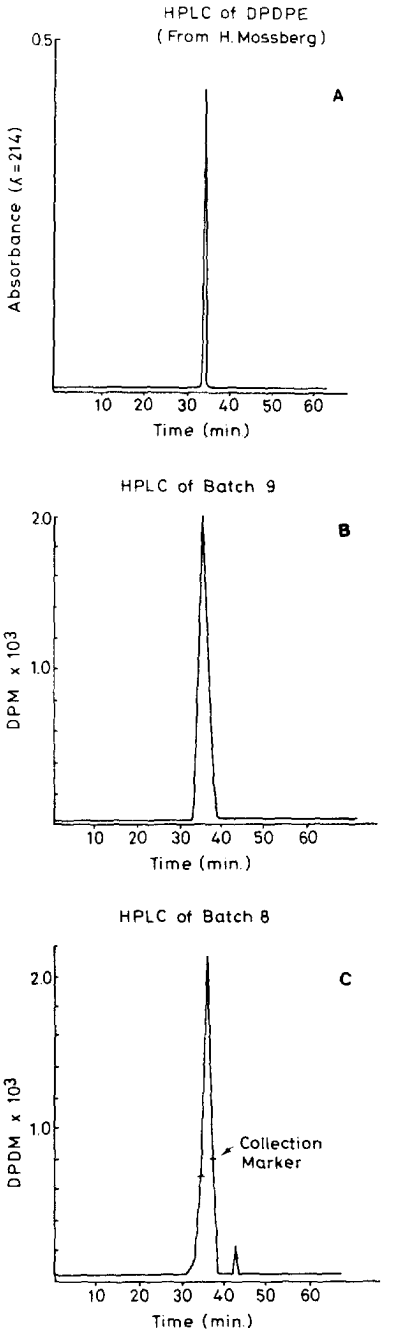

Figure 2. HPLC Chromatograms for DPDPE Batches

RESULTS

The Scatchard plot from the "LIGAND" analysis for DPDPE binding to rat brain diencephalic tissue for Amersham batch 8 is shown in figure 1(A). The best "LIGAND" fit for this data was a two receptor model with a K_{D1} of 2.4 nM and a K_{D2} of 13.8 nM. The corresponding B_{max} values were 44 and 189 pM respectively.

The Scatchard plot from the "LIGAND" analysis for binding of the major component of batch 8 (which had the same elution volume as authentic DPDPE, figure 2(A) and (C)) to diencephalic tissue is shown in figure 1(B). The best "LIGAND" fit was for a single receptor site having a K_D of 7.6 nm with a B_{max} of 146 pM.

The Scatchard plot from the "LIGAND" analysis of the binding of most batches of DPDPE was typified by batch 9 as shown in figure 1(C). The HPLC chromatogram of this material is shown in figure 2(B). This gives rise to a single receptor model with a K_D = 17 nM and a B_{max} = 157 pM.

CONCLUSIONS

Amersham DPDPE, batch 8, contained an easily removable impurity which altered the binding characteristics of this material.

The binding of purified DPDPE to diencephalic sections of rat brain is consistent with a single receptor model having a K_D = 7.6 nM and a B_{max} of 146 pM.

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ACKNOWLEDGMENTS

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COMPARISON OF THE OPIOID ANTAGONIST PROPERTIES OF A CYCLIC SOMATOSTATIN ANALOG IN VITRO AND IN VIVO

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INTRODUCTION

We have recently developed a conformationally constrained analog of somatostatin-14 called CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH2) which shows a high degree of affinity and selectivity for the mu opioid receptor in binding assays using homogenized rat brains (Pelton et al. 1985; 1986; Gulya et al. 1986). The purpose of these investigations was to determine the opioid pharmacology of CTP in the guinea pig isolated ileum, mouse isolated vas deferens, and in analgesic and gastrointestinal transit time tests in mice.

MATERIALS AND METHODS

In Vitro Bioassays: The guinea pig ileum longitudinal muscle-myenteric plexus (GPI) and mouse vas deferens (MVD) were used to determine the mu/kappa and mu/delta selectivity of CTP respectively. The GPI (Kosterlitz et al. 1970) and MVD (Hughes et al. 1975) were run according to standard procedures.

In Vivo Bioassays: CTP was tested for antagonism of mu, delta and kappa selective agonists in two models of analgesia in male ICR mice (20-25 g), the hotplate and abdominal stretch tests. The hotplate (55°C) test was run according to the methods of Porreca et al. (1984). For the abdominal stretch test, mice received intrathecal (5 ul) distilled water or drug, followed immediately by an intraperitoneal (i.p.) injection of 1.2% acetic acid. Mice were then observed for stretching behaviors for 5 min.

The rate of gastrointestinal transit in mice was determined by the geometric center method as described by Porreca et al. (1984).

Peptides: The following peptides were used as mu, delta and kappa selective agonists respectively: PLO17 (Chang et al, 1983), DPDPE (Mosberg et al. 1983) and U50,488H (VonVoigtlander 1983). PLO17 and DPDPE were purchased from Peninsula Labs., Belmont CA., and U50,488H was purchased from Upjohn, Kalamazoo, MI. CTP was synthesized as previously described (Pelton et al. 1986). All peptides were dissolved in distilled water.

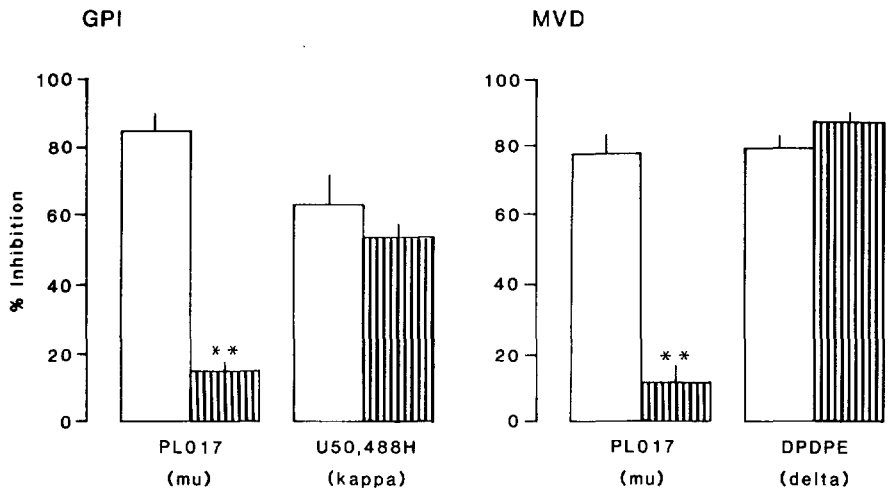

FIGURE 1. The effect of pretreatment with 1.0 uM CTP on the inhibitory actions of PL017 (0.1 uM) and U50,488H (0.1uM) in the guinea pig ileum and of PL017 (1.0 uM) and DPDPE (0.01 uM) in the mouse vas deferens. Significant differences between control (open bars) and CTP treated (hatched bars) are indicated by **, p<0.01, n=4.

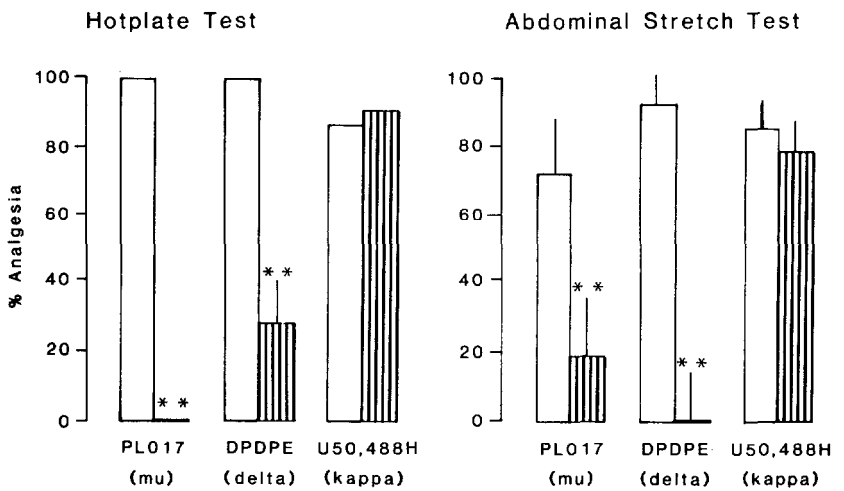

FIGURE 2. The effect of icv distilled water (open bars) or icv 1.0 ug CTP (hatched bars) pretreatment on the analgesic actions of PL017 (0.3 ug, icv). DPDPE (3.0 ug, icv) or U50,488H (0.1 ug, icv) in the hotplate test and PL017 (0.3 ug, it). DPDPE (1.0 ug, it) or U50,488H (0.1 ug, it) in the abdominal stretch test. Significant differences are indicated by **, p< 0.01, n = 6/dose.

RESULTS

In Vitro Bioassays: In the GPI, pretreatment with CTP antagonized the actions of PLO17 but not U50,488H (figure 1). The actions of PLO17 were also blocked by CTP in the MVD. In contrast, CTP did not antagonize the effects of DPDPE in this tissue (figure 1).

In Vivo Bioassays: PLO17, DPDPE and U50,488H given intracerebroventricularly (icv) all produced increases in the latency to display escape behaviors in the hotplate test (figure 2). The increased latency caused by U50,488H appeared to be related to the production of immobility rather than true analgesia. Pretreatment with CTP antagonized the analgesic actions of PLO17 and DPDPE in the hotplate test, but did not block the actions of U50,488H (figure 2). Schild analysis of the interaction of supraspinal CTP with PLO17 or DPDPE showed that CTP antagonized PLO17 in a competitive fashion (pA2 11.9 ± 1.3, slope -0.85 ± 0.5) but antagonized DPDPE in some complex, noncompetitive fashion (slope -0.55 ± 0.3). Likewise, in the abdominal stretch test, CTP again blocked the analgesic actions of PLO17 and DPDPE, but did not block that produced by U50,488H (figure 2). The actions of all 3 agonists were reversed by naloxone in both analgesic tests (data not shown).

Both PLO17 and DPDPE also slowed the rate of gastrointestinal transit after spinal administration to mice (table 1). While pretreatment with CTP antagonized the antitransit effects of PLO17, it did not block the effects of DPDPE (table 1). Naloxone (1.0 ug, it) antagonized the antitransit effects of both agonists (data not shown)

Table 1. The effect of intrathecal CTP (1.0 ug) pretreatment on the rate of gastrointestinal transit.

	% Inhibition of Gastrointestinal Transit	
Pretreatment	PLO17 (3 ug, it)	DPDPE (3 ug, it)
vehicle	60 ± 16	40 ± 8
CTP	26 ± 10*	34 ± 9

*$p<0.05$; n=5/dose.

DISCUSSION

Results from the GPI and MVD studies indicate that CTP possesses mu selective antagonist properties with no delta or kappa antagonist actions. These findings support the conclusions of Pelton et al. (1985, 1984; Gulya et al. (1984, who have shown that CTP displays a high degree of selectivity for the mu receptor in binding assays using homogenized rat brains.

The selective antagonism of mu but not delta related inhibition of gastrointestinal transit shows that CTP retains its mu selectivity in vivo. And, as expected from the results of the GPI studies, CTP had no kappa antagonist properties when tested in vivo.

Despite the consistent mu selectivity of CTP (no delta or kappa antagonism) seen in the binding assays, GPI, MVD and gastrointestinal transit time tests, CTP antagonized the analgesia evoked by both mu and delta agonists. The antagonism of mu related analgesia was shown to be of a competitive nature while that of the delta agonist was not competitive. These findings may indicate that certain mu and delta receptors which mediate analgesia are functionally coupled, and that through its interaction with the mu receptor, CTP can also block delta related analgesia.

In conclusion, CTP appears to produce mu selective antagonism both in vitro and in vivo. CTP may prove useful in studying the possible link between analgesic mu and delta receptors, and may also help in determining if mu and delta receptors which mediate other functions are also coupled.

ACKNOWLEGEMENTS

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H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2: A POTENT AND SELECTIVE ANTAGONIST FOR MU OPIOID RECEPTORS

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ABSTRACT

H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2(CTOP) exhibited high affinity (IC_{50} = 2.80 nM) in displacing [^{3}H]naloxone binding (nH = 0.89 ± 0.1) and showed an exceptional selectivity for mu opioid receptors with an IC_{50}(DPDPE)/IC_{50}(naloxone) ratio of 4,840, while it displayed very low affinity for somatostatin receptors (IC_{50} = 22,700 nM) in rat brain binding assays. [^{3}H]CTOP was recently custom synthesized (spec. act.: 84 Ci/mmol) and evaluated for its in vitro binding properties towards the mu opioid receptors in rat brain membrane preparations. Association and dissociation of [^{3}H]CTOP binding to mu opioid receptors were rapid at 25°C with a kinetic K_d value of 0.67 nM. Saturation experiments gave apparent K_d value of 1.11 nM and B_{max} value of 136 ± 13 fmol/mg prot at 25°C. Specific [^{3}H]CTOP binding was inhibited by a number of different opioid and opiate ligands. Among them, putative mu opioid receptor-specific ligands, such as naloxone, naltrexone and CTOP inhibited the binding with high affinity, while delta opioid receptor-specific compounds or non-opioid drugs inhibited specific [^{3}H]CTOP binding with low affinity or they were ineffective.

INTRODUCTION

In an attempt to study the selectivity, potency and conformational requirements of somatostatin-related peptides for the mu opioid receptor, we prepared a series of cyclic conformationally restricted penicillamine (β,β-dimethylcysteine) containing somatostatin octapeptide analogues by standard solid phase synthetic methods and tested them for their ability to inhibit specific [^{125}I]CGP 23,996 (des-Ala^1-,Gly^2-[desamino-Cys^{3}Tyr^{11}]-dicarba^{3,14}-somatostatin), [^{3}H]naloxone or [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin) binding in rat brain membrane preparations: Our most potent and mu receptor-selective octapeptide CTOP was recently custom labeled and its binding characteristics were evaluated toward the mu opioid receptors.

MATERIALS AND METHODS

[^{3}H]CTOP (84 Ci/mmol), [^{3}H]naloxone (57 Ci/mmol) and [^{3}H]DPDPE (33.6 Ci/mmol) were from New England Nuclear. [^{125}I]CGP 23,996, a relatively nonhydrolysable analogue of somatostatin, was prepared according to Czernik and Petrack (1983). Inhibition studies employing [^{125}I]CGP 23,996, [^{3}H]naloxone or [^{3}H]DPDPE were carried out as described elsewhere (Pelton et al. 1985a, b; Gulya et al. 1986). [^{3}H]CTOP binding was carried out in 50 mM Na/K-PO_4

Table 1. IC_{50} value and selectivity of CTOP for somatostatin, mu- and delta opioid receptors in rat membrane preparation

Radiolabeled ligand	CTOP IC_{50} (nM)	n_H	Ratio[1]	Ratio[2]
[^{125}I]CGP 23,996	22,700 (13,000-30,000)	N.D.	8,107	-
[^{3}H]naloxone	2.80 (2.29-4.22)	0.89±0.1	-	-
[^{3}H]DPDPE	13,522 (10,980-16,860)	1.10±0.1	-	-

IC_{50} values represent the geometric mean with the range of values from at least three separate experiments done in duplicates shown in parentheses. The average Hill values represent the Hill coefficients from the unweighted best-fits of the computer-generated regression curves. N.D. = not determined.
[1] IC_{50}(somatostatin)/IC_{50}(mu); [2] IC_{50}(delta)/IC_{50}(mu).

buffer (pH 7.4 at 25°C) containing 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA) and 100 uM phenylmethylsulfonylfluoride (PMSF). For kinetic studies or experiments involving the inhibition of [^{3}H]CTOP binding, a total concentration of 0.5 nM [^{3}H]CTOP was incubated with 400-450 ug protein. For saturation experiments, twelve concentrations of [^{3}H]CTOP ranging from 0.1 nM to 16 nM were used. 1 uM naltrexone was used to determine nonspecific binding. The reaction was terminated by rapid vacuum filtration through 0.1% polyethylenimine-pretreated Whatman GF/B glass filters. The filters were then rinsed three times with 3 ml saline solution and dried. Scintillation fluid (6 ml) was added and the samples were counted by liquid scintillation spectrophotometry (44% efficiency).

RESULTS AND DISCUSSION

The potency and selectivity of CTOP on inhibiting somatostatin, mu- and delta opioid receptors are shown in table 1. Specific [^{125}I]CGP 23,996 and [^{3}H]DPDPE bindings were inhibited by CTOP with low affinity, while [^{3}H]naloxone binding was displaced with high affinity from an apparently single population of binding sites (nH = 0.89 ± 0.1). The exceptional selectivity of CTOP toward mu opioid receptors (IC_{50}(delta)/IC_{50}(mu) = 4,840) is the result of its high affinity for mu opioid receptors combined with very low affinity for the delta sites. No substantial interaction to somatostatin receptors was observed at low concentrations of CTOP. Because of its high affinity and selectivity for the mu opioid receptor binding sites, it seemed to be the ligand of choice for further characterization of this class of opioid receptors using direct radioligand binding studies.

Table 2. Determination of the nonspecific binding of [^{3}H]CTOP using selected mu opioid compounds in rat brain membranes at 25°C

Displacer drug	Nonspecific binding (% of total)
naloxone	37
naltrexone	39
naloxonazine	40
DAG0	34
PL017	38
CTOP	24

[^{3}H]CTOP (0.5 nM) was incubated with 400-450 ug protein in 50 mM Na/K-PO_4 buffer (pH 7.4 at 25°C) containing 5 mM $MgCl_2$, 1 mg/ml BSA and 100 uM PMSF for 120 min at 25°C. Nonspecific binding was determined in the presence of different putative mu opioid receptor-selective drugs at a final concentration of 1 uM.

Table 3. Inhibition of specific [^{3}H]CTOP binding by selected opioid and non-opioid compounds in rat brain membrane preparations

Drug	IC_{50} (nM)	K_i (nM)	n_H
opioids			
naltrexone	0.37 (0.33-0.42)	0.26	1.112 ± 0.06
naloxone	0.97 (0.59-1.64)	0.67	1.084 ± 0.08
CTOP	4.62 (3.52-4.19)	2.19	1.344 ± 0.25
DAGO	1.33 (0.78-2.05)	0.92	0.826 ± 0.12
morphine	7.32 (6.06-8.14)	5.08	0.713 ± 0.04
PLO17	44.1 (34.1-70.1)	30.4	0.707 ± 0.03
DADLE	14.3 (10.5-21.0)	9.9	0.817 ± 0.13
DSLET	46.5 (31.1-63.4)	32.1	0.883 ± 0.20
DPDPE	845 (645-1,246)	583	0.711 ± 0.11
non-opioids (at 10 uM)	% of control		
somatostatin	94		
substance P	70		
phenoxybenzamine	69		
picrotoxin	68		
atropine	86		

The inhibition of [^{3}H]CTOP binding by 1 uM naltrexone was used to define nonspecific binding (table 2) to the mu opioid receptor since CTOP inhibited [^{3}H]CTOP binding an additional 15% from a putative non-opioid site.

Results of kinetic experiments performed to determine association (k_{+1}) and dissociation (k_{-1}) rate constants at 25°C show that specific [^{3}H]CTOP binding reached steady-state after 45 min of

incubation (k_{+1} = 2.04 M^{-1} min^{-1}), while dissociation of specific [^{3}H]CTOP binding was rapid ($t_{1/2}$=4.40 min) with a k-1 value of 1.62x10^{-1} min^{-1}. The kinetic K_d was found to be 0.67 nM at this temperature.

Computer-assisted nonlinear least squares regression analyses of saturation isotherms of specific [^{3}H]CTOP binding gave an apparent dissociation constant (K_d) of 1.11 nM and a B_{max} value (maximal number of binding sites) of 136 $\pm$ 13 fmoles/mg protein in rat brain membranes.

Inhibition curves were obtained for several opioid and opiate compounds as well as non-opioid drugs in order to determine the specificity of [^{3}H]CTOP binding to mu opioid receptors (table 3). [^{3}H]CTOP binding was displaced with high affinity (K_i values from 0.26 nM to 2.19 nM) by opioid antagonists such as naloxone, naltrexone and CTOP from a single population of binding sites, while putative mu opioid agonists such as DAGO (Tyr-D-Ala-Gly-NMePhe-Gly-ol), morphine and PLO17 ([NMePhe^3, D-Pro^5]morphiceptin) had n_H values significantly less than one, indicating the presence of multiple affinity states of mu opioid receptors. Delta opioid receptor-specific compounds like DADLE ([D-Ala^2, D-Leu^5]enkephalin), DSLET ([D-Ser^2]Leu-enkephalin-Thr) or DPDPE Inhibited [^{3}H]CTOP binding with much lower affinity (K_i values from 9.90 to 583 nM) and Hill coefficients less than one. Non-opioid compounds were very weak in displacing [^{3}H]CTOP binding, or they were ineffective.

In summary, we designed and synthesized a somatostatin-related conformationally restricted octapeptide analogue highly specific for the mu opioid receptor site. This peptide antagonist has high affinity and exceptional selectivity for this class of opioid receptors virtually without any interaction to other neurotransmitter systems at lower concentrations. This octapeptide might be the ligand of choice for further characterization of mu opioid receptors.

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OPIOID AGONIST AND ANTAGONIST EFFECTS OF CONFIGURATIONAL STEREOISOMERS OF 3-(DIMETHYLAMINO)-2,2-DIMETHYL-7-HYDROXY-1-TETRALOL

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ABSTRACT

The opioid agonist and antagonist effects of two configurational aminotetralin stereoisomers, namely, the cis-(MRSAL) and trans-(RMG) diastereo isomers of 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralol were studied using the guinea pig ileum longitudinal muscle preparation. MRSAL demonstrated opioid antagonist activity but failed to show agonist effects. In contrast, RMG showed agonist activity which was partially reversed by NX and MRSAL. RMG also exhibited weak antagonist activity towards DHM, NM and DADLE. These results showed that MRSAL was an opioid antagonist while RMG possessed weak mixed opioid agonist-antagonist activities.

INTRODUCTION

Aminotetralins are considered A, B ring structural analogs of morphine. Martin and Kandeel (1973) showed that these compounds had opioid agonist activity. Subsequently, Fries and Bertelli (1982) synthesized aminotetralin analogs which were capable of producing mixed opioid agonist-antagonist activities. More recently, we have reported on several structural modifications of aminotetralins (Lippman et al. 1985) , the most potent being 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralone (J). In vitro and in vivo testing of J showed only mu agonist activity. Reduction of the C-1 carbonyl on J to a hydroxyl group resulted in an opioid antagonist, MRSAL (Lippman et al. 1985/1986).

Recently we purified the diastereo isomers of 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralol. We now report on the pharmacological properties of these two isomers on the guinea pig ileum longitudinal muscle (GPI).

MATERIALS AND METHODS

Male guinea pigs (400-500 g) were decapitated and the ileum longitudinal muscle was removed and prepared as described by Kosterlitz et al. (1981). The longitudinal muscle with its myenteric plexus was mounted in a 5 ml tissue bath (37°C) between two platinum electrodes containing oxygenated (95% 0_2 :5% CO_2) Krebs-bicarbonate buffer solution with the following composition (mM): NaCL, 118.40; KCl, 4.75; $CaCl_2$, 2.54; $MgSO_4$, 1.20; KH_2PO_4, 1.19; $NaHCO_3$, 25; glucose, 11.10; choline chloride, 20 uM; and pyrilamine maleate, 0.125 uM. The muscle strip was connected to a Grass transducer (FT.03) and isometric contractions were induced by a Grass Model S44 stimulator at 0.1 Hz, 0.25 msec, 80-90v and recorded on a Grass Model 7B polygraph. The tension was set at 1.0 g. After equilibrium was reached the

compounds were added to the bath and the effects recorded for 5 min. In agonist experiments the maximum effect (5 min) of the compound was recorded, then naloxone (NX) was added to the bath. In antagonist experiments, the compounds were incubated for 5 min before the agonists prototypes dihydromorphine (DHM), normorphine (NM) and DADLE were added to the bath.

The diastereo isomers were assigned relative configurations by FT-NMR and mass spectroscopy.

RESULTS

The effects of MRSAL and RMG on the electrically stimulated contraction of the GPI are shown in table 1. RMG caused a maximal decrease

TABLE 1. Effects of MRSAL and RMG in the electrically-stimulated guinea pig ileum.

Concentration (uM)	% Change in Effect (mean ± S.E.)			
	N	MRSAL	N	RMG
42.5	19	3.21 ± 0.5	--	NT
85.0	55	9.94 ± 1.1	18	21.6↓ ± 1.7
170.0	7	11.34 ± 1.2	5	17.6↓ ± 2.0

NT= not tested

in contraction of 21% at 85 uM, while MRSAL caused increases in contractions of 3-11% at concentrations of 42.5-170 uM. The maximal agonist effect of RMG was partially antagonized by NX (53%) and MRSAL (63%) (table 2). suggesting that RMG had some opioid agonist

Table 2. Antagonism of RMG agonist activity by naloxone and MRSAL.

RMG (uM)	N	% Decrease	Antagonist	Concn	% Blockade
85.0	7	21.6 ± 1.7	NX	176 nM	53.9 ± 2.9
170.0	3	17.6 ± 2.0	NX	704 nM	44.3 ± 3.0
85.0	4	20.7 ± 1.9	MRSAL	85 uM	63.3 ± 3.1

% is expressed as the mean ± S.E.

activity. Both compounds were able to antagonize selected opioid agonists (table 3). However, MRSAL was more potent than RMG. MRSAL was able to antagonize nearly equally NM and DHM by 89% and DADLE by

81%, while RMG antagonized NM by 65%, DHM by 47% and DADLE by 37%. The above data demonstrated that MRSAL was an opioid antagonist devoid of agonist activity, while RMG had mixed opioid agonist-antagonist activities at equiconcentrations of the two isomers.

Table 3. Effect of Opioid Antagonist Activity of MRSAL and RMG Against Selected Opioid Agonists in guinea pig ileum.

Opioid Antagonist	N	Opioid Agonist	% Blockade
MRSAL	14	NM	89.9 ± 1.9
MRSAL	4	DHM	89.0 ± 1.8
MRSAL	7	DADLE	81.1 ± 1.8
RMG	4	NM	65.5 ± 7.7
RMG	6	DHM	47.8 ± 3.8
RMG	3	DADLE	37.5 ± 1.9

Concentration used for MRSAL and RMG was 85 uM. Results are expressed as the mean ± S.E.

DISCUSSION

We had previously demonstrated that 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralone (J) to be a mu opioid agonist. When the C-1 carbonyl on J was reduced to a hydroxyl, at least two diastereo isomers were formed as shown in figure 1. When these isomers were

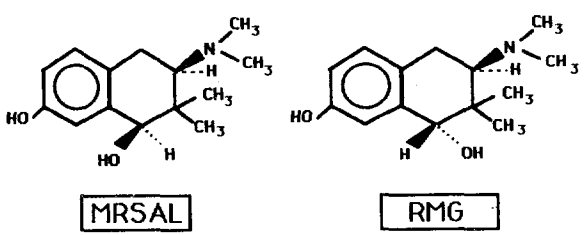

FIGURE 1. Cis-(MRSAL) and trans-(RMG) isomers of 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralol.

tested in the GPI bioassay in this study, it was clear that the agonist activity present in J was significantly decreased by this very slight alteration in the molecular structure. The trans-isomer (RMG) inhibited the muscle contraction only 21% at 85 uM of which 53% was reversed by NX as compared to 70% inhibition by J at 30 uM and was completely reversed by NX (Matos et al. this volume). The cis-isomer (MRSAL) was devoid of agonist activity and instead increased slightly the muscle contraction of the ileum. This latter effect is similar to that of NX as has been shown by many investigators. When these two isomers were tested against prototype opioid

agonists, both isomers showed antagonist activity.

The orientation of the hydroxyl group about the C-1 position appeared to be important for both agonist and antagonist activities. If the hydroxyl was trans to the nitrogen, then agonist and antagonist activities were conferred. Whereas if the hydroxyl was cis, only antagonist activity was observed. In addition the cis-isomer reversed the opioid agonist activities of DHM, NM and DADLE more effectively than did the trans-isomer. Thus, it appeared that the preferred configuration for antagonism of opioid agonists was the cis-isomer.

Since the optical isomers of these diastereo isomers were also present, final conclusions about antagonism of opioid agonists by these compounds must await final determination of the absolute configuration of the active isomer. Nevertheless, since the cis-isomer effects in the GPI were very similar to that of NX, it is possible that these two antagonists are acting via similar mechanisms at the level of the mu-opioid receptor. If this is true, then these small molecules might be good pharmacological tools to be used in increasing our knowledge about the interaction of opioid agonists and antagonists at the molecular level of the opioid receptor(s).

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The authors wish to thank Dr. Jim Shoolery and Varian Scientific for their assistance in obtaining and interpreting the FT-NMR data.

OPIOID RECEPTOR EFFECTS OF TWO 3-AMINO-2,2-DIMETHYL-TETRALIN ANALOGS IN GUINEA PIG ILEUM LONGITUDINAL MUSCLE

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ABSTRACT

Two substituted analogs of 3-amino-2,2-dimethyltetralin, namely 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralone HBr (J) and 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralol (MRSAL), were evaluated for opioid agonist and antagonist activity using the electrically driven guinea pig ileum longitudinal muscle preparation (GPI). Compound J appeared to be an opioid agonist with a preference for mu receptors while MRSAL was an opioid antagonist with-little selectivity for mu or kappa receptors,

INTRODUCTION

Aminotetralins with opioid analgesic activity were first studied by Martin et al. (1969). Subsequently, Kandeel and Martin (1973) reported a number of aminotetralins with potent opioid agonist activity. Such compounds also have the potential for opioid antagonist as well as agonist activity. In the present report, two new aminotetralin analogs, J and MRSAL (figure 1), were studied for both opioid agonist and antagonist activities using

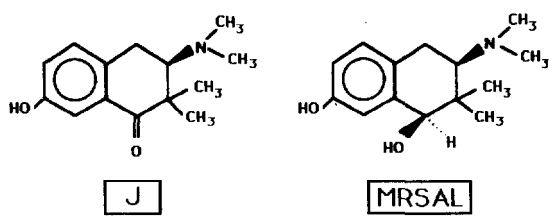

FIGURE 1. Aminotetralin chemical structures.

the electrically stimulated GPI. Opioid receptor selectivity for the agonist was studied by using naloxone (NX) as a prototype antagonist. The potency of MRSAL antagonism was evaluated by using agonist prototypes such as N-normorphine HCl (NM), dihydromorphine (DHM), ethylketocyclazocine methanesulfonate (EKC) and eptides such as dynorphin 1-13 (DYN 1-13) and (D-alanine^2, D-leucine^5) enkephalin (DADLE).

MATERIALS AND METHODS

Male guinea pigs (400-550 g) were decapitated and ileums

removed. Each longitudinal muscle with myenteric plexus attached was prepared as described by Kosterlitz et al. (1970) from a 6-cm segment 10-15 cm proximal to the ileocaecal valve using the method of Su et al, (1981). The muscle was set up between two platinum electrodes in a 5-ml bath containing oxygenated (95% O_2:5% CO_2) Krebs bicarbonate buffer solution with the following composition (mM): NaCl 118.4, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.2, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.1 plus choline chloride 20 µM and pyrilamine maleate 0,125 µM. The strip was connected to a transducer, allowed to equilibrate with constant electrical stimulation (0.1 Hz, 0.25 msec, 80-90 V) and isometric contractions polygraphically recorded. The resting tension was 1 g. After equilibrium, the agonists were added to the bath and the effects recorded until the maximum effect had been reached. The antagonists were either preincubated for 3 min (NX) and 5 min (MRSAL) or added after the agonist.

RESULTS AND DISCUSSION

The results of this study demonstrate that J behaved as an opioid agonist producing a concentration-response related decrease in contractions with the effects antagonized by NX. On the other hand, MRSAL demonstrated significant opioid antagonist activity in a manner similar to that of NX. Figure 2 shows the concentration-effect agonist curves for NM (mu-receptor prototype) and NM

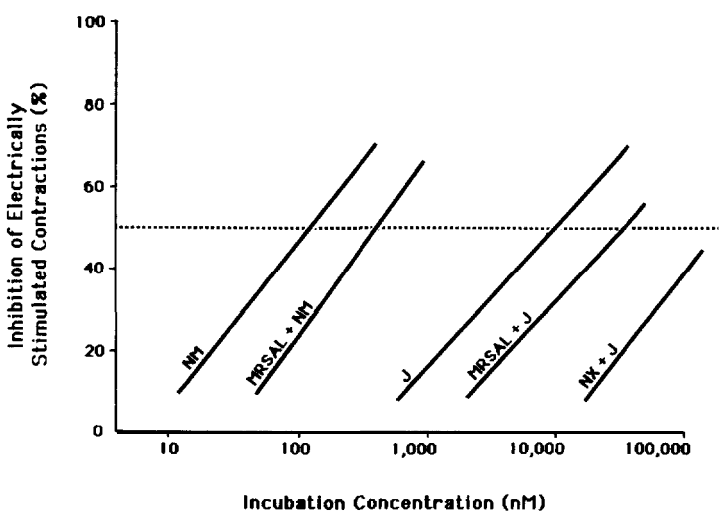

FIGURE 2. Agonist capacity in GPI. Slope (b) values are: NM 38.6 (r-0.62, N = 300), MRSAL + NM 42.9 (r = 0.86, N = 50), J 34.1 (r = 0.74, N = 174), MRSAL + J 33.5 (r = 0.93, N = 49), NX + J 38.8 (r = 0.90, N = 57).

TABLE 1: Calculated pA2 values for NX and MRSAL antagonism of selected opioid agonists

Antagonist	Agonist	N	pA2 ± S.E.M.
NX	NM	15	8.40 ± 0.06
NX	EKC	14	7.62 ± 0.08
NX	J	30	8.17 ± 0.07[1]
MRSAL	NM	28	5.41 ± 0.02
MRSAL	DHM	21	5.53 ± 0.05
MRSAL	EKC	25	4.50 ± 0.07
MRSAL	J	20	5.09 ± 0.04
MRSAL	DYN 1-13	24	5.10 ± 0.05
MRSAL	DADLE	20	5.22 ± 0.03

[1]Significantly different from corresponding values for NX + NM ($P < 0.05$) and NX + EKC ($P < 0.001$).

antagonism by MRSAL. In addition, the concentration-effect curve for J is shown and the antagonism of J by NX and MRSAL. The concentrations (nM) of the agonists causing 50% inhibition of maximum response (IC_{50}) as calculated from the respective regression-line equations are: NM 110.2, DHM 45.1, EKC 1.3, DYN 1-13 1.8, DADLE 19.9 and J 9625.0. Compound J was completely and reversibly antagonized by low concentrations of NX (88 nM) suggesting a specificity for mu receptors (pA2 = 8.17, table 1). The pA2 values for MRSAL antagonism of selected opioid agonists are also shown in table 1. MRSAL demonstrated low affinity but behaved as a full antagonist of opioid receptors. Its highest affinity was seen toward mu rather than kappa opioid receptors. Prototypes selective for mu receptors (NM, DHM) and J were antagonized with concentrations of 21.2 µM while kappa agonists (EKC, DYN 1-13) required higher concentrations such as 85 µM.

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PREPARATION OF /^{3}H/-OXYMORPHAZONE AND ITS BINDING TO RAT BRAIN MEMBRANES

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ABSTRACT

Oxymorphazone is a 14-hydroxydihydromorphinone derivative which contains C-6 hydrazone group and hence could serve as an irreversible lahel for opioid receptors.
^{3}H-oxynorphazone was synthesized by the reaction of ^{3}H-oxymorphone with excess hytirazine, with 640 GBq/mmol specific radioactivity. Both the unlabelled compound and the tritiated ligand show high affinity to mu and kappa opiate receptor subtypes in rat brain membranes. TWO binding sites were detected by equilibrium binding studies. About 60% of the ^{3}H-oxymorphazone specific binding is irreversible at 10nM ligand concentration. Preincubation of the membranes with unlabelled oxymorphazone resulted in an irreversible blockade of the high affinity ^{3}H-naloxone binding sites.

INTRODUCTION

Using ligands of high specific radioactivity two classes of binding sites - low and high affinity - is detectable for mu and delta opioid receptor subtypes. 14-Hydroxydihydromorphinone hydrazones and azines, first synthesized by Pasternak et al.(1980) have been proved to be a useful tools in elucidation of their pharmacological role and involvement in opiate analgesia.
For the detailed investigation of the mechanism and kinetics of the irreversible binding tritiated oxymorphazone was needed.
We have synthesized ^{3}H-oxymorphazone and studied its binding characteristics on rat brain membranes.

MATERIALS AND METHODS

Synthesis of 8-^{3}H-oxymorphazone:

a/ Synthesis of 8-^{3}H-oxymorphone: The precursor molecule (14-hydroxy-codeinone) was prepared by the method of Iiyima et al.(1978), and O-demethylated by BBr_3 to give 14-hydroxy-morphinone, which was tritiated in the psesence of PdO catalyst.
b/ Synthesis of 8-^{3}H-oxymorphazone:
The product was reacted with tenfold excess of hydrazine at room temperature. Specific radioactivity of 640 GBq/mmol was obtained.

Chemicals:

^{3}H-EKC was purchased from New England Nuclear (specific activity: 0.74 TBq/mmol), ^{3}H-DAGO was from Amersham (specific activity: 1.67 TBq/mmol), ^{3}H-DALE (1.37 TBq/mmol) and ^{3}H-naloxone (3.09 TBq/mmol) was synthesized by Toth (1982,1985). DADLE was purchased from Sigma, morphine was a commercial product. Unlabelled oxymorphazone was synthesized by our laboratory, all other chemicals were of analytical grade.

Membrane preparation:

Crude membrane fraction from rat (PVG/C) brain was prepared as described earlier (Simon et al. 1986). Protein content was estimated by the method of Bradford (1976).

Binding Assay:

Membrane suspension in 50 mM Tris-HCl buffer, (pH=7.4) was incubated with ^{3}H-oxymorphazone in the presence or absence of unlabelled ligands in a final volume of 1 ml. Specific binding was defined as the difference between the bound radioactivity in the absence and presence of unlabelled ligand. Incubation was started by addition of the membrane protein and stopped by rapid filtration of the mixtures through Whatman GF/C filters, which were presoaked in 10 µM unlabelled oxymorphazone solution for 1 hour.
Radioactivity was determined in toluene based scintillation cocktail by LKB Minibeta Liquid scintillation spectrometer.

Irreversibility studies:

500 ul of membrane suspension was incubated with 200 µl ^{3}H-oxymorphazone solution in the presence or absence of excess levorphanol (10 µM)in a final volume of 2 ml. After 90 min incubation at 0°C samples were diluted with ice cold Tris-HCl buffer and centrifuged. The pellets were resuspended in 30 ml buffer, incubated for 10 min and recentrifuged. This washing step was repeated 4 times. 1 ml aliquots of the homogenized final pellets were filtered through presoaked GF/C filters. The washing experiments with unlabelled ligands were performed as previously described (Szucs et al. 1983).

RESULTS AND DISCUSSION

The binding reached equilibrium at about 90 min at 0°C. All further experiments were carried out at 0°C in order to minimize the possibility of azine formation.
Dissociation of the radiolabelled ligand was extremely slow as measured by dilution or after addition of excess of reversible (levorphanol) or irreversible (unlabelled oxymorphazone) compounds (data not shown).
Scatchard analysis of ^{3}-H-oxymorphazone saturation curve is shown on fig.1. On the basis of computerized fit of data two classes of binding sites were identified with dissociation constants of 0.62 and 28 nM, and B_{max}: 18 and 134 fmol/mg respectively.

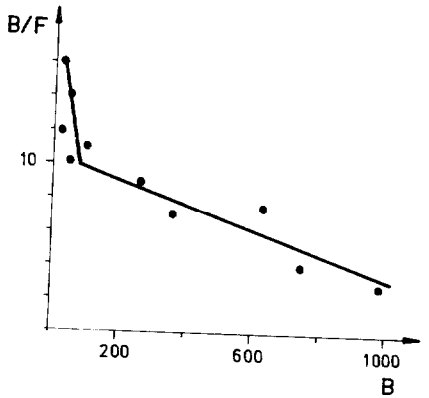

FIGURE 1. Scatchard plot of ^{3}H-oxymorphazone binding.
Rat brain membranes were incubated at 0°C for 90 min with ^{3}H-oxymorphazone in the presence or absence of 10 µM unlabelled oxymorphazone. B: bound ligand (fmol/mg protein) F: free ligand (nM).

Competition experiments were carried out with ^{3}H-oxynorphazone and with sub-type specific tritiated ligands (Kosterlitz et al. 1951). From these data it became evident that oxymorphazone exhibits high affinity for mu IC_{50}:0,4 nM) and kappa (IC_{50}:2 nM) sites and extremely low affinity for delta (IC_{50}:2000 nM) sites.

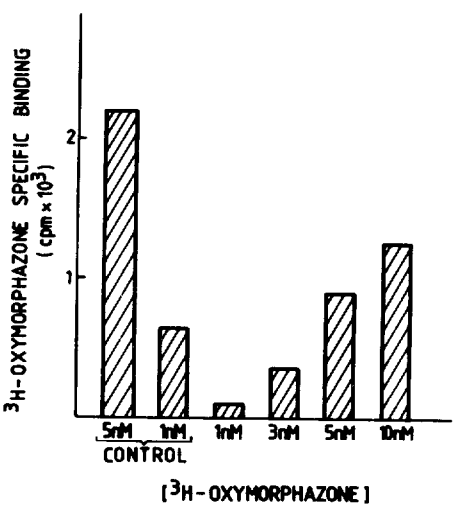

FIGURE 2. Irreversibility of ^{3}H-oxymorphazone binding.
Irreversible labelling of rat brain membranes was carried out as described in Materials and Methods. Control values repres nt specific binding of ^{3}H-oxymorphazone on membrane preparations incubated with buffer and washed in the same way.

As shown in fig. 2. the binding of ^{3}H-oxymorphazone is irreversible in about 20% at 1nM isotope concentration despite of extensive washings, and this irreversible binding greatly increased at higher concentrations. The irreversible binding blocks mainly the high affinity (mu,) opioid binding site as shown in fig. 3.

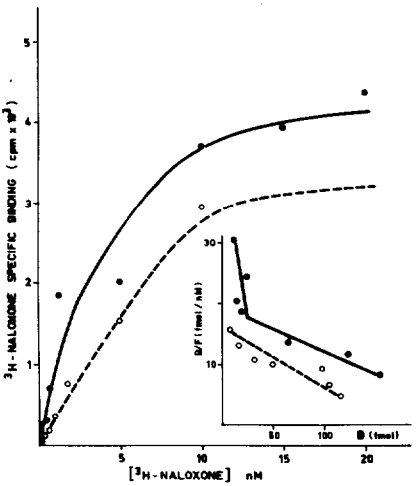

FIGURE 3. Scatchard analysis of ^{3}H-naloxone binding.
Rat brain membranes preincubated with 10 µM oxymorphazone were labelled with naloxone after extensive washing (•). Control was treated in the same way without addition of oxymorphazone (o).

In this case the membrane was incubated with unlabelled oxymorphazone (10 µM) and the remaining ^{3}H-naloxone binding was measured. These results are in agreement of earlier investigations (Pasternak et al. 1980).

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COMPARISON OF THE OPIOID ACTIVITY OF CAM[1] AND KAPPA AGONISTS

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ABSTRACT

CAM has been shown to produce opiate agonist and antagonist activities. Based on its potent agonist activity in rabbit vas deferens and the low potency of naloxone to antagonize its agonist effect in the guinea pig ileum and mouse vas deferens, CAM can be considered as kappa agonist. It is further supported by its diuretic effect and the estimated "apparent pA_2" values with naloxone and Mr-2266BS. However, in the rabbit ear artery and cat nictitating membrane, ethylketazocine (EK) produced agonist activity, while CAM produced antagonist activity, Also, EK differs from CAM in mouse vas deferens in the presence of 4-aminopyridine. It is concluded, that CAM might be an agonist on kappa type (subtype) of opiate receptors, although its spectrum of activity was found to be different from EK or hremazocine.

INTRODUCTION

The most widely accepted classification of opioid receptors (OR) is mu, kappa and delta (Iwamoto and Martin 1981, Lord et al. 1977). Previously we reported the peculiar spectrum of activity of CAM, producing strong agonist and antagonist effects in several in vivo and in vitro tests /Knoll 1977, Furst and Knoll 1982, 1985). In an attempt to define OR subtype(s) involved in the effects of CAM, various isolated organ preparations, containing different OR-s were used, together with some in vivo tests. The sensitivity to naloxone (NX) and Mr-2266BS was also studied in various assays.

MATERIALS AND METHODS

The effects of the test substances on OR-s in isolated tissue preparations were compared using the electrically stimulated myenteric plexus-longitudinal muscle of the guinea pig ileum (GPI), electrically stimulated mouse, rat and rabbit vasa deferentia (MVD, RVD and LVD, resp.), electrically stimulated isolated cat nictitating membrane (CNM) and rabbit ear artery (REA) with the following parameters; 0.1 Hz, 1 ms (GPI,RVD); 0.01 Hz, 1 ms (CNM); 0.05 Hz, 1.0 ms (LVD) 3 pulses of 10 Hz, 1.0 ms per 10 s (MVD); 5 pulses of 5 Hz, 1.0 ms per 60 s (REA). Supramaximal voltage of field stimulation was used in all experiments. The antinociceptive activity of the test substances was assessed using the acetic acid writhing test, in the mouse. Urine output measures were carried out according to Leander (1983a). Intrathecal injections (i.t.) were made to rats as described by Yaksh and Rudy (1976).
Drugs: Morphine HCl, normorphine, N-cyclopropylmethylnorazidodihydroisomorphine (Alkaloida, Hungary), naloxone HCl (Endo), ethylketazocine EK (Sterling Winthrop), Mr-2266BS (Boehringer, Ingelheim) hremazocine

[1] N-cyclopropylmethylnorazidodihydroisomorphine

HCl, BR (Sandoz) 4-aminopyridine 4-AP (Sigma), Thiobutabarbital-Natrium (INACTIN, Byk-Gulden, Hamburg), [D-Ala^2,Leu^5] enkephalin (DALE), [D-Ala^2, D-Leu^5] enkephalin (DADL), [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGO). (Central Res. Inst. for Chemistry, Budapest, Hungary)

RESULTS AND DISCUSSION

The rabbit vas deferens has been shown to be especially useful for identifying kappa agonists. Such compounds are potent inhibitors of electrically induced contraction in this tissue, whereas mu and delta agonists are virtually ineffective (Oka 1981). CAM vas found to be agonist, similarly to EK and other kappa agonists (table 1). For the antagonism of the agonist action of CAM and EK in GPI, 10-11 and in MVD 6-7 times more NX was required than was needed to

TABLE 1. The agonist activities of CAM and EK in isolated organs (IC_{50}; nM ± S.E.M.)

	GPI	MVD control	MVD 4-AP[1]	LVD	CNM
CAM	0,3±0.07	1.7±0.2	*	18.3±1.6	**
EK	1.2±0.22	3.9±0.4	19.8±5.3	5.7±0.3	3.8±21.0

*the maximum inhibition <40%
**agonist activity undetectable
[1]with 4-AP $2x10^{-5}$M
n:5-12

antagonize the effect of normorphine. In RVD, CAM and EK had no agonist activities but antagonized the effect of DALE. However, in REA and CNM, EK produced opiate agonist action while CAM showed only antagonist activity. In CNM the agonist actions of morphine, DALE and EK were antagonized by CAM. In REA the mu agonists normorphine and DAGO did not show agonist effect. The delta agonist DADL was

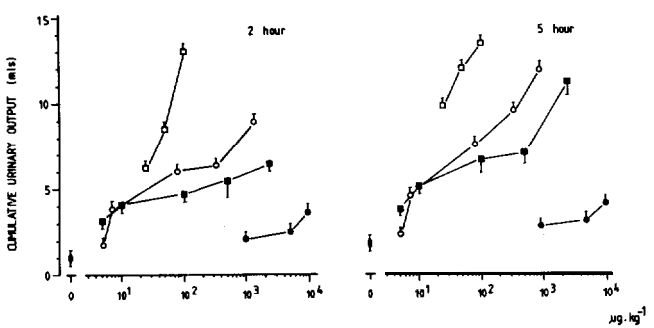

FIGURE 1. The effects of various doses of CAM s.c.(■) i.t.(□), nalorphine s.c.(●), bremazocine, s.c.(○) on cumulative urine output at 2 hr (left) and 5 hr (right) in the Sprague Dawley rats weighing between 160-220 g. The points and bars are the mean ± S.E. for 8-14 rats. Controls above 0 (⬮).

potent agonist indicating a lack of mu receptors in this preparation supporting the existence of enkephalinergic receptors as it was first suggested by Knoll (1976). The difference observed between the effects of CAM and EK was further supported by the facts that in MVD in the presence of 4-AP ($2x10^{-5}$M) which increased the electrically evoked noradrenaline release (Friedmann et al. 1994) CAM proved to be only partial agonist. When the maximal agonist effect of CAM was already reached (<40%) DADL and EK further reduced the contractions. In the presence of 4-AP, CAM ($2.5x10^{-7}$M) diminished the marked agonist actions of DADL and EK. Recently, it has been shown (Slizgi and Ludens 1982, Leander 1983a) that kappa opioid agonists -such as EK, BR, etc. - have a marked diuretic effect in rat, and it was antagonized by opioid antagonists in a potency order which indicated that the effects were due to an action at kappa OR-s. Figure 1 shows the effect of CAM on urine output in normally hydrated rats, compared to other drugs. CAM produced dose related (0.01-2.5 mg kg^{-1}) diuresis similarly to BR, by s.c. administration, This effect was more pronounced when CAM was given i.t. This diuretic effect was antagonized by kappa antagonist dose of NX(10 mg kg^{-1}, 0.5-2.5 mg kg^{-1} dose of Mr-2266BS, and blocked by 20 mg kg^{-1} morphine administration (table 2). Mr-2266BS was more potent than NX in antagonizing the

TABLE 2. Antagonism of CAM (0.5 mg kg^{-1} s.c.) induced urination. Data are the mean±S.E. in mls for 8-15 rats.

dose mg kg^{-1} of antagonist	0.5 mg kg^{-1}CAM + antagonists 2 hr	5 hr
+ 0 (control)	6.6±0.3	7.7±0.6
+ 1 naloxone	5.6±0.8	6.8±0.6
+10 naloxone	3.4±0.5	4.7±0.4
+2.5 Mr-2266BS	1.19±0.2	2.3±0.25
+20 morphine	1.73±0.2	5.5±3.6

diuretic effect of CAM, as it was found against BR and U-50488H, reported by Leander (1983b). It is interesting, however, that while the diuretic effects of two other kappa agonists: proxorphan or BR are not affected significantly by morphine, supposingly because of

TABLE 3. Sedative effects of morphine, CAM and bremazocine and their antagonism by naloxone and Mr-2266BS (n=10 rat/dose)

		morphine	CAM	bremazocine
ED_{50} (mg kg^{-1} i.v.)		0.7	0.28	0.036
AD_{50} (mg kg^{-1} s.c.)	naloxone	1.2	3.8	3.5
	MR-2266BS	0.1	0.1	0.3

The ED_{50} is defined as the dose that induced a fivefold increase of control sleeping time. The AD_{50} is defined as the dose of the antagonist needed to reduce by 50% the narcosis potentiating effect of equi-active doses (ED_{90}) of the agonist.

their mu opioid receptor antagonist activity (Leander 1983b). The effect of CAM was found to be suppressed by morphine in spite of its strong mu antagonist activity demonstrated by several tests, as it was published elsewhere (Furst and Knoll 1982, 1985) and in this paper. The analgesic effect of CAM in the writhing test was found to be 37 times stronger than that of morphine (ED_{50} s.c.: 0.015 and 0.43 mg kg^{-1} resp.) The pA_2 for naloxone antagonism of morphine(6.9) was much higher than that for CAM (5,6). Conversely, Mr-2266BS a reported kappa antagonist (Romer et al. 1980), has a higher pA_2 for CAM (6.2) than for morphine (5.8) in the acetic acid induced writhing test. This observation also supports the concept, that CAM and morphine exert their analgesic effect through different receptors. As another possible measurement of kappa activity, we also evaluated the sedative effects of this class of drugs, as the prolongation of barbiturate narcosis (table 3.) BR was observed as the most potent drug, while morphine the less active one. The reversal of these effects by naloxone and Mr-2266BS is also presented in table 3. It is remarkable, that Mr-2266RS was 10 times more potent than NX in antagonizing morphine and BR induced prolongation of sleeping time. Our data demonstrate that the opioid agonist effects of CAM are mediated by similar type of receptor (kappa or subtype of kappa receptors) as the effects of classical kappa agonists: EK or BR in GPI, LVD and MVD preparations and in the sedation, diuresis and writhing test. However, CAM failed to show agonist activity in CNM and REA and we could not elicit full effect in MVD in the presence of 4-AP in tests where the classical kappa agonist drugs e.g. EK or BR proved to be agonists. These results indicate the existence of OR population of kappa receptor subtype/s/ or others/?/ which are stimulated by EK or BR but not by CAM. In the RVD, BR, CAM and EK in some analgesic-tests CAM (Furst and Knoll 1985) and BR (unpublished observation) proved to be antagonists while in some assays, where CAM showed antagonistic activity (CNM, REA and MVD in the presence of 4-AP) EK did not (mu, delta, kappa subtype). In the latter two tests BR also exhibited antagonist activity (unpublished data).

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COMPARATIVE EFFECTS OF DYNORPHIN A(1-9) PYRROLIDINE ANALOGS ON ISOLATED TISSUES

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ABSTRACT

Three dynorphin A(1-9) pyrrolidine analogs were synthesized by substituting isoleucine at position 8 with alanine (Ala), D-alanine (D-ala), and D-leucine (D-leu), and assayed for their effect on electrically induced twitches of the isolated guinea pig ileum and mouse vas deferens. All three dynorphin A(1-9) pyrrolidine analogs caused inhibition of the evoked twitches of the two preparations dose-dependently that was reversed by naloxone. However, the substitution by alanine or D-alanine resulted in a decrease in potency on guinea pig ileum when compared to dynorphin A(1-9) pyrrolidine and substitution by D-leucine caused a considerable loss of potency in both mouse vas deferens and guinea pig ileum. Although these substitutions reduced overall potency, a change of potency ratio towards more delta selectivity resulted.

INTRODUCTION

It has been shown that incorporating amino-acids at different positions of dynorphin A(1-13) and its fragments greatly modifies potency and affinity of the resulting peptides to multiple opiate receptors. For example, Chavkin and Goldstein (1981) reported that substitutions at arg^{7} and lys^{11} reduce selective binding of dynorphin in the guinea pig ileum. Also, Rezvani et al. (1984) evaluated seven dynorphin A(1-9) analogs on the guinea pig ileum and mouse vas deferens and concluded that protection of the carboxy terminal with a pyrrolidine group results in prolongation of dynorphin A(1-9) action. Furthermore, Turcotte et al. (1984) replaced the amino acids in positions 1-12 of dynorphin A(1-13) by ala and concluded that only replacement at position 8 results in a higher potency of the peptide in the mouse vas deferens. In order to investigate whether other substitutions at position 8 will change the potency of dynorphin (1-9) we have synthesized three dynorphin A(1-9) pyrrolidine analogs by substituting iso-leucine at position 8 with

alanine, D-alanine or D-leucine and evaluated their effects on the guinea pig ileum and mouse vas deferens. The results are presented in this communication.

MATERIALS AND METHODS

Ala[8] dynorphin A (1-91, D-ala[8] dynorphin A(1-9) and D-leu[8] dynorphin A(1-9) were synthesized by the solid phase method and the pyrrolidine group was attached by treatment of the peptide-resins with pyrrolidine-DMF (1:1 mixture). The peptides were purified by reverse-phase column chromatography using C-18 resin. The purity was checked by HPLC, TLC and electrophoresis.

The isolated longitudinal muscle with the attached myenteric plexus from the guinea pig ileum was mounted in a chamber containing Ringers' solution and the excised mouse vas deferens in Krebs' solution. The tissues were stimulated electrically and the induced twitches recorded. Agonists were added to the media in increasing concentrations and a dose response curve was plotted. The concentration which inhibits maximally induced contractions by one-half (IC_{50}) was then determined from the dose response curve.

RESULTS

High performance liquid chromatography showed the three peptides to be pure as evidenced by a single peak for each (figure 1).

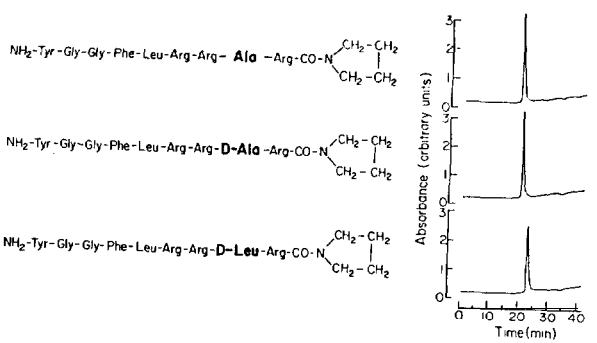

FIGURE 1: High performance liquid chromatography of three dynorphin A(1-9) pyrrolidine analogs

All three dynorphin A(1-9) pyrrolidine analogs inhibited electrically induced twitches of the guinea pig ileum and the mouse vas deferens dose-dependently. Ala[8] dynorphin A(1-9) pyrrolidine showed the highest potency in the guinea pig ileum, while D-ala[8] dynorphin A(1-9) pyrrolidine and Ala8 dynorphin A (1-9) pyrrolidine showed the highest potency in the mouse vas deferens. However, based on their relative activities in both tissues the D-ala[8] analog exhibited more selective delta activity. D-leucine[8] dynorphin A (1-9) pyrrolidine showed the lowest potency both in the guinea pig ileum and mouse vas deferens. The IC_{50} and relative potencies of three peptides in guinea pig ileum and mouse vas deferens are shown in Table 1. The effect of all three compounds could be blocked by naloxone.

TABLE 1. Potency of dynorphin A(1-9) pyrrolidine analogs on the guinea pig ileum and mouse vas deferens

Peptide	IC_{50} nM		
	GPI	MVD	Ratio G/M
ALA[8]dynorphin A(1-9) pyrrolidine	200	75	2.6
D-ALA[8]dynorphin A(1-9) pyrrolidine	750	75	10
D-LEU[8] dynorphin A(1-9) pyrrolidine	250	1250	0.2
Dynorphin A(1-9) pyrrolidine	7.5	160	0.04
Dynorphin A(1-13)	0.8	2.8	0.2

DISCUSSION

Our results demonstrate that all three modified dynorphin A(1-9) pyrrolidine analogs inhibit electrically induced twitches of the guinea pig ileum and mouse vas deferens in a dose-dependent manner. However, the substitution of iso-leucine by alanine or D-alanine decreases potency in the guinea pig ileum but increases potency in the mouse vas deferens. These results are consistent with those obtained by Turcotte

et al. (1984) who observed an increase in potency for dynorphin A(1-13) in mouse vas deferens by replacing iso-leucine with alanine at position 8. Other substitutions on dynorphin A(1-13) resulted in decreased potency in both the guinea pig ileum and mouse vas deferens. For example, replacement of residues at positions 1,2, and 4 inside the leu-enkephalin core causes a decrease in biological activity (Turcotte et al. 1984), and positions 6 and 7 are found to be the most important site outside the enkephalin moiety (Chavkin et al.1982) for biological activity.

With respect to substitution at position 8, replacement of iso-leucine by D-leucine causes a decrease of potency of the resulting peptides both in the guinea pig ileum and mouse vas deferens. On the other hand, substitution of iso-leucine by alanine or D-alanine, seems to favor delta-like activity, since the alteration decreases potency in the guinea pig ileum much greater than in the mouse vas deferens.

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^{125}I-DPDYN, MONOIODO [D-PRO^{10}]- DYNORPHIN (1-11), IS AN EFFECTIVE AND USEFUL TOOL FOR THE STUDY OF KAPPA OPIOID RECEPTORS.

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ABSTRACT

Iodination of the κ -selectfve peptide DPDYN, [D-Pro^{10}]-dynorphin (1-11), has been performed. The non radioactive monoiodo derivative of DPDYN retains κ-selectivity (κ /μ = 48 and κ/δ = 140), despite a general but moderate decrease in affinity. Radioiodination of DPDYN leads to the monoiodinated peptide (S,A 700-800 Ci/mmol) which interacts specifically and reversibly with the κ-sites in guinea-pig cerebellum membranes with high affinity (K_D = 0.12-0.18 nM). In guinea-pig brain (μ~δ~κ') and rabbit cerebellum (κ'<<μ), ^{125}I-DPDYN discriminates between κ- and other (μ, δ') binding sites. We have used this new labelled probe for the direct, precise and rapid (exposure time less than 100 hours) visualization of κ'-sites in guinea-pig and rabbit cerebellar slices using autoradiography.

INTRODUCTION

Biochemical and pharmacological studies on a particular opioid receptor type (μ, δ or κ) are greatly facilitated by use of selective ligands. DPDYN ([D-Pro^{10}]-dynorphin (1-11)), derived from dynorphin A, displays high affinity and selectivity for the κ opioid receptor type (Gairin et al. 1984). So, it was of interest to obtain a labelled K-probe derived from this selective ligand. Here we would like to show that the radiolabelled monoiodo derivative ^{125}I-DPDYN appears to be an effective and useful tool for the biochemical studies on kappa opioid receptors.

MATERIALS AND METHODS

Preparation of iodinated derivatives of DPDYN.

Radioiodination of DPDYN was performed as described elsewhere (Gairin et al. 1986). Non radioactive iodination was performed according to the same method however on milligram quantities.

Binding studies

Crude membrane fractions were prepared according to Meunier et al. (1983). Affinities of DPDYN and of its non radioactive mono and diiodo derivatives at μ, δ and κ sites were determined by measuring their inhibitory effects respectively on the binding of [^{3}H]-DAGO (1.5 nM) and [^{3}H]-DSLET (2.5 nM) in rat brain membranes and of [^{3}H]-bremazocine (0.5 nM) in guinea-pig cerebellum membranes at 25°C in tris-HCl pH 7.4. Saturation and kinetic binding studies of ^{125}I-DPDYN to κ-sites were carried out on crude guinea-pig cerebellum membranes at 25°C as previously described (Gairin et al. 1986).

Autoradiographic studies

After preincubation at room temperature in 50 mM tris-HCl buffer pH 7.4, slices (20 μm section) of guinea-pig or rabbit cerebellum were incubated with ^{125}I-DPDYN for 40 min. at 25°C in the presence (non specific binding) or absence (total binding) of 1μM unlabelled

peptide. Sections were then washed (6 x 40 min.) in cold tris-HCl buffer -2 % BSA and quickly dried. Autoradiograms were exposed for 3-5 days at 4°C.

RESULTS AND DISCUSSION

Binding properties of mono an diiodo derivatives of DPDYN

As shown in table 1, incorporation of one iodine atom on the tyrosyl phenolic ring of DPDYN results in a decrease of affinity for κ binding site (K_I = 0.38 nM). Interestingly, weaker affinities are also observed for μ- and δ- binding sites, so that κ- selectivity (κ/μ = 48 and κ/δ = 140) is preserved. Incorporation of an additional bulky iodine atom (diiodo derivative) leads to a further decrease in affinity and selectivity of the peptide for κ-binding sites.

TABLE 1. Binding selectivity profiles[a] of DPDYN and of its non radioactive mono- and diiodo- derivatives.

peptide	Binding site μ	δ	κ
DPDYN	62	233	[10.49]
monoiodo-DPDYN	48	139	[9.42]
diiodo-DPDYN	25	102	[8.57]

[a]Number in brackets is -log K_i(nM) at the preferred (κ) site. Other numbers are binding selectivities defined as the ratio of K_i at each non-preferred site over K_i at the preferred (κ) site.

Saturation equilibrium and kinetic binding studies

Equilibrium binding experiments have been performed at 25°C in guinea-pig cerebellum membranes, containing essentially (> 80 %) κ-binding sites. The linear Scatchard transformation of the data indicates that ^{125}I-DPDYN interacts specifically with one (kappa) single class of site with high affinity (K_D = 0.12 nM).The maximum binding capacity value (Bmax = 120 fmol/mg protein is quite comparable to the ones observed by Robson et al. (1984) (110 fmol/mg protein) or Frances et al. (1985) (180 fmol/mg protein) in the same tissue preparation. The low proportion of other opioid sites (μ or δ) was not detected.

TABLE 2. Parameters of the binding of ^{125}I-DPDYN in guinea-pig cerebellum membranes at 25°C.

	parameter	value
thermodynamic	Bmax	118 ± 11 fmol/mg protein
	K_D	0.12 ± 0.01 nM
kinetic	k_a	1.9 ± 0.2 x 10^7 $M^{-1}s^{-1}$
	k_d	3.5 ± 0.2 x 10^{-3} s^{-1}
	$K_D=k_d/k_a$	0.18 nM

Equilibrium of the binding of ^{125}I-DPDYN (0.2 nM) to guinea-pig brain membranes is reached after 10-15 minutes at 25°C. In the presence of

an excess of unlabelled peptide, ^{125}I-DPDYN dissociates from its complex with the receptor, as a result of a reversible binding process. The linear semi-logarithmic representation of the dissociation kinetic data indicates a first-order reaction. The calculated association and dissociation rate constants (k_a,k_d) are reported in table 2. It is interesting to note that K_D values from kinetics or equilibrium experiments are very close to each other (0.18 and 0.12 nM) and in good agreement with the K_I value (0.38 nM) of the non radioactive monoiodo derivative at κ-sites.

Specific binding of ^{125}I-DPDYN (0.15 nM) to guinea-pig brain and rabbit cerebellum membranes

In guinea-pig brain, containing equal proportions of μ-, δ- and κ- sites, ^{125}I-DPDYN (0.15 nM) binds selectively to κ-sites. As shown in table 3, the binding capacities of the labelled peptide are rigorously comparable without (B (μ + δ + κ) = 73 ± 9 fmol/mg protein) or with suppression of μ- and δ- binding (B(κ) = 71 ± 9 fmol/mg protein). No specific binding at μ- or δ- sites can be detected after suppression of κ- binding. A comparable binding pattern is obtained in the rabbit cerebellum containing essentially (> 75 %) u-binding sites and low content (< 20 %) of κ- sites.

TABLE 3. Binding capacity (fmol/mg protein) of ^{125}I-DPDYN (0.15nM) at 25°C pH 7.4 to crude membrane fractions of guinea-pig brain and rabbit cerebellum in presence (single binding site) or absence (multiple binding sites) of selective (μ,δ or κ) unlabelled ligand[a].

	multiple binding sites	single binding site		
	μ + δ + κ	μ	δ	κ
guinea-pig brain	73±9	n.d.	n.d.	71±9
rabbit cerebellum	63±10	n.d.	n.d.	47±4

[a] μ-, δ- or K-binding were respectively suppressed by 200nM DAGO, 100 nM DSLET or 3 nM DPDYN. n.d.: not detectable.

These results clearly show that ^{125}I-DPDYN at 0.15 nM selectively discriminates between κ- and other (μ, δ) sites in nerve tissues containing various proportions of opioid binding sites.

Autoradiographic studies

Figure 1 shows that κ-binding sites labelled with ^{125}I-DPDYN (0.3 nM) are localized mainly in the molecular layer of the guinea-pig cerebellum (A,B). This result is in complete agreement with that found by Robson et al. (1984) in the same tissue. In the rabbit cerebellum, very similar autoradiograms are obtained with (C,D) or without (E,F) suppression of μ- and δ- binding. Interestingly, in the rabbit cerebellum, the ^{125}DPDYN binding sites are restricted to the Purkinje cell layer while in the guinea-pig cerebellum they are present throughout the molecular layer. All these results clearly confirm the κ-selectivity of the labelled probe.

In summary, ^{125}I-DPDYN can be considered as an useful tool for the study of κ- opioid receptor, particularly for their direct (no suppression of μ- and κ- binding is needed), precise and rapid

(exposure time less than 100 hours) visualization in nerve tissues by autoradiography.

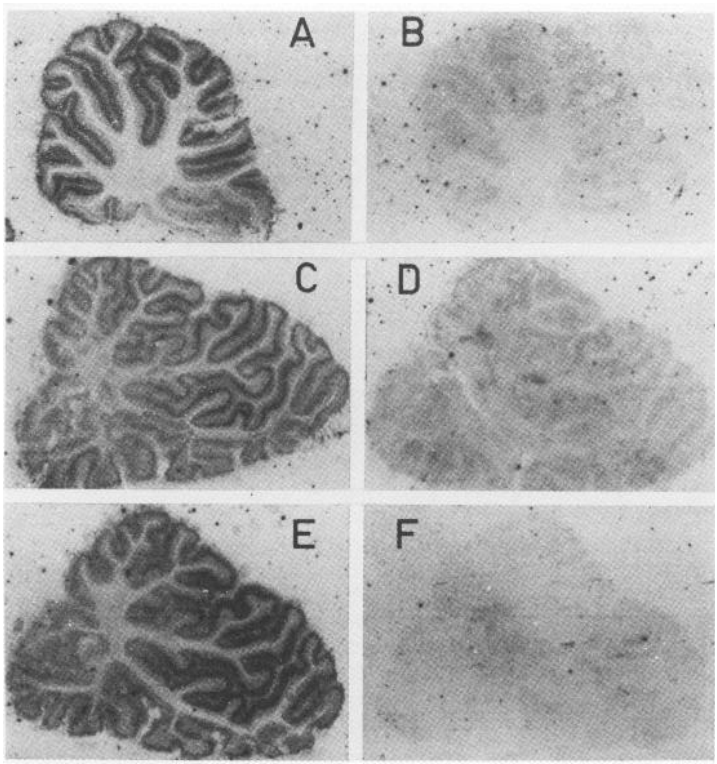

FIGURE 1: Autoradiographic localization of ^{125}I-DPDYN (0.3nM) binding sites to sagittal sections of guinea-pig (A,B) and rabbit (C,D,E,F) cerebellar slices. Left side of the figure (A,C,E) represents total binding and right side (B,D,F) non specific binding. Autoradiograms of rabbit cerebellum slices were obtained in presence (C,D) or absence (E,F) of 100nM DAGO + 200nM DSLET (suppression (or not) of μ- and **δ-** binding).

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INFLUENCE OF Y-20003, AN ANALGESIC AGENT, ON THE ENDOGENOUS OPIOID PEPTIDE SYSTEM IN RATS

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ABSTRACT

Changes of endogenous opioid peptide system by pain stimuli were examined using adjuvant-induced arthritic rats. Following the 5th day after adjuvant inoculation, met-enkephalin(ME)-like peptide content in plasma decreased, while after the 12th day, the peptide content increased in adrenal medulla. A significant correlationship was found between pain threshold and plasma ME-like peptide content. Y-20003, a new nonsteroidal agent without inhibition of prostaglandin biosynthesis, showed an analgesic effect which was significantly antagonized by naloxone or Win 44,441-3, suggesting that endogenous opioid peptide system may be involved in the analgesic mechanism of Y-20003.

INTRODUCTION

It has been reported that the contents of opioid peptides in plasma or cerebrospinal fluids decreased in patients with chronic pain (Panerai et al. 1982, Simonnet et al. 1986), although the physiological significance was unclear. On the other hand, it has been also reported that β-endorphin(B-ED) content in human plasma was increased by diclofenac, a nonsteroidal antiinflammatory agent, (Martini et al. 1984). β-END content in rat pituitary gland was reduced and the content in rat hypothalamus was increased by this agent (Sacerdote et al. 1985), suggesting that β-END might be involved in the analgesic mechanism of diclofenac, in addition to inhibition of prostaglandin synthesis. Furthermore, it has been known that in vitro experiments, indomethacin enhanced release of β-END from neurointermediate lobe of the rat pituitary gland (Knepel et al. 1985). In the present study, it was examined what changes of the endogenous opioid peptide contents could be shown in adjuvant-induced arthritis of the rat, a model of chronic pain diseases, and then whether the analgesic action of Y-20003 [10-amino-6-methyl-1,2,3,4,7,8,9,10-tetrahydrobenzo(f)quinolin-3-one] in the rat could be mediated through the endogenous opioid peptide system or not.

MATERIALS AND METHODS

Male rats (Lewis strain) were used. Animals were intradermally injected at the base of the tail with 0.1 ml of adjuvant. The adjuvant was a 0.5 % suspension of dead tubercle bacilli (R35H5) in liquid paraffin. The foot was pressed to measure pain threshold and the foot volume was determined by volumetry before and 5, 12 and 20 days after inoculation. In addition, the adrenal medulla, incisor pulps and blood were collected. Plasma from the blood, including heparin and actinonin(20 μM), were deproteinized with HCl-ethanol. Adrenal

TABLE 1. % Changes of endogenous opioid peptide contents after adjuvant inoculation

Opioids	Tissues	Days after adjuvant inoculation			
		0	5	12	20
ME-like peptides	adrenal medulla	100.0 ± 13.6(7)	110.0 ± 9.5(7)	191.9 ± 17.9(7)**	186.8 ± 9.7(7)**
	tooth pulp	100.0 ± 5.1(6)	90.8 ± 5.5(6)	96.5 ± 5.8(7)	98.2 ± 7.1(7)
	plasma	100.0 ± 6.1(5)	63.2 ± 4.3(7)**	59.8 ± 4.1(7)**	47.5 ± 4.9(7)**

**p<0.01, significantly different from the corresponding 0 day value, respectively. Each value represents the mean ± SEM. Numbers in parentheses are the numbers of animals used.

TABLE 2. Influences of Y-20003 on pain threshold and foot volume

	Treatments	Days after adjuvant inoculation			
		15	16	18	20
pain threshold (Δ g)	control	-63.1 ± 0.6(10)	-64.8 ± 15.7(10)	-57.7 ± 13.7(10)	-48.3 ± 16.9(10)
	Y-20003	-61.1 ± 13.0(10)	-10.2 ± 16.7(10)*	-10.1 ± 15.6(10)*	-9.4 ± 12.7(10)*
foot volume (ml)	control	1.84 ± 0.06(10)	1.98 ± 0.06(10)	1.87 ± 0.07(10)	1.95 ± 0.08(10)
	Y-20003	1.87 ± 0.05(10)	1.93 ± 0.05(10)	1.94 ± 0.06(10)	2.01 ± 0.07(10)

*p<0.05 significantly different from the corresponding control value, respectively. Each value represents the mean ± SEM. Numbers in parentheses are the numbers of animals used. Y-20003 was daily administered in a dose of 50 mg/kg/day, p.o. from 15th day after adjuvant inoculation.

medulla and tooth pulps were homogenized with 0.1 N HCl. These materials were centrifuged and the supernatants were lyophilized. The lyophilized materials were radioimmunoassayed to measure the ME-like peptide contents, as described previously (Kudo et al. 1983).

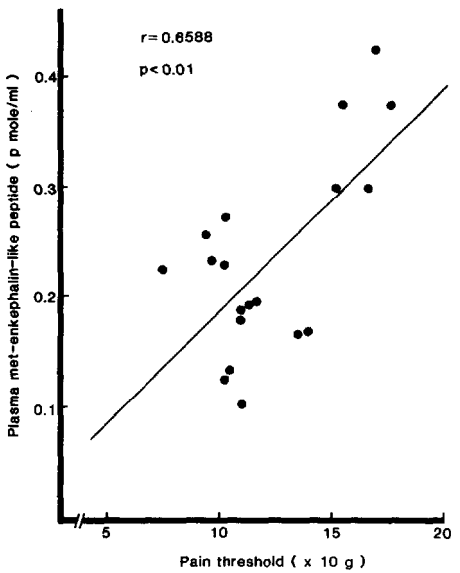

FIGURE 1. Correlationship between pain threshold and plasma ME-like peptide content. The data from control, 12th and 20th days after adjuvant inoculation were plotted.

TABLE 3. Antagonism of naloxone or Win 44,441-3 against the analgesic effect of Y-20003

Treatments	Doses(mg/kg)	n	Δ g of pain threshold
control	0	10	-75.1 ± 21.3
Y-20003	50	10	- 7.2 ± 16.9*
Y-20003 + naloxone	50 + 2	10	-55.0 ± 14.0#
Y-20003 + Win 44,441-3	50 + 1	10	-78.6 ± 15.6##

*p<0.05, significantly different from the control. #p<0.05 and ##p<0.01, significantly different from the Y-20003 group. Each value (Δ g) represents the mean ± SEM. Y-20003 was orally administered. Naloxone and Win 44,441-3 were injected subcutaneously 10 min before Y-20003. The data are from those of 18th day after adjuvant inoculation.

RESULTS AND DISCUSSION

In the present study, it was examined what changes of endogenous opioid peptide contents could be shown in adjuvant-induced arthritis of the rats. It was found that pain threshold decreased and foot volume increased significantly following the 12th day after adjuvant inoculation. These indices did not show any changes at the 5th day, suggesting that the day was on the latent stage of the arthritis (data not shown). However, the content of plasma ME-like peptides were significantly reduced following 5th day, while the content of adrenal medullary ME-like peptides was elevated after the 12th day(table 1). These results may suggest that a long term of lowering of plasma opioid peptide levels is necessary to lower the pain threshold. Furthermore, it is conceivable that at the initial stage of adjuvant inoculation(5th day), the lowering of the peptide level is due to activation of some degrading enzymes in plasma, followed by compensatory promotion of opioid peptide processing in adrenal medulla since 12th day. In fact, as shown in fig. 1, a significant correlationship was found between pain threshold and plasma ME-like peptide content. On the other hand, in the present study, we investigated whether analgesic action of Y-20003 which did not inhibit prostaglandin biosynthesis could be mediated through the endogenous opioid peptide system in the adjuvant-induced arthritis or not. The pain threshold, but not the foot volume, was recovered near the normal level after Y-20003(table 2). The analgesic effect of Y-20003 was antagonized by naloxone or Win 44,441-3, a κ-antagonist(table 3). In addition to these results, we have previously certified that adrenal medullary ME-like peptide content was markedly reduced and in contrast, plasma ME-like peptide content showed a tendency to be increased by Y-20003 in normal rats(data not shown). From these findings, it may be concluded that 1) plasma opioid peptide levels are closely related to pain threshold in the arthritic rats and 2) the analgesic action of Y-20003 on the adjuvant-induced arthritis is mediated through endogenous opioid peptide.

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PROHORMONE PROCESSING REACTIONS: DISCRETE STAGES IN THE FORMATION OF β-ENDORPHIN RELATED PEPTIDES

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ABSTRACT

Analysis of lipotropin from the pars intermedia of bovine pituitary showed that it contained the full COOH-terminal sequence of β-endorphin. In contrast the β-endorphin related peptides exhibited a high degree of C-terminal proteolysis. The implications of these findings are discussed with reference to the cellular compartments where the processing reactions take place.

INTRODUCTION

It is well appreciated that peptide hormones and transmitters are formed by proteolytic cleavage of prohormone Precursors and their formation may involve the proteolysis of more than one peptide bond. It is not known, however, whether these cleavages take place simultaneously and competitively or whether they take place independently and in a predetermined order. The formation of β-endorphin 1-27 offers an example of a prohormone fragment that requires two processing reactions for its release, one cleavage to free the NH_2-terminus of the peptide and the second to release the COOH-terminus. We present evidence that the two reactions take place consecutively. They may occur in different cellular compartments.

METHODS

Peptides were extracted from 4 bovine anterior pituitaries or pars intermedia by homogenisation in acid acetone (Smyth et al. 1978). The extracts were dried and fractionated by gel exclusion chromatography on Sephadex G75 (100 x 2cm) in 20% acetic acid. Lipotropin and β-endorphin related peptides were located by RIA with a β-endorphin antiserum (Zakarian and Smyth 1979) and the lipotropin containing fraction was re-chromatographed under the same conditions to ensure complete removal of the β-endorphin related peptides.

To determine whether the lipotropin molecules contained the full 31 residue sequence of β-endorphin or were shortened at the COOH-terminus, the lipotropin containing fractions were subjected to citraconylation and trypsin digestion. This procedure allowed limited proteolytic cleavage to take place at arginine residues, thus releasing the C-terminal sequence. After removal of

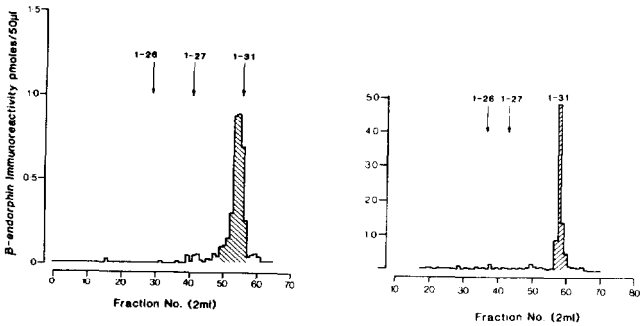
the citraconyl groups, the corresponding forms of β-endorphin were identified chromatographically. The lipotropin obtained from 2 pituitaries was reacted with 40µl of citraconic anhydride (in 160µl dioxan) in 2ml of 400mM sodium phosphate at pH 9. The reaction mixture was vortexed until no further change in pH was observed, the pH being maintained by automatic addition of 1M-NaOH. The reaction mixture was then treated with TPCK trypsin (2µg) and digestion carried out at 37°C for 16 h. The reaction was terminated by addition of acetic acid and gel exclusion chromatography was employed to resolve β-endorphin related peptides from residual lipotropin. The peptides were located by RIA with a β-endorphin antiserum, the citraconyl groups being removed by the exposure to acetic acid during chromatography. The β- endorphin containing fraction was resolved by ion exchange chromatography on SP-Sephadex C25 (sodium form) in 50% acetic acid with a sodium chloride gradient (0 to 0.5M, mixer volume 1OOml). The peptides related to β-endorphin again were located in the eluted fractions by RIA.

The lipotropin fractions from the two regions of pituitary, obtained by gel exclusion chromatography, were further examined by HPLC (Smyth 1984) using a C18 column in 10mM HCl with an acetonitrile gradient (0 to 25% in 2 min, and 25 to 50% in 25 min). Aliquots of the eluted fractions were dried in vacuo and the lipotropin located by RIA.

RESULTS AND DISCUSSION

The lipotropin fractions obtained from both the anterior pituitary and pars intermedia, after citraconylation and trypsinisation, gave rise only to β- endorphin 1-31; none of the shortened forms of β- endorphin β-endorphin 1-26 and β-endorphin 1-27) were formed (figure 1). Similarly no evidence for the presence of shortened forms of lipotropin could be detected by HPLC; only a single immunoreactivity component was observed. It can be concluded that lipotropin is generated in both regions of pituitary in a form that contains the full 31 residue sequence of β-endorphin. The results show that the proteolytic processing reaction that releases the N-terminus of lipotropin goes to completion and the lipotropin is converted to β-endorphin before any detectable C-terminal proteolysis takes place.

It is well known that the β-endorphin related peptides that are present in the pars inter-media exhibit a high degree of C-terminal proteolysis (Smyth and Zakarian 1980). The major peptides are the 26 and 27 residue forms together with the corresponding N-acetyl derivatives, whereas the 31 residue form of β-endorphin in the pars intermedia is only a minor component. The present



(a) (b)

FIGURE 1. Ion exchange chromatography of β-endorphin forms released by tryptic digestion of citraconylated lipotropin from (a) bovine pars intermedia and (b) anterior pituitary. The peptides were chromatographed as described in the text.

results show, in marked contrast, that lipotropin in the pars intermedia and in the anterior pituitary exhibits no C-terminal shortening of the peptide chain. It is clear that the processing reactions that release lipotropin (Reaction 1), and β-endorphin from lipotropin (Reaction 2), take place and go to completion before proteolysis at the C-terminus commences. It could be that the combined rates of Reactions 1 and 2 are several orders of magnitude greater than the rate of Reaction 3. Alternatively the final processing step may take place in a different cellular compartment from that where lipotropin is formed, or it may take place in the same compartment but under different conditions. It seems likely, furthermore, that the enzyme that generates lipotropin and β-endorphin is different from the enzyme that is involved in C-terminal shortening of the peptide chain.

The molecular events involved in the conversion of lipotropin to β-endorphin 1-27 are seen to comprise a series of three cleavage reactions which take place consecutively. Thus shorter forms of lipotropin do not

$$\text{POMC} \xrightarrow{1} \text{LPH 1-91} \xrightarrow{2} \beta\text{-end 1-31} \xrightarrow{3} \beta\text{-end 1-27}$$

seem to occur to a measurable extent in the pituitary and the conversion of lipotropin to β-endorphin 1-31 appears to be a mandatory step which must take place before C-terminal proteolysis of the peptide chain can

occur.

The results suggest that processing reactions take place in ordered sequence, each step going to completion before the succeeding step commences. The ordered processing could, of course, be related to conformational restriction of certain cleavage sites which would determine the nature of the fragments produced; alternatively the cleavage reactions may be catalysed by different enzymes. In either case the possibility should be considered that proteolytic processing reactions may be regulated independently.

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BRIDGE PEPTIDE IS A CLEAVAGE PRODUCT OF PRO-DYNORPHIN PROCESSING IN THE RAT ANTERIOR PITUITARY

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ABSTRACT

Bridge peptide, the sequence of amino acids which joins alpha-neo-endorphin and dynorphin A 1-17, was determined to exist as a free molecule in the rat anterior pituitary. Gel filtration chromatography revealed the presence of the free peptide as well as a high molecular weight intermediate of 8-9 kilodaltons.

INTRODUCTION

Pro-dynorphin (PRO-DYN) is the common precursor to a series of leucine-enkephalin extended opioid peptides, including dynorphin (DYN) A 1-17, DYN A 1-8, DYN B 1-13 and alpha-neo-endorphin (for review see Dores et al. 1984). These products are generated when PRO-DYN undergoes a series of proteolytic cleavages at basic amino acid pairs. The order in which these events occur is not well understood. However, nucleotide sequence analysis of cloned complementary DNA disclosing the primary sequence of PRO-DYN (Kakidani et al. 1982) combined with knowledge of Lys-Arg as a cleavage site permits certain predictions. One of these predictions would be the liberation of the amino acid sequence which lies in between alpha-neo-endorphin and DYN A 1-17, and is referred to in this text as "bridge peptide."

The bridge peptide sequence is highly heterogeneous across species (Civelli et al. 1985). In rat, it is composed of 23 amino acids, while porcine and human bridge peptide are 21 and 19 amino acids, respectively. However, the 6 amino acids which compose ethe C-terminal of bridge peptide are conserved across species.

PRO-DYN end product immunoreactivity is known to be present in the anterior lobe of the pituitary (cf. Khachaturian et al. 1986) and has been characterized as existing mainly in high molecular weight intermediates (Seizinger et al. 1984). Two intermediates have been described, an 8 kdal molecule with alpha-neo-endorphin in its C-terminal and a 6 kdal molecule with DYN A 1-17 in its N-terminal. This would suggest the presence of free bridge peptide in the anterior pituitary. This study was undertaken to verify if bridge peptide occurred as an intact entity.

METHODS

A radioimmunoassay (RIA) to bridge peptide was developed. Porcine bridge peptide synthesized by Dr. Richard Houghten (Scripps Clinic, La Jolla, CA) was conjugated to thyroglobulin and injected s.c. to rabbits. The labelled antigen for this RIA was iodinated (I^{125}) rat

bridge peptide (rat bridge peptide was also synthesized by Dr. Houghten). The iodination was carried out in the presence of chloramine-T and the unreacted cold, peptide was separated from labelled bridge peptide by HPLC. The RIA was carried out in a 300 ul volume, consisting of 2 parts - 150 mM PO buffer (pH 7.2) with 0.1% BSA, 0.9% NaCl, 0.06% triton and 0.01% sodium azide; 1 part - methanol: 0.1 N HCl (1:1, v/v). The assay was incubated 24 hrs at 4°C and bound labelled antigen was separated from free labelled antigen by the addition of 100 ul of 0.5% bovine gamma globulins and 500 ul of 20% polyethylene glycol. Under these conditions the antibody was used at a final dilution of 1/18,000. The limit of sensitivity, defined here as the amount of unlabelled antigen required to displace 10% of labelled antigen, was 30 fmoles. The antibody was determined to be C-terminal directed.

Fifty anterior pituitaries from adult male Sprague-Dawley rats were homogenized in 10 ml of methanol: 0.1 N HCl (1:1, v/v). The extracts were chromatographed on Sephadex G-50, superfine column (1.5 X 90 cm) in 1% formic acid and 0.01% BSA. Two ml fractions were collected, lyophilized and resuspended in methanol: 0.1 N HCl (1:1, v/v). Aliquots of these fractions were analyzed for bridge peptide immunoreactivity.

RESULTS AND DISCUSSION

Gel filtration chromatography of rat anterior pituitaries combined with the bridge peptide RIA revealed the presence of two peaks; Peak I and Peak II, (Table I).

TABLE I. Distribution of bridge peptide immunoreactivity (IR) in the rat anterior pituitary after gel filtration chromatography.

	Apparent MW (kilodaltons)	Bridge Peptide-IR[1] (fmoles)	% of Total Bridge Peptide-IR
Peak I	8-9	864	7.0
Peak II[2]	2-3	11,475	93.0

[1]Sum of immunoreactivities measured in fractions of Peaks I and II.

[2]Co-migrates with rat bridge peptide.

Ninety-three percent of the bridge peptide immunoreactivity was contained in Peak II, a Peak which co-eluted with synthetic rat bridge peptide standard. Seven percent of the immunoreactivity was contained in Peak I, a higher molecular weight species of 8-9 kdal. When accounting for column recovery (80%) and the number of pituitaries chromatographed, one rat anterior lobe would contain approximately 280 fmoles of free bridge peptide. However, the total content of bridge peptide is most likely higher, since we observed a

high molecular weight species with bridge peptide immunoreactivity (Peak I). It is possible that this molecule contains bridge peptide with a blocked C-terminal, i.e. extended with DYN A 1-17. If this were the case, the amount of immunoreactivity detected for Peak I, would be largely underestimated since our antibody was determined to be C-terminal directed and a C-terminal extended bridge peptide is poorly crossreactive.

The biological effects of bridge peptide are not known and will have to be explored. This peptide does not contain the Tyr-Gly-Gly-Phe amino acid sequence associated with opioid function. Modulation of opioid function, however, may still be possible. It is not known what role bridge peptide fulfills in regards to existing in the same precursor molecule as the leucine-enkephalin extended opiate peptides known as dynorphins.

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A PROCESSING ENZYME FOR PRODYNORPHIN DERIVED PEPTIDES

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ABSTRACT

Endo-oligopeptidase A known to hydrolyse the Phe^5-Ser^6 bond of bradykinin and the Arg^8-Arg^9 bond of neurotensin has been shown to produce, by a single cleavage, $leucine^5$-enkephalin from small prodynorphin derived enkephalin-containing peptides. The specificity constants (k_{cat} /k_m) obtained for the hydrolysis of bradykinin, neurotensin arnd dynoprphin B are of the same order, suggesting that the substrate amino acid sequence is not the only factor determining the cleavage site of this enzyme.

INTRODUCTION

Biologically active peptides are generated from large inactive precursor molecules by proteolytic cleavages. It has been proposed that biologically active sequences bracketed by pairs of basic amino acid residues are liberated by the action of a trypsin-like endopeptidase followed by a carboxypeptidase B type exopeptidase (Steiner et al. 1980). In this study, we provide evidence that this pattern of sequential cleavage may not be the only mechanism for liberating enkephalin from low molecular weight enkephalin-containing peptides. Previous reports have described the purification and characterization of endo-oligopeptidase A, a 75000 Dalton thiol peptidase (Camarqo et al. 1973; Carvalho and Camargo 1081) which hyrolyses the Phe^5-Ser^6 bond of bradykinin (Camargo et al. 1973), the Arg^8-Arg^9 bond of neurotensin (Carmargo et al. 1983) and liberates $methionine^5$-enkephalin (ME) from BAM 12P by cleavage at the Met^5-Arg^6 position (Camargo et al. 1985). Following this latter observation, we report here that bovine brain endo-oligopeptidase A can produce, by a single cleavage, $leucine^5$-enkephalin (LE) from small precursor molecules.

MATERIALS AND METHODS

Endo-olipopeptidase A assay

Endo-oligopeptidase A used for enzyme assays was highly purified by immunoaffinity chromatography (data not shown). Enzyme assays. were performed in a final volume of 100 µl of 17.5 mM Tris-HCl pH 7.6 containing 7 µg of endo-oligopeptidase A (specific activity 1370 µmol/min/mg towards bradykinin), 0.35 mM dithiothreitol and 10-20 nmol of the required peptide. Samples were incubated at 37°C and the

reaction terminated by addition of 5 ul of concentrated H_3PO_4. Aliquots from each incubation medium were analysed by reverse phase HPLC over a C18 µBondapak column (4.6 x 250 mm; Millipore Corporation). Peptides were separated by an initial 3 min isocratic elution in 0.1% H_3PO_4 pH 2.7 containing 10% (v/v) acetonitrile followed by a 15 min linear gradient of 10-50% (v/v) acetonitrile in the 0.1% H_3PO_4 pH 2.7, at a flow rate of 2 ml/min. Reaction products were monitored by optical density at 214 nm.

RESULTS

The ability of endo-oligopeptidase A to hydrolyse several enkephalin-containing prodynorphin derived peptides was studied. With dynorphin(DYN)-B as substrate the product formed was LE, demonstrating that this peptide is hydrolysed at the Leu^5-Arg^6 bond (fig.1A; table 1). Furthermore, the amount of LE released accounted for all the LE contained within DYN-B fig.1B. LE formation was inhibited in a concentration dependent manner by bradykinin, indicating that the purified endo-oligopeptidase A contained only one enzyme activity.

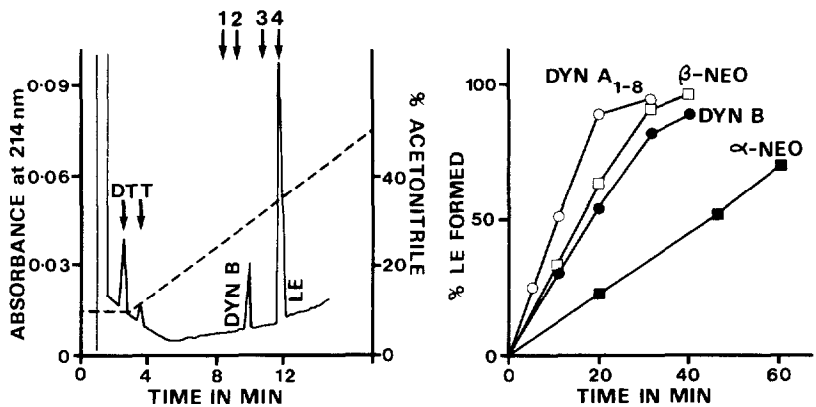

fig. 1. Hydrolysis of peptide substrates by purified endo-oligopeptidase A was analysed by reverse phase HPLC with (A) a typical chromatogram showing the production of LE from DYN-B and (B) the formation of LE from DYN-A_{1-8}, (○—○), β-NEO (□—□), DYN-B(●—●) and α-NEO (■—■). Standards : Tyr-Gly-Gly-Phe (1), LE-Arg^6-Arg^7 (2) LE-Arg^6 (3) and LE (4).

Similar results were obtained using the prodynorphin derived peptides DYN-A_{1-8}, α-neoendorphin(NEO) and β-NEO. LE formation again accounted for around 100 % of the total peptide LE content, except in the case of α-NEO where this could not be measured due to the slower rate of hydrolysis over the time course of the experiment (fig. 1B; table 1). The complementary basic peptides produced by endo-oligopeptidase A hydrolysis of these species eluted as single peaks on another HPLC system described elsewhere (Camargo et al. 1985), substantiating the

observation that cleavage occurs only at one position. In contrast, the related peptides DYN-A_{1-17} and DYN-A_{1-13} were not detectably cleaved by endo-oligopeptidase A. Since smaller peptides DYN-A_{1-6} (LE-Arg^6) and LE itself were also found to be resistant to cleavage by this enzyme, whereas the heptapeptide DYN-A_{1-7} was hydrolysed to liberate Tyr-Gly-Gly-Phe, but not LE, these results indicate that in general, the optimum for the release of LE is between 8-13 amino acid residues and that substrate size may be important in determining both the cleavage position and the susceptibility tc hydrolysis.

TABLE 1

Prodynorphin peptides hydrolysed by endo-oligopeptidase A

DYN A_{1-7}	Tyr-Gly-Gly-Phe↓Leu-Arg-Arg
DYN A_{1-8}	Tyr-Gly-Gly-Phe-Leu↓Arg-Arg-Ile
DYNE B	Tyr-Gly-Gly-Phe-Leu↓Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
α-NEO	Tyr-Gly-Gly-Phe-Leu↓Arg-Lys-Tyr-Pro-Lys
β-NEO	Tyr-Gly-Gly-Phe-Leu↓Arg-Lys-Tyr-Pro

Arrows indicate the observed cleavage points. The sequences of LE, LE-Arg^6, DYN-A_{1-13} and DYN-A_{1-17}, which were resistant to hydrolysis, are not shown.

Specificity of endo-oligopeptidase A

These results indicate an optimum substrate size for catalysis by endo-oligopeptidase A. However, the requirement for amino acids around the subceptible bond is unclear since the enzyme can cleave the Phe^5-Ser^6 bond in bradykinin (Camargo et al. 1973, the Arg^8-Arg^9 bond in neurotensin (Camargo et al. 1983) and the Leu^5-Arg^6 bond in DYN-B, where the amino acid residues found surrounding the cleavage sites bear little similarity. Therefore, the hydrolysis of these peptides was further investigated using a >20000 fold purified enzyme. As shown in table 2 all three peptides prove good substrates for this enzyme, with km values in the lower µM range. In addition the high specificity constants (k_{cat}/k_m) are not strikingly different between substrates, suggesting that the primary amino acid sequence of the peptides alone is not responsible for determining the cleavage site.

TABLE 2

Specificity of endo-oligopeptidase A

	Km (µM)	kcat (s^{-1})	kcat/Km ($M^{-1}s^{-1}$)
Bradykinin	8.78	9.8	1.12×10^6
Neurotensin	12.49	1.55	0.28×10^6
DYN-B	5.23	21.52	4.11×10^6

Endo-oligopeptidase A catalysis of bradykinin, neurotensin and DYN-B was stopped when less than 15 % of the substrates had been hydrolysed and the products analysed over HPLC. Assays were conducted in triplicate at 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 µM substrate concentrations. k_m and k_{cat} values were obtained using Lineweaver-Burk plots with initial rates expressed as molar rates.

DISCUSSION

Recently it has been reported that the processing of dynorphin precursors into dynorphins may occur via a single-step reaction involving cleavage at a single arginine (Wallace et al. 1984; Devi and Goldstein 1985) which differs from the general mechanism of trypsin-like followed by carboxypeptidase-B like hydrolysis (Steiner et al. 1980). Since several biologically active peptides are now known to be released by proteolysis at cleavage sites other than dibasic residues (Devi and Goldstein 1985; Niall et al. 1982; Kreil et al. 1980), it may be that processing does not necessarily follow the trypsin-carboxypeptidase B-like enzyme hypothesis. In this study it has been shown that endo-oligopeptidase A, an enzyme which displays selectivity towards small intermediates, could be involved in this type of processing scheme and, more particularly, in the processing of the prodynorphin precursor.

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ASSAY AND BIOCHEMICAL CHARACTERIZATION OF A DYNORPHIN CONVERTING ENZYME IN HUMAN CEREBROSPINAL FLUID

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ABSTRACT

An endopeptidase hydrolyzing dynorphins A and B and alpha-neo-endorphin at the Arg^6-Arg^7 or Arg^6-Lys^7 bonds, was partially purified from human cerebrospinal fluid and further characterized by various biochemical techniques including HPLC gel permeation (UltroPac TSK G3000SW) and ion exchange (TSK DEAE-3SW) chromatography. A procedure for quantitative analysis of the enzyme in individual CSF samples is also described. The activity in lumbar CSF of women in late pregnancy was significantly lower than that in control samples.

INTRODUCTION

The endogenous opioid peptides alpha-neo-endorphin, dynorphin A (Dyn A) and dynorphin B (Dyn B) derive from a common precursor polypeptide, proenkephalin B. As with other neurotransmitters or neuromodulators their actions are likely to be under enzymatic control. Several peptidases capable of converting dynorphins have been reported (e.g. Devi and Goldstein 1984; Mizuno and Matsuo 1984). We recently reported an endopeptidase in human cerebrospinal fluid (CSF) capable of releasing the N-terminal hexapeptide Leu-enkephalin-Arg^6 from dynorphin A (Nyberg et al. 1985). The enzyme, identified as a serine protease, cleaves at pairs of consecutive basic amino acid residues and showed close similarity with an endopeptidase previously purified from yeast (Mizuno and Matsuo 1984). Here we describe further biochemical characterization of this activity and a procedure for its quantitation in individual CSF samples.

MATERIALS AND METHODS

Materials. Reference peptides were from sources previously noted (Nyberg et al. 1985). Labelling of dynorphin B with ^{125}I was performed as described (Christensson-Nylander et al. 1985). The chromatographic material (SP-Sephadex) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The peptidase inhibitors bestatin and phosphoramidon were from Sigma and captopril from Squibb (Princeton, NJ, U.S.A.). All other chemicals and solvents were of analytical reagent grade from commercial sources.
Enzyme preparation. The dynorphin converting endopeptidase was purified from pooled human CSF by ion exchange

TABLE 1. Conversion of proenkephalin B derived peptides by the purified CSF endopeptidase[1].

Peptide	Structure	Release of Leu-enkephalin-Arg[6] (pmol/min)
Dyn A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg--Ile-Arg-Pro-Lys-Leu-Lys-Trp--Asp-Asn-Gln	12.3
Dyn A (1-8)		4.1
Dyn B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg--Gln-Phe-Lys-Val-Val-Thr	10.1
Alpha-neo-end.	Tyr-Gly-Gly-Phe-Leu-Arg-Lys--Tyr-Pro-Lys	7.4

[1] Peptides (10 uM) were incubated in triplicates with the CSF enzyme (1 mg/ml) and aliquots were removed at different times and analyzed by RIA for the hexapeptide peptide product. For other details see text.

chromatography, hydrophobic interaction chromatography, and molecular sieving (Nyberg et al. 1985).

High performance liquid chromatography (HPLC). The HPLC system (LKB Produkter AB, Bromma Sweden) was used for gel permeation (TSK G3000SW, 7.5 x 600 mm), ion exchange (TSK DEAE-3SW, 7.5 x 150 mm) and reversed phase (Spherisorb C-18, 5u, 4.6 x 250 mm) chromatography. Eluted fractions were collected and analyzed for enzyme activity. All columns were calibrated with peptide and protein standards.

Enzyme assay. All incubations were performed in Eppendorf tubes at 37°C. Enzyme fractions or pure CSF (20 ul) were incubated with a mixture of ^{125}I-labelled (about 5000 cpm) and unlabelled (40 pmol) dynorphin B in a final volume of 40 ul Tris-HCl (20 mM, pH 7.8) in the presence of 100 uM bestatin, 10 uM captopril, and 20 uM phosphoramidon. The reaction was terminated by dilution with 1.5 ml cold pyridine(0.018 M)-formate(0.1 M) buffer (pH 3.2) before product separation on SP-Sephadex minicolumns. The generated enkephalyl hexapeptide was eluted with 0.35 M pyridine-formate buffer (pH 4.4) and collected for counting. In assays of individual CSF samples incubations (in triplicates) were allowed to proceed for 60 min. Product formation was also monitored by radioimmunoassay (RIA) (Nyberg et al. 1985).

RESULTS

Fig. 1A illustrates the the UV-profile and the pattern of

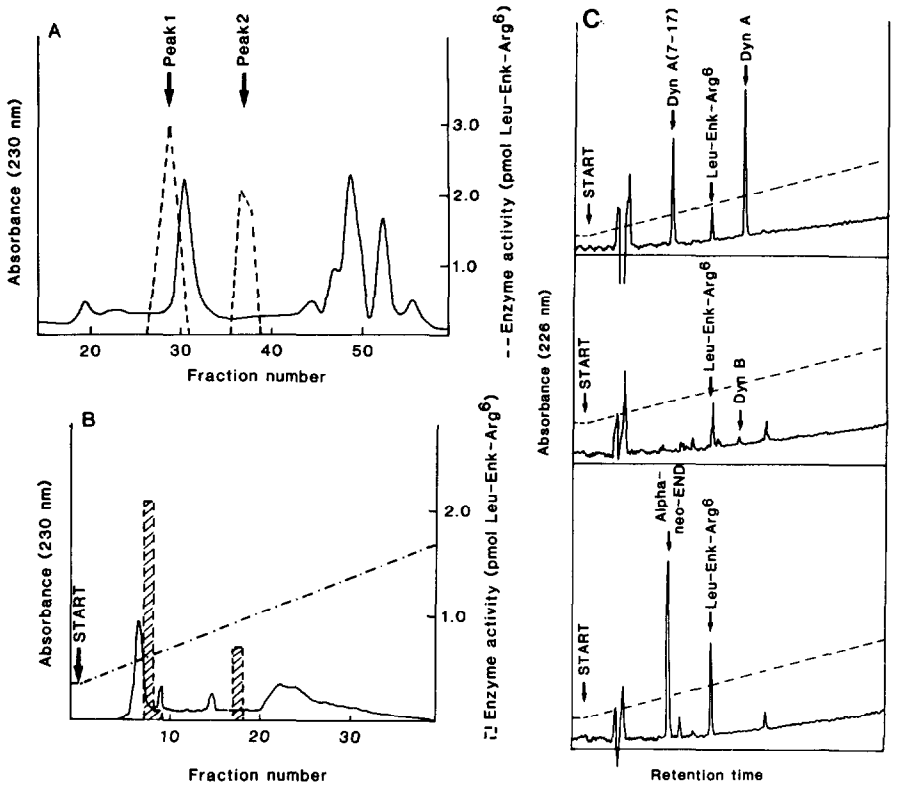

FIGURE 1. (A) HPLC gel permeation chromatography of a crude CSF sample (200 ul). The column was eluted with 40 mM ammonium acetate (pH 7.0) and fractions of 0.5 ml were collected at a flow rate of 30 ml/h. (B) HPLC ion exchange chromatography of peak 2 material. Gradient, 0.05-0.25 M NaCl in 20 mM Tris-HCl (pH 7.2); fraction size, 1 ml; flow rate, 60 ml/h. (C) RP-HPLC reaction mixtures of the CSF enzyme and Dyn A, Dyn B or alpha-neo-endorphin. The column was eluted at a flow rate of 1 ml/min with a 40 min linear gradient of acetonitrile (15-45%) containing trifluoroacetic acid (0.04%).

dynorphin converting activity obtained after HPLC gel filtration of a crude CSF sample. As earlier shown (Nyberq et al. 1985)) the enzyme activity was resolved in two major peaks, one eluting ahead of albumin (> 68 kdaltons) and the other at a position corresponding to 40 kdaltons (peak 2). In this study, material corresponding to peak 2 was subjected to further characterization. On HPLC ion exchange chromatography (Fig. 1B), peak 2 separated into two distinct fractions. The major fraction eluted as a comparatively basic protein, thus showing similar properties as those of the yeast enzyme (Mizuno

and Matsuo 1984). This enzyme fraction was capable of releasing the N-terminal fragment, Leu-enkephalin-Arg^6, from Dyn A, Dyn B and alpha-neo-endorphin, as shown in Fig. 1C, as well as from Dyn A (1-8). The enkephalyl hexapeptide appeared as a major product in all experiments. The formation of this fragment was confirmed by amino acid analysis. Studies performed with RIA amounts of substrate indicated a different rate of conversion of the various proenkephalin B products (Table 1). Dyn A and Dyn B were converted at a higher rate than alpha-neo-endorphin and Dyn A (1-8). A K_M-value of 0.45 mM for conversion of Dyn B was estimated for the purified enzyme.

The enzyme could be analyzed in less than 100 ul CSF samples. Values obtained by product quantitation with RIA parallelled those obtained by the labelled substrate procedure, which was used here. The enzyme activity was analyzed in lumbar CSF of normal women of fertile age and of women in late pregnancy. Mean levels in the pregnant group (6.7 units, n = 12) were significantly lower ($p < 0.01$) than those in the nonpregnant subjects (11.7 units, n = 10, one unit was defined as the release of 1 pmol Leu-enkephalin-Arg^6 per ml CSF and min).

DISCUSSION

The enzyme described here acts on the proenkephalin B family of opioid peptides with specificity towards consecutive pairs of basic amino acid residues. The enzyme more efficiently converted Dyn A than e.g. its (1-8) fragment suggesting that the sequences beyond the local cleavage site are important. We have therefore named it a dynorphin converting enzyme. The lower levels found in CSF of pregnant women is of interest since they correlate negatively to the increased levels of opioid peptides observed in this group (Lyrenäs et al. to be published). It should be noted that the product, Leu-enkephalin-Arg , maintains opioid activity but with different receptor preference. Therefore, this enzyme may not only terminate the action of dynorphin peptides but also generate a fragment with different activity profile.

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PRESENCE OF ENKEPHALIN PRECURSOR IN MOLLUSCAN NEURAL TISSUE EXTRACT

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ABSTRACT

Mytilus pedal ganglia extract was treated sequentially with TPCK-trypsin and carboxypeptidase B. The treated sample and an untreated sample were purified separately with Sep-Pak C_{18} and subjected to HPLC. Fractions with R_t's corresponding to the Met-, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 were assayed for enkephalin activities by displacement studies using pedal ganglia membrane and ^{3}H-DAMA. The data showed the activities in the regions of Met-, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 of the treated sample increased over that of the untreated sample by 2.3, 2.5 and 1.6 folds respectively. These results provided strong evidence for the presence of enkephalin precursor material in Mytilus.

INTRODUCTION

Recently, we reported the isolatio and identification of Met-, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 in Mytilus pedal ganglia (Leung and Stefano 1984; Stefano and Leung 1984). These results demonstrated directly the presence of an enkephalinergic system in the invertebrates. In addition, this system must be similar to the mammalian one. Direct biochemical evidence, obtained by HPLC-RIA technique, for the presence of enkephalin in shore crab has also been reported by Jaros et al. (1986). Thus, the opioid system must be developed at a very early stage of evloution.

Based upon current knowledge, all known opioid peptides are processed from three precursors--proenkephalin, prodynorphin and pro-opiomelanocortin. The nucleotide sequences as well as the amino acid sequences for all three precursors had been deduced by cDNA technique (Noda et al. 1982; Nakanishietal. 1979; Kakidani et al. 1982). Comparison of the gene and protein sequences reviewed tremendous homology among the precursors (Horikawa et al. 1983). It is, therefore, likely that all three precursors share a common evolutionary origin.

Structurally, all opioid peptides in the precursors are bracketed by a pair of basic amino acids on both the N- and C-side. This indicated a similar processing scheme for all three opioid precursors. Opioid peptides can be processed from their precursors by sequential treatment with trypsin and carboxypeptidase B (Lewis et al. 1980. The isolation and identification of Met-enkephalin-Arg^6-Phe^7 in Mytilus is especially strong evidence for the presence of proenkephalin-like precursor in this organism. This is true since the heptapeptide represents the last seven amino acid sequence

in the mammal proenkephalin. In this present report data will be presented which strongly suggested the existence of opioid precursor material in Mytilus pedal ganglia.

MATERIALS AND METHODS

Subtidal Mytilus were harvested from Long Island Sound at Northport, N.Y. Pedal ganglia from 425 fresh animals were excissed and stored at -160° C before use. The ganglia were homogenized with a Brinkman Polytron Homogenizer with four 3-sec bursts in 7.5 ml of 50 mM Tris-HCl buffer, pH 8.0; containing 0.1 M NaCl and 10 mM $CaCl_2$. The homogenate was centrifuged at 10,000xg for 10 min at 4° C and the supernatant was filtered with a 0.22 um membrane. The protein content of the supernatant was determined by Bio-Rad (Richmond, CA.) dye assay. TPCK-Trypsin was added to an aliquot of the filtrate in an amount equal to 1% of the protein content. This mixture was incubated overnight at 37° C. The enzyme activity was inactivated by heating at 80° C for 15 min. After clarification by centrifugation, carboxypeptidase B was added in an amount equal to 2% of the protein content. It was incubated at 37° C for 4 hr and then inactivated as described earlier.

The incubation mixture was purified by Sep-Pak C_{18} cartridge (Waters). The cartridge was activated first with methanol and water. After the sample was injected into the column, it was rinsed with 1% acetic acid and then eluted with methanol. The eluant was lyophilized, dissolved in water and filtered through a 0.45 um membrane. An aliquot of the extract without enzymatic treatment was processed in similar manner for use as the control,

Purified samples of the treated and untreated extracts were fractionated by HPLC on a Brownlee RP-300 reverse-phase column. The HPLC system used was a Waters model 840. The solvent system used was consisted of 0.1% trifluoroacetic acid (TFA) as A and 80% acetonitrile in 0.1% TFA as B. The column was initially eluted isocratically for 5 min with 20% B and followed by linear gradient of 20-80% B in 20 min. One-ml fractions of both samples were collected. Fractions with R_t's coresponding to Met-, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 were subjected to displacement assay using Mytilus pedal ganglia membrane and ^{3}H-DAMA. The displacement assay procedure was the same as described previously (Leung and Stefano 1984).

RESULTS AND DISCUSSION

The chromatograms, monitored at 280 um, of the untreated and treated samples are shown in figure 1. The untreated sample contained numerous small peaks and several major peaks (fig. 1A). After enzyme treatment significant changes in the peak pattern was observed (fig 1B). As a result of the enzymatic actions some of the major peaks were significantly reducedin size. Results from the displacement assays are also represented in figure 1. The displacement activities in fractions from the untreated sample are in general significantly lower than those in the treated sample.

The combined displacement activities found in the Met-, Leu-enkephalin and Met-enkephalin-Arg^{6}-Phe^{7} regions as compared to the ^{3}H-DAMA standard were 35, 23 and 30% respectively. The combined activities in these same regions in the treated sample were 81, 57 and 48%. They represented an .pa increased of 2.3, 2.5 and 1.6 folds of activities in Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg^{6}-Phe^{7}, respectively, in the treated sample over that of the untreated. These data strongly suggested the presence of opioid precursor material in the Mytilus pedal ganglia extract.

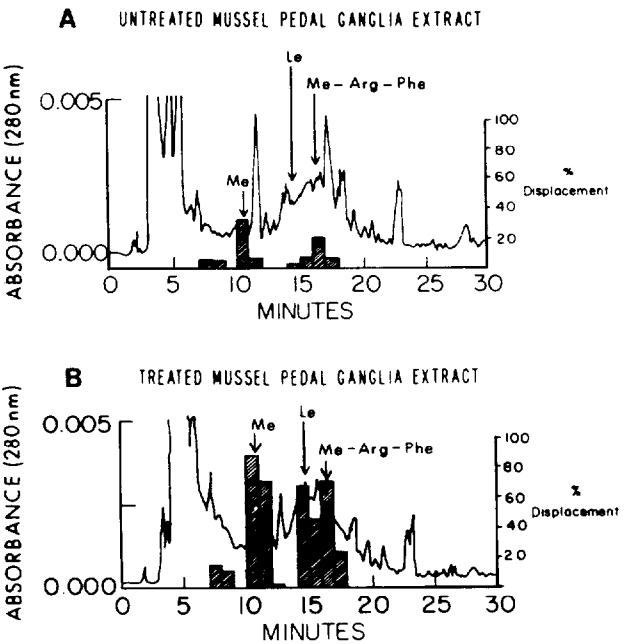

FIGURE 1. HPLC and displacement assays of untreated (A) and treated (B) samples of Mytilus pedal ganglia extracts. Procedure were as described in the text. One hundred percent displacement is ^{3}H-DAMA bound in the presence of 10 uM levorphanol minus ^{3}H-DAMA bound in the presence of 10 UM dextrorphan.

Evidence has been gathered which demonstrate the presence of opioid system in the invertebrates. Great-structural and physiological similarities also exist between the vertebrate and invertebrate opioid peptides (review, Leung and Stefano 1986). This preliminary report provides additional support in this area by showing the existence of enkephalin precursor in Mytilus. Even though only limited information is available concerning the precursor material in Mytilus, one significant similarity with the mammalian precursors is observed. Since the enkephalin peptides in the Mytilus precursor can be released by trypsin and carboxypeptidase B they must be

bracketed by basic amino acids as in the mammalian precursor. However, it is not known if the amino acids at both ends are paired and if more than one precursor is present in the tissue.

In conclusion, our current and earlier findings clearly demonstrated the existence of an opioid mechanism in Mytilus. The enkephalin peptides in Mytilus areidenticaltothose of mammals. As in the mammals, the enkephalin peptides in Mytilus appear to exist in a precursor form when first synthesized. The enkephalin peptides in the Mytilus precursor are also bracketed by basic amino acids and can be released by trypsin and carboxypeptidase B treatment. These results suggest that the entire opioid mechanism, including the structure of the precursor and the processing scheme, is a product of early evolutionary development. Its importance as a signal system is emphasized by its conservation through evolution.

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INACTIVATION OF [LEU^5]-ENKEPHALIN IN THREE ISOLATED PREPARATIONS: RELATIVE IMPORTANCE OF AMINOPEPTIDASE, ENDOPEPTIDASE-24.11 AND PEPTIDYL DIPEPTIDASE A

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ABSTRACT

Enkephalin had been shown to be almost exclusively hydrolyzed by three peptidases in the previous studies. In the present investigation, the relative importance of three enzymes in the inactivation of [Leu^5]-enkephalin was examined in three isolated preparations. Results showed that amastatin-sensitive aminopeptidase played the greatest role in both guinea-pig ileum and rat vas deferens while it played the similar role to either phosphoramidon-sensitive endopeptidase-24.11 or captopril-sensitive peptidyl dipeptidase A in mouse vas defer-ens.

INTRODUCTION

Three distinct enzymes, aminopeptidase, endopeptidase-24.11 ("enkephalinase"), and peptidyl dipeptidase A (angiotensin converting enzyme), had been shown to inactivate enkephalin in three isolated preparations, guinea-pig ileum (Aoki et al. 1984), mouse vas deferens (Aoki et al. 1986), and rat vas deferens (Cui et al. 1986). Additionally, the enkephalin-hydrolyzing aminopeptidase, endopeptidase-24.11, and peptidyl dipeptidase A had been reported to be inhibited almost completely with either 100 μM of bestatin or 1 μM of amastatin, either 1 μM of thiorphan or 1 μM of phosphoramidon, and 1 μM of captopril, respectively (Aoki et al. 1984; Aoki et al. 1986; Cui et al. 1986). Moreover, approximately 98% of enkephalin had been shown to remain intact when enkephalin was incubated with an ileal membrane fraction for 60 min at 37°C in the presence of three peptidase inhibitors, amastatin, captopril, and thiorphan (Hiranuma and Oka 1986). Thus, enkephalin had been demonstrated to be almost exclusively hydrolyzed by three distinct enzymes. In the present investigation, the relative importance of three enzymes in the inactivation of [Leu^5]-enkephalin was examined in three in vitro isolated preparations, guinea-pig ileum, mouse vas deferens, and rat vas deferens.

MATERIALS AND METHODS

Chemicals

A gift, gratefully received, was captopril from Sankyo Company (Tokyo, Japan). Amastatin, phosphoramidon and [Leu^5]-enkephalin were purchased from Peptide Institute, Inc. (Minoh, Japan).

In vitro isolated preparations

The vasa deferentia from mice or rats, and the myenteric plexus-lon-

gitudinal muscle strip of guinea-pig ileum were prepared and set up for electrical stimulation as described previously (Oka et al. 1982). The % inhibition of the stimulated muscle twitch produced by enkephalin was plotted against the log concentration of the opioid to estimate the IC_{50} (concentration of the opioid peptide to produce 50% inhibition of the twitch). When the effect of the peptidase inhibitor on the potency of enkephalin was studied, the inhibitor was given at least ten minutes before the enkephalin administration. The % difference shown in the tables was calculated from the formula % difference = [(IC_{50} before additional treatment - IC_{50} after additional treatment) / IC_{50} before additional treatment] x 100. The significance of % differences between IC_{50} values of two adjacent groups was determined by the paired Student's t-test.

RESULTS AND DISCUSSION

[Leu^5]-Enkephalin significantly inhibited the electrically-evoked contractions of guinea-pig ileum. Since its IC_{50} value in one preparation was sometimes significantly different from that in another, the IC_{50} values in the absence and the presence of the peptidase inhibitor were estimated in the same preparation.

TABLE 1. The greater enhanced effects of amastatin than either phosphoramidon or captopril on the inhibitory potency of [Leu^5]-enkephalin in guinea-pig ileum

Inhibitors	IC_{50} (nM)	Ratio of potency	% Difference
None	220 ± 73	1	73.3 ± 2.0***
Amastatin	55.0 ± 13	3.82 ± 0.31	
None	275 ± 81	1	16.9 ± 1.9***
Phosphoramidon	227 ± 68	1.20 ± 0.084	
None	194 ± 59	1	16.4 ± 1.7***
Captopril	165 ± 43	1.18 ± 0.093	

Each peptidase inhibitor at the final concentration of 1 μM was given 10 min before the enkephalin administration. Each value represents the mean ± S.E. of 4 experiments.
***P < 0.01.

The inhibitory potency of [Leu^5]-enkephalin was significantly enhanced by the pretreatment of the guinea-pig ileum with either amastatin, phosphoramidon or captopril (table 1). The magnitude of the enhancement by amastatin was significantly higher than that by either phosphoramidon or captopril (table 1). This shows that amastatin-sensitive aminopeptidase plays the major role in the inactivation of exogenously given enkephalin in guinea-pig ileum, being consistent with the previous report (Geary et al. 1982).
In the mouse vas deferens, the inhibitory potency of [Leu^5]-enkephalin was also significantly enhanced by each peptidase inhibitor (table 2). The magnitude of the enhancement by amastatin, however, was similar to that by either phosphoramidon or captopril in mouse vas deferens (table 2). This shows that three enkephalin-hydrolyzing peptidases play the similar role in the inactivation of

[Leu^5]-enkephalin in mouse vas deferens.

TABLE 2. The similar magnitude of the enhanced effects of three peptidase inhibitors, amastatin, phosphoramidon and captopril, on the inhibitory potency of [Leu^5]-enkephalin in mouse vas deferens

Inhibitors	IC_{50} (nM)	Ratio of potency	% Difference
None	9.54 ± 1.5	1	
Amastatin	4.76 ± 0.56	2.01 ± 0.19	47.5 ± 4.6***
None	11.7 ± 1.7	1	
Phosphoramidon	7.19 ± 0.98	1.63 ± 0.061	37.7 ± 2.5***
None	10.7 ± 1.7	1	
Captopril	5.39 ± 0.61	1.93 ± 0.16	45.3 ± 4.0***

Each peptidase inhibitor at the final concentration of 1 μM was given 10 min before the enkephalin administration. Each value represents the mean ± S.E. of 8 experiments. ***P < 0.01.

TABLE 3. The highest, the intermediate, and the lowest magnitude of the enhanced effects of amastatin (A), phosphoramidon (P), and captopril (C), respectively, on the inhibitory potency of [Leu^5]-enkephalin in rat vas deferens pretreated with the residual two peptidase inhibitors

Inhibitors	IC_{50} (μM)	Ratio of potency	% Difference
P + C	59.2 ± 13	1	
P + C + A	2.16 ± 0.19	37.3 ± 7.9	96.5 ± 1.2***
C + A	9.45 ± 2.1	1	
C + A + P	5.43 ± 1.6	1.99 ± 0.15	46.6 ± 3.9***
A + P	1.95 ± 0.44	1	
A + P + C	1.43 ± 0.26	1.32 ± 0.093	22.8 ± 6.1*

The mixture of peptidase inhibitors was given 10 min before the enkephalin administration at the final concentration of 1 μM each. Each value represents the mean ± S.E. of 4 experiments. *P < 0.05, ***P < 0.01.

In the rat vas deferens, the presence of the enzyme with low enkephalin-hydrolyzing activity was sometimes difficult to detect when the residual enzyme with high enkephalin-hydrolyzing activity was not inhibited in the preliminary experiments. When the enkephalin-hydrolyzing ability of a particular enzyme among three enzymes was examined, therefore, the residual two enzymes were inhibited in advance. The IC_{50} values of [Leu^5]-enkephalin in the rat vas deferens pretreated with three peptidase inhibitors were significantly lower than those pretreated with any combination of two peptidase inhibitors (table 3). The data in table 3 show that amastatin-sensitive aminopeptidase, phosphoramidon-sensitive endopeptidase-24.11 and captopril-sensitive peptidyl dipeptidase A play, respectively, the

greatest, the intermediate and the least role in the inactivation of [Leu^5]-enkephalin in rat vas deferens.

Previous studies (Aoki et al. 1984; Aoki et al. 1986; Cui et al. 1986) have shown that, when three peptidase inhibitors are given individually to the bath after the enkephalin administration, the enkephalin-induced inhibition of contractions of the isolated preparation is enhanced every time by the administration of each peptidase inhibitor although each inhibitor by itself does not inhibit the contractions at all when an opioid peptide is absent in the bath. Additionally, the observation that the inhibition produced by peptidase inhibitors is completely reversed by naloxone, an opioid antagonist, indicates that the inhibition must be caused by the increased amount of the enkephalin in the vicinity of opioid receptors by the administration of each peptidase inhibitor. Therefore, previous results (Aoki et al. 1984; Aoki et al. 1986; Cui et al. 1986) have indicated that three enkephalin-inactivating peptidases are all located very close to opioid receptors and produce a significant concentration difference of enkephalin between the surrounding organ bath and the vicinity of opioid receptors. Additionally, these enzymes are suggested not only to inactivate the exogenously given enkephalin in the vicinity of opioid receptors but also to play important roles in the termination of the action on opioid receptors of the enkephalin released from presynaptic nerve terminals. Interestingly, the results in the present investigation have shown that the relative importance of the [Leu^5]-enkephalin-inactivating enzymes is dependent on the tissue examined. The significance of the difference in the relative importance of the inactivating enzymes from one tissue to another remains to be elucidated in the present investigation.

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CHARACTERIZATION OF ENKEPHALIN DEGRADATION IN RAT PLASMA

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ABSTRACT

Approximately 80% of the hydrolysis of [leu]enkephalin in rat plasma can be attributed to bestatin-sensitive aminopeptidase activity, and an additional 5% is due to angiotensin converting enzyme. Thiorphan-sensitive enkephalinase hydrolysis of [leu]enkephalin could not be detected in plasma. On the other hand, 2-d-ala-1-[leu]enkephalin is metabolized approximately 35% by an unidentified bestatin-sensitive enzyme and approximately 15% by thiorphan-sensitive enkephalinase in rat plasma, while captopril-sensitive angiotensin converting enzyme is without measurable activity against this substrate.

INTRODUCTION

We reported previously (Martinez et al. 1985) that a very high correlation exists in rats between the rate of [leu]enkephalin (LE) hydrolysis in plasma and the latency to escape on the first trial in an active avoidance task. The activity of the plasma enzyme system is altered by shock experience, suggesting that the enzyme system may influence behavior by regulating the concentration of [leu]enkephalin, or its substrate, in blood and hence at a receptor. The specific enzymes involved in this relationship are as yet unidentified.

Data from brain tissue suggest a major role for aminopeptidases and a dipeptidyl carboxypeptidase ("enkephalinase") in the cerebral metabolism of endogenous and exogenously-administered enkephalin (Schwartz et al. 1983; Hambrook et al. 1976; Hazato et al. 1983). Studies by Hambrook et al. (1976) implicate the same enzymes in rat plasma as well. In vitro studies with the guinea pig ileum attribute enkephalin hydrolysis in this tissue to the combined action of aminopeptidases, enkephalinase, and angiotensin converting enzyme (ACE) (Aoki et al. 1984). In the isolated perfused rat lung, aminopeptidases and ACE, but not enkephalinase, are responsible for enkephalin metabolism (Gillespie et al. 1985). In the present study we characterize in more detail the enzymes associated with the hydrolysis of LE in rat plasma.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300-400 gm) were anesthetized with pentobarbital and implanted with femoral artery cannulae. In vitro enkephalin metabolism was measured in blood samples collected two days later through the indwelling cannulae. For this assay, plasma was rapidly separated, incubated 5 min at 37 deg C to permit

metabolism of endogenous enkephalin, and then combined in 115 or 230 µl aliquots with one or more of the following inhibitors: bestatin (prepared in saline to achieve final concentrations of 5 - 1500 µM), thiorphan (prepared in 5% N,N-dimethyl formamid in saline to achieve final concentrations of 0.1 - 10 µM), and captopril (prepared in saline to achieve final concentrations of 0.1 - 10 µM).

Following a 15 min incubation period, either ^{3}H-LE (3 or 6 µM, specific activity 43.6 Ci/mmol) or ^{3}H-2-d-ala-1-LE (3 or 6 µ1, specific activity 58 Ci/mmol) was added to the reaction mixture. Aliquots of plasma were removed at several time points after label addition; the reaction was terminated in these aliquots with two volumes of 0.1% trifluoroacetic acid in methanol. Unmetabolized LE or 2-d-ala-1-LE was separated from its combined metabolites by thin layer chromatography, according to the method of Ziring et al. (1983), and quantified by liquid scintillation counting.

RESULTS

The half-life of LE in rat plasma was determined to be 2.4 min, which is in close'agreement with the 2.5 min half-life determined by Hambrook et al. (1976) in a vas deferens bioassay. The plasma half-life of 2-d-ala-1-LE was between 3.5 and 7.5 hr.

The aminopeptidase inhibitor bestatin, in concentrations between 5 and 1500 µM, produced concentration-dependent decreases in the rate of LE metabolism, to a maximum of 80% inhibition. The dipeptidyl carboxypeptidase ("enkephalinase") inhibitor thiorphan, in concentrations up to 10 µM, was without detectable effect on the rate of LE hydrolysis. The combination of bestatin and thiorphan produced no further inhibition beyond that attributable to bestatin alone. On the other hand captopril (0.1 - 10 µM), an ACE-selective inhibitor, when combined with bestatin (500 µM), produced an additional concentration-dependent decrease in LE hydrolysis. The maximal inhibition produced by this combination was approximately 25% beyond that produced by bestatin alone. See Table 1 for summary of these data.

A different pattern of enzyme activities was revealed when 2-d-ala-1-LE replaced LE as substrate for the reaction. Although this substrate is reportedly insensitive to aminopeptidase degradation in brain (Llorens et al. 1982), a concentration-dependent decrease in 2-d-ala-1-LE hydrolysis was seen in the presence of bestatin (50 - 1500 µM), to a maximum of approximately 30 - 40% inhibition (although in one animal more than 90% inhibition was observed). Thiorphan (0.1 - 10µM) produced small concentration-dependent decreases in hydrolysis, to a maximum of 10 - 20% inhibition. The combination of bestatin (500 µM) and thiorphan (0.1 - 10 µM) produced no further inhibition beyond that attributable to bestatin alone. ACE hydrolysis of 2-d-ala-1-LE was not detectable, since captopril (0.1 - 10 µM) produced no measurable inhibition. These data are summarized in Table 1.

TABLE 1: Effects of Inhibitors on Enkephalin Metabolism

Substrate	Inhibitor (Highest concentration; µM)	Concentration Dependent Inhibition?	Maximum % Inhibition (Estimate)
^{3}H-LE	bestatin (1500)	yes	80
	thiorphan (10)	no	0
	best (500) + thio (10)	no	80
	best (500) + cap (10)	yes	85
^{3}H-2-d-ala-1-LE	bestatin (1500)	yes	35
	thiorphan (10)	yes	15
	captopril (10)	no	0
	best (500) + thio (10)	no	35

Combined inhibitor studies = bestatin (best) at a single concentration, with thiorphan (thio) or captopril (cap) in increasing concentrations

DISCUSSION

These data indicate that bestatin-sensitive aminopeptidase activity accounts for approximately 80% of the [leu]enkephalin hydrolysis in rat plasma. This is in agreement with the report by Venturelli et al. (1985), based on the concentrations of resulting metabolites, that aminopeptidases hydrolyze approximately 75% of the enkephalin in rat blood. Approximately 5% of the LE in blood is metabolized by captopril-sensitive ACE, while thiorphan-sensitive enkephalinase activity could not be detected. This pattern of enzyme activities is similar to that in rat lung (Gillespie et al. 1985), but different from that in brain and guinea pig ileum (Schwartz et al. 1983; Hambrook et al. 1976; Hazato et al. 1983; Aoki et al. 1984). The enzyme(s) responsible for the remaining 15% of LE metabolism in blood are as yet unidentified.

Our data also suggest an unusual pattern of enzyme activities against the 2-d-ala-1-LE substrate. This substrate reportedly is selective for enkephalinase and insensitive to aminopeptidase activity in brain tissue (Llorens et al. 1982), and its hydrolysis in brain is potently inhibited by thiorphan (Roques et al. 1983). In addition, brain enkephalinase is not inhibited by bestatin (van Amsterdam et al. 1983). However, in our studies, only 10 - 20% of the hydrolysis of

2-d-ala-1-LE could be attributed to a thiorphan-sensitive enkephalinase; 30 - 40% of the hydrolysis was due to an unidentified bestatin-sensitive enzyme, captopril-sensitive ACE activity could not be detected, and the source of the remaining 40 - 60% of the degradation is unknown.

Taken together, these data indicate that rat plasma has its own unique pattern of enkephalin hydrolysis. The enzymes active in plasma may be isoenzymes to the enzymes found in other tissues, differing from these latter enzymes in their sensitivities to inhibitors. Alternatively, the plasma enzymes may only appear to be different because of differences in conformation or in the availability of a substance that competes with, or alters the binding of, [leu]enkephalin or the inhibitors to the enzymes.

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CHARACTERIZATION OF THE ENKEPHALIN-DEGRADING AMINOPEPTIDASE OF NEUROBLASTOMA (N1E-115) CELL MEMBRANES

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ABSTRACT

A membrane-bound enkephalin (ENK) -hydrolyzing amino-peptidase (AP) was partially purified from neuroblastoma (clone N1E-115) cell membranes; enzyme activity was assayed by determination of the leu-enkephalin (LENK) degradation product, tyrosine (Tyr), with HPLC. The enzyme was extracted with Triton X-100, resolved by anion-exchange chromatography and further purified by gel filtration. The overall purification was about 100-fold with a yield of 43%. The apparent Mr value of the AP by gel filtration in the presence of 0.3% Triton X-100 was approx. 400 kDa. In the absence of detergent the apparent Mr value was about 305 kDa. In the elution buffers, where Triton X-100 was omitted, the peptidase activity was lost. The enzyme had a Km of 0.13 mM and a Vmax of 450 nmoles per mg protein per min at 25°C for LENK and exhibited little sensitivity to bestatin (IC50: 200 µM) and puromycin (IC50: 500 µM), but it was strongly inhibited by amastatin (IC50: 8 µM). The enzyme is an amphiphilic membrane protein; the native primary structure is preserved only in the 'detergent form'. It seems to be AP N (EC 3.4.11.2) with an optimum of pH 7.2 to 7.4. We assume that this AP plays the important role in inactivating ENKs on the neuronal level.
The ENK-degrading AP, partially purified from primary rat brain astrocyte cell membranes, exhibited a smaller apparent Mr value (130 kDa) and a higher sensitivity to amastatin (IC50: 0.4 µM), bestatin (IC50: 90 nM) and puromycin (IC50: 5 µM) than did the N1E-115 enzyme. From these data we conclude that there are at least two membrane-bound APs responsible for the ENK degradation in the brain.

INTRODUCTION

In this paper we report the existence of an AP N like enzyme in membranes of neuroblastoma cells (N1E-115) and of a bestatin-sensitive AP in cell membranes of primary astrocytes of 2-day old rats. In case of the neuronal enzyme the entire AP molecule in the cell membranes of the NlE-115 cells is extracted by the neutral detergent Triton X-100 leading to an amphiphatic form called the detergent form. The catalytically active site of the enzyme can also be solubilized by proteolytic cleavage at the hydrophilic-hydrophobic junction of the protein chain (protease form). Both forms of the AP have been

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purified and some of its properties have been investigated and compared. The comparison includes the partially purified glial AP, in which a detergent and protease form was not distinguishable.

MATERIALS AND METHODS

(Tyr-3,5-3H)-LENK (36.5 Ci/mmol) was purchased from DuPont (NEN, Dreieich, Germany). Mono Q monobeads and superose 6 were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). LENK, bestatin and puromycin were from Sigma Chemical Co. (Munich, Germany). Mouse neuroblastoma cells, clone N1E-115, were grown as monolayers. The media were changed three times a week and the experiments performed at the confluent stage, usually 7 days after plating. Primary astrocytes of 2-day old rats were cultured as previously described (Lentzen and Palenker 1983). After washing the cells were scraped off in 10 mM dipotassium phosphate buffer, pH 7.4, containing 1 mM EDTA with 2.8 µg aprotinin/ml and homogenized in a Potter-Elvehjem homogenizer (glass/Teflon). The homogenate was centrifuged at 4°C at 1000 x g for 15 min, the 1000 g supernatant at 8000 x g for 20 min, the 8000 g supernatant at 40000 x g for 30 min. The 40000 g pellet was washed once with 10 mM dipotassium phosphate buffer, pH 7.4, containing 1 mM EDTA with 2.8 µg aprotinin/ml and once more in dipotassium phosphate buffer without EDTA. The pellet was resuspended in 10 mM dipotassium phosphate buffer (40 mg/ml) containing 1 mM DT, 1 mM PMSF, 100 nM pepstatin A, 0,3% Triton X-100, 20% ethylene glycol, 2.8 µg aprotinin /ml, pH 7,7, solubilized at 4°C for 1 h and subsequently centrifuged at 4°C at 100000 x g for 1 h. The 100000 x g supernatant was loaded on an anion-exchange column (Mono Q, Pharmacia, Uppsala, Sweden) and eluted with 10 mM dipotassium phosphate, 20% ethylene glycol, 1 mM DT, 1 mM PMSF and 0.3% Triton X-100 and an NaCl gradient from 0 to 1 M with a flow rate of 1 ml/min. The pooled enzyme fractions of the Mono Q chromatography were concentrated by ultrafiltration and applied to a Superose 6 column (Pharmacia; 1.0 x 30 cm). Protein was eluted with 10 mM dipotassium phosphate buffer containing 0.1 M NaCl, 0.3% Triton X-100, 20% ethylene glycol, pH 7.4, at a flow rate of 0.5 ml/min. 40 µl of enzyme fractions were preincubated with 40 µl dipotassium phosphate buffer, pH 7.4, for 15 min at 25° C and further incubated with 3H-LENK (20 µl) for 15 min. The incubation was stopped with 10 µl of 1 N HCl and by deep-freezing of the samples in liquid nitrogen. The 3H-labeled Tyr was detected as described (Lentzen and Palenker 1983).

RESULTS AND DISCUSSION

The enzyme was purified within 10 h after homogenization of the cells. The optimal time was 60 min for

solubilizing the AP from the neuroblastoma cell membranes incubated with 0.3 % Triton X-100 at 4°C. Under these conditions 95% of the whole AP activity was solubilized. In the supernatant after solubilization only one activity peak was detected using anion-exchange chromatography. At a concentration of 0.25-0.35 M NaCl about 77% of the whole activity loaded on the column was eluted. In the absence of detergent in the elution buffer, the peptidase activity was lost. The relatively naked protein molecules tend to bury their hydrophobic regions by clustering together, forming large aggregates under the loss of enzyme activity. The fractions from anion-exchange chromatography were loaded after concentrating by ultrafiltration on a gel filtration column. The Mr value of the enzyme in the presence of Triton X-100 was approx. 400 kDa. The following proteins were used for calibration: thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, aldolase 158 kDa. After solubilization, using Triton X-100 as detergent, the hydrophobic domain binds a micelle of detergent, thereby increasing the molecular weight by about 95 kDa, corresponding to approx. 150 molecules of detergent. Therefore, the Mr value of the native enzyme after gel filtration seems to be 305 kDa. The Michaelis constants, Km and Vmax, for LENK as substrate was calculated by Lineweaver-Burk analysis using substrate concentrations from 1.8 to 800 µM. The Km and Vmax for LENK were 125 µM and 450 nmoles per mg protein per min at 25°C. The AP inhibitors, amastatin (IC50: 8 µM), bestatin (IC50: 200 µM) and puromycin (IC50: 500 µM) had inhibitory effects. The enzyme is active between pH 5.9 and 8.2 with maximal activity for LENK hydrolysis between pH 7.2 and 7.4. These properties of the enzyme are not in accordance with the studies about ENK-degrading APs in synaptosomal preparations (Hui et al. 1983; Hersh 1985). However, they are in agreement with the studies of Maroux et al. (1973), Gray and Santiago (1977), Kenny and Maroux (1982) and others on the AP N from microvilli from intestinum and kidney of pigs and rats. Experiments of Gros et al. (1985) suggest, that AP N is present in the brain. If solubilization and purification of the enzyme was made without suitable protease inhibitors or if trypsin was added to the detergent extract, some catalytically active fractions were isolated in the anion-exchange chromatography, which showed activity both in the presence and in the absence of the detergent in the elution buffer. We selected a low-molecular weight activity peak (66 kDa) for determination of the kinetic parameters for the substrate LENK and for estimation of the IC50 values for the inhibitors bestatin and puromycin. This fraction showed a six-fold higher affinity (Km: 25 µM) and more sensitivity against bestatin (IC50: 0.6 µM).

Our studies with the so-called detergent- and protease form of the enzyme demonstrate that there obviously exists an amphiphatic integral membrane protein

comparable to the AP N. Correspondingly this peptidase has a lipohilic, catalytically inactive anchor in the membrane and a hydrophilic catalytically active head projecting into the extracellular space. The hydrophilic head can be cut off by proteases like trypsin and can be further fragmented dependent on the incubation time. It was surprising that the hydrophilic part of the AP shows a lower Km value and lower inhibitor constants than did the detergent form of the enzyme. We suggest that this enzyme is the membrane-bound, ENK hydrolyzing, neuronal AP, which must be distinguished from a glial AP.

We isolated an AP from cell membranes of primary astrocytes. Solubilization and purification of the glial enzyme were performed as described above for the neuronal enzyme. As in the case of the neuronal enzyme the glial AP is an membrane protein as several solubilization trials with different detergent concentrations revealed. Using anion-exchange chromatography, only one AP activity peak was eluted at a sodium chloride concentration of 0.3 M. In contrast to the neuronal AP the glial enzyme was catalytically active independent on the presence or absence of Triton X-100 in the elution buffer. The pooled Mono Q fractions containing the AP activity were concentrated by ultrafiltration and then further purified by gel filtration. After calibrating the column with standard proteins, this peptidase corresponded to a molecular weight of approx. 130 kDa.

The enzyme had an optimum of about pH 6.7. Its affinity to the substrate LENK with a Km of 14 µM is relatively high. It was strongly inhibited by amastatin (IC50: 0.4 µM), bestatin (IC50: 90 nM) and puromycin (IC50: 5 µM). Similar results for a synaptosomal bestatin-sensitive AP were reported by Hui et al. (1983), Hersh (1985) and Giros et al. (1986). In contrast to the neuronal enzyme, a detergent- and protease form of the glial AP is not distinguishable. (This work was supported by the DFG).

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THE ENDOGENOUS TRIPEPTIDE TYR-GLY-GLY AS AN EXTRACELLULAR METABOLITE OF ENKEPHALINS IN RAT BRAIN : ORIGIN AND METABOLISM

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ABSTRACT

The tripeptide Tyr-Gly-Gly (YGG) was established as an endogenous constituent in rat brain. Its origin from enkephalin neurons is suggested by its regional distribution paralleling that of (Met^5)-enkephalin (YGGFM), its decrease following kainate-induced ablation of the striato-pallidal neurons and its enhanced formation following depolarization of pallidal slices. Enkephalinase (EC 3.4.24.11) is selectively responsible for endogenous YGG formation in vitro and in vivo.

INTRODUCTION

Using a sensitive RIA (Llorens-Cortes et al. 1985b) YGG, a potential metabolite of opioid peptides (OP), was characterized as an extraneuronal constituent of mouse brain (Llorens-Cortes et al. 1985a).
In this present work we have characterized YGG in rat brain regions and explored the hypothesis that it may originate from OP by assessing the effects of lesions of a known enkephalin pathway and studying its formation in depolarized slices.

MATERIALS AND METHODS

In most cases male Wistar rats were killed by decapitation, brain regions dissected out in the cold in less than 3 min and homogenized in 0.4 N $HClO_4$. Experiments with slices of globus pallidus were performed as described (Giros et al. 1986).
RIAs for YGGFM and YGG were performed as described (Gros et al. 1978; Llorens-Cortes et al. 1985a)

RESULTS AND DISCUSSION

YGG immunoreactivity was detected in all regions of rat brain (table 1) and appears to correspond to authentic YGG as indicated by its chromatographic patterns in various systems (namely HPLC on C_{18} µBondapak columns). Its levels in striatum did not differ when rats were killed by decapitation or microwave irradiation, indicating that it was not formed artefactually by hydrolysis of larger peptides brought into contact with peptidases at the homogenization step.
In most brain areas YGG levels corresponded to about 10 % of YGGFM levels (table 1). This parallel regional distribution is consistent with the idea tnat the tripeptide constitutes a metabolic product of enkephalins or related OP.
Ablation of proenkephalin-A-containing neurons in the striato-pallidal pathway elicited by intrastriatal kainate was accompanied by significant decreases in YGG in both the injected and the projection areas (table 2).

TABLE 1: Regional distribution of YGG and YGGFM immunoreactivities in rat brain

REGIONS	YGG	YGGFM (pmol/mg protein)	YGG / YGGFM
Globus pallidus	3.50 ± 0.40	38.2 ± 0.38	0.09 ± 0.01
Substantia nigra	1.71 ± 0.25	10.0 ± 0.80	0.17 ± 0.03
Caudate putamen	1.01 ± 0.11	14.8 ± 0.16	0.07 ± 0.01
Hypothalamus	0.47 ± 0.03	12.7 ± 0.70	0.04 ± 0.01
Cerebral cortex	0.45 ± 0.05	3.62 ± 0.30	0.12 ± 0.01
Hippocampus	0.29 ± 0.02	2.40 ± 0.28	0.12 ± 0.02
Brainstem	0.05 ± 0.01	0.46 ± 0.03	0.11 ± 0.04
Cerebellum	0.02 ± 0.02	0.10 ± 0.01	0.20 ± 0.02

Means ± S.E.M. of data from 5 to 18 experiments.

TABLE 2: Effect of kainic acid injections on YGG-ir and YGGFM-ir levels in caudate-putamen and globus pallidus

ANALYSED REGION	YGG LEVELS (pmol/mg protein)	YGGFM LEVELS
Caudate putamen		
Control side	1.06 ± 0.11	19.7 ± 0.8
Injected side	0.77 ± 0.09*	15.4 ± 1.3=
Variation	- 27 %	- 23 %
Globus pallidus		
Control side	4.12 ± 0.36	28.2 ± 1.9
Injected side	2.09 ± 0.21**	19.7 ± 1.3
Variation	- 49 %	- 30 %

* P < 0.05 ; ** P < 0.01 ; *** P < 0.001 compared to control

Kainic acid (1.5 µg/1 µl), dissolved in distilled water adjusted to pH 7.4 was microinjected into the right anterior caudate-putamen at a rate of 0.6 µl/min. Animals were sacrificed by decapitation 14 days later.
Means ± S.E.M. of data from 18 separate experiments.

When pallidal slices were incubated in the absence (not shown) or the presence (table 3) of bestatin, an aminopeptidase inhibitor preventing the hydrolysis of YGG (De la Baume et al. 1983), the tripeptide was predominantly found in the incubation medium. This extracellular localization of YGG is consistent with data from subcellular fractionation studies in mouse brain (Llorens-Cortes et al. 1985a). K^+-induced depolarization of the slices elicited a strong elevation of YGG in the medium (in which it was identified by HPLC) as well as in slices (in which it might be locates in the extracellular space). The amount of YGGFM released by the K^+ stimulus (corresponding to the difference of YGGFM tissue levels at 5 mM K^+ and 50 mM K^+, respectively) was notably inferior to the increase of the sum (YGGFM + YGG) recovered in the medium (table 3). This indicates that released peptides other than YGGFM, i.e. presumably other OP derived from proenkephalin A also contribute in YGG formation.

TABLE 3: Effects of a depolarizing stimulus on tissue and incubation medium levels of YGG-ir and YGGFM-ir of pallidal slices

CONDITIONS	LEVELS IN SLICES (pmol/mg protein)		LEVELS IN MEDIUM (pmol/mg protein)	
	YGGFM	YGG	YGGFM	YGG
5 mM K^+	23.8 ± 0.9	1.2 ± 0.2	0.8 ± 0.1	2.6 ± 0.5
50 mM K^+	17.9 ± 0.2	2.1 ± 0.2	2.6 ± 0.2	10.2 ± 0.7
Difference	- 5.9 ± 1.0	+ 0.9 ± 0.3	+ 1.8 ± 0.2	+ 7.6 ± 0.9

*P < 0.05 ; ** P < 0.01 ; *** P < 0.001 compared to 5 mM K^+
Slices were preincubated for 10 min in the presence of 100 µM bestatin), and incubated for a further 5 min with 50 mM K^+, when stated. At the end of incubations, slices were separated from medium by a short centrifugation and peptides extracted into $HClO_4$ were radioimmunoassayed in both fractions.
Means ± SEM from 6 experiments

In the presence of Thiorphan in increasing concentrations, the K^+-induced rise in YGG was progressively abolished while the recovery of YGGFM in the incubation medium rose to reach the amount of YGGFM released from the slices (figure 1). The IC_{50} value of Thiorphan (9 nM) was consistent with its effect being se;lectively mediared by inhibition of enkephalinase. In addition acetorphan (10 mg/kg), a parenterally-active derivative of Thiorphan, strongly reduced YGG levels in various brain regions (by 55-70 % after 1h), establishing enkephalinase as the YGG forming enzyme in vivo as well as in vitro. This peptidase selectively cleaves amide bonds comprising the amino group of en aromatic (or hydrophobic) amino acid residue which is consistent with the idea that OP (which all have in common the YGGF sequence) are precursors of YGG. Also the fact that enkephalinase is an ectoenzyme in brain (De La Baume et al. 1983) is consistent with

the idea that YGG is formed after release of OP, derived from the proenkephalin A precursor since other OPs are poor substrates.

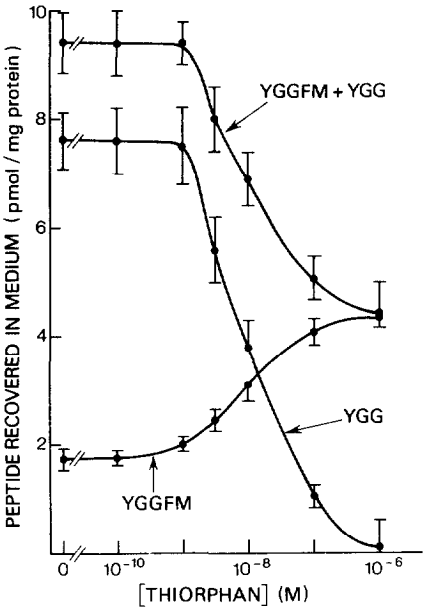

FIGURE 1: Effects of Thiorphan, en "enkephalinase" inhibitor, on levels of YGG and YGGFM imnunoreactivities in the incubation medium of K^+-depolarized slices of rat globus pallidus. Pallidal slices were preincubated for 10 min in the presence of 100 µM bestatin and increasing concentrations of Thiorphan and then exposed for 5 min to 50 mM K^+. After centrifugation, YGG and YGGFM were radioimmunoasssyed in media.

In conclusion, all data were consistent with the idea that YGG represents an extracellular metabolite of endogenous OPs derived from proenkephalin-A for which its measurement may constitute an index of release.

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EVALUATION OF STRIATAL ENKEPHALIN RELEASE IN VIVO FROM STEADY STATE LEVELS AND TURNOVER RATES OF THE TRIPEPTIDE TYR-GLY-GLY

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ABSTRACT

The tripeptide Tyr-Gly-Gly (YGG), an extraneuronal metabolite of opioid peptides (OP) derived from proenkephalin A is in a highly dynamic state in mouse striatum. Inhibition of its synthesis by Thiorphan reduced its levels with a t 1/2 of 12 min. Inhibition of its degradation by bestatin elicited its rapid accumulation consistent with a t 1/2 of enkephalins in the one-hour range. Pentobarbital anesthesia markedly reduced its steady state level end turnover rates.

INTRODUCTION

Using a sensitive RIA, we recently characterized YGG as an endogenous constituent of mo$_u$se (Llorens-Cortes et al. 1985a,b) and rat brain tissues (Giros et al. this volume). Endogenous YGG appears to be formed extraneuronally by enkephalinase degradation of the OPs derived from the proenkephalin A precursor because i) its regional distribution parallels that of YGGFM, ii) its levels decrease after ablation of these neurons iii) its extracellular localization has been shown by subcellular fractionation and studies with brain slices; iv) its level rises considerably as a result of depolarization of proenkephalin A neuron terminals, which are abundant in slices from globus pallidus, and v) inhibitors of the ectoenzyme enkephalinase inhibit YGG formation selectively.
We have presently attempted to evaluate YGG turnover in mouse striatum by measuring its rates of decline and rise following inhibition of enkephalinase and aminopeptidase respectively. To validate these measurements under conditions of altered neuronal activity, we assessed the effects of pentobarbital anesthesia which is known to modify the activity of a variety of neuronal systems.

MATERIALS AND METHODS

Male Swiss mice were killed after i.c.v. administration of saline or peptidase inhibitors. The striata were dissected out in less than 2 min, homogenized in 0.4 N $HClO_4$ and YGG measured in extracts by a RIA (Llorens-Cortes et al. 1985a,b).

RESULTS AND DISCUSSION

Thiorphan elicited a monoexponential decline of striatal YGG with a fractional rate constant of 3.5 $\pm$ 0.5 hr corresponding to a t 1/2 of 12 $\pm$ 2 min end a turnover-rate of 18 $\pm$ 3 pmol/mg protein/hr (figure 1). A similar rate of decline was found with 1 mg/kg acetorphan a parenterally-active enkephalinase irhibitor (Lecomte et al. 1986) (not shown).

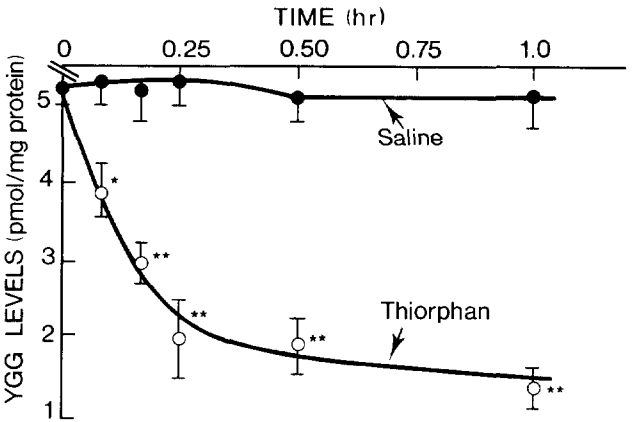

FIGURE 1: Decline of YGG levels in mouse striatum after administration of the enkephalinase inhibitor Thiorphan.
Mice were given saline or 100 ug of Thiorphan i.c.v. and were killed at different times after injection. Values are means ± S.E.M. of five to eight experiments.
* P < 0.05 ; ** P < 0.01 versus the respective controls.

After bestatin administration, striatal YGG increased linearly for 15-20 min and then reached a plateau at about twice its steady-state level (figure 2). The initial rate of YGG accumulation was 18 ± 3 pmol/mg protein/hr.

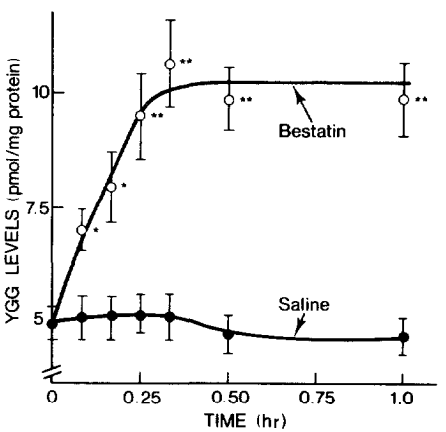

FIGURE 2: YGG accumulation in mouse striatum after administration of the aminopeptidase inhibitor bestatin.
Amimals received saline or 150 ug of bestatin i.c.v. and were killed at different times after injection. Values are means ± S.E.M. of 5-11 experiments.
*P < 0.05 ; ** P < 0.01 versus the respective controls.

From these two consistent estimations it may be inferred that YGGFM release and its degradation into YGG occur at a rather high rate, implying that the turnover of striatal enkephalins is comparable to that of "classical" neurotransmitters. This conclusion is somewhat unexpected since the t 1/2 of neuropeptides, although never reported before, is often assumed to be longer due to processes of ribosomal synthesis, maturation and axonal transport which precede their exocytosis.

However the above values should be considered with caution since they are based upon a series of assumptions that may not be verified. Peptidase inhibition may not be complete immediately after treatments and may trigger feedback control mechanisms regulating OP release. Bestatin blocks the degradation of not only YGG but also of OPs. Hence the fairly good agreement between the two estimations of YGG turnover rate may be to a certain extent fortuitous. Nevertheless the observation (Llorens-Cortes et al. 1986) that a similar value is found for the rate of YGGFM accumulation in an extrasynaptosomal fraction of mouse striatum following complete blockade of its metabolism (by Thiorphan plus bestatin) strenghthen our conviction that these represent reasonable estimates of the rate of enkephalin release in vivo.

In mice treated with pentobarbital the striatal YGG levels were found to decrease progressively, a plateau being reached after 30 min (figure 3) without significant modification of YGGFM levels (not shown).

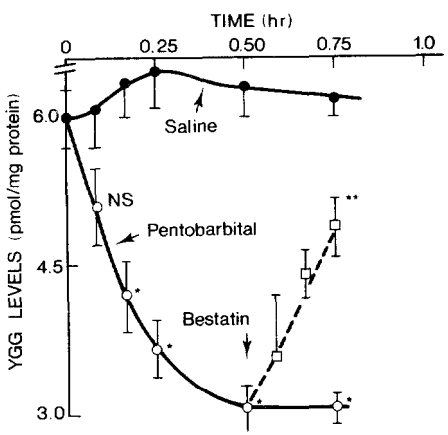

FIGURE 3: Steady-state levels end bestatin-induced accumulation of YGG in mice anesthetized with pentobarbital.
Mice were given pentobarbital i.p. at 60 mg/kg either alone (solid line) or with 150 ug of bestatin, administered 30 min later i.c.v. (broken line) and were killed at various intervals thereafter. Values are means ± S.E.M. of 5-15 experiments.
N.S. non significant
* $P < 0.01$ versus saline ; ** $P < 0.01$ versus pentobartital alone.

When this new steady-state level was reached, administration of bestatin elicited, for the next 15 min, linear accumulation of YGG at a rate (pmol/mg protein/hr) of 7 ± 1 versus 18 ± 2 in controls.
A similar effect was observed when mice were anesthetized with chloral. This decrease in steady-state YGG level during anesthesia seems attributable to impairment of enkephalin release and of YGG formation shown with peptidase inhibitors. Barbiturates reduce the activity of several classes of aminergic neurons. As far as we know, this is the first time that their effects on peptidergic neuron activity have been reported. The drastic reduction in the activity of enkephalin neurons, shown by the present data, might be relevant to the hyperalgesic and anticonvulsive effects of barbiturates since enkephalins induce opposite effects.
Hence the steady-state level of YGG appetis to constitute a useful index of changes in enkephelin neuron activity in the brain, just as the levels of certain extraneuronal metabolites reflect changes in monoamine neuron activity. Measurement of this simple index offers the great advantage of avoiding the administration of any metabolic inhibitor. A similar experimental approach could conceivably be applied to other classes of cerebral neuropeptides as soon as their metabolic pathways are identified.

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ISOLATION AND CHARACTERIZATION OF ACTH-RELATED PEPTIDES FROM PORCINE PITUITARIES

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ABSTRACT

We report on the isolation of new variants of porcine ACTH with corticotropic activity. Two of these variants differ in their amino acid sequence from porcine ACTH at one position (Arg8). This implies that they cannot be derived from the known precursor proopiomelanocortin (POMC).

INTRODUCTION

The adrenocorticotropic hormone (ACTH) is synthesized as part of its precursor POMC. In the pituitary intermediate lobe ACTH 1-39 can be processed to α-MSH (acetyl-ACTH 1-13-amide) and CLIP (ACTH 18-39). None of these peptides possesses corticotropic activity.
However, recently corticotropic active fragments of ACTH were isolated from porcine anterior pituitaries and identified as ACTH 1-38, ACTH 1-37, ACTH 7-39 and ACTH 7-38 (Ekman et al. 1984).
We supposed that there could be more and yet shorter ACTH-variants showing significant biological activities. We will report on the isolation and sequence determination of five new ACTH-variants from porcine pituitaries, two of them differing in their primary structure from the corresponding ACTH-sequence.

MATERIALS AND METHODS

Isolation of ACTH-like peptides

Fresh porcine pituitaries were obtained from a slaughterhouse. They were extracted and prepurified as described by Schleyer et al. (1976) and by additional gel filtration on Fractogel TSK HW-40 (S) (Merck, F.R.G.). The resulting fraction P-LF II D3 was further chromatographed in a number of successive reversed phase HPLC-systems.
The semipreparative system using an Ultrasphere ODS column (250x10 mm, particle size 10µ, Beckman, U.S.A.) yielded three main peaks eluting in a gradient of 0.2 % TFA and acetonitrile. These main fractions were further chromatographed in one or two analytical HPLC-systems using either a 250x4.6 mm column packed with Ultrasphere ODS 10µ and a gradient of 0.2 % TFA and acetonitrile and/or a 250x4 mm column packed with Hypersil WP 300 butyl 5µ (Shandon, U.K.) and a gradient of 50 mM

potassium phosphate pH 2.5 and acetonitrile. This resulted in 6 pure fractions F1 to F6. All peptides were detected by their UV-absorption at 210 and 280 nm.

Peptide mapping

Fractions F1 to F6 were digested by TPCK-treated trypsin (Sigma, F.R.G.), staphylococcus aureus protease (Miles, U.S.A.), proline specific endopeptidase (Miles, U.S.A.) and mouse submaxilliary protease (Sigma, F.R.G.) following slightly modified standard procedures.
The digests were subjected to reversed phase HPLC on a 250x4 mm column packed either with HD-Gel (Gynkotek, F.R.G.) or Hypersil WP 300 butyl 5µ applying a gradient of 0.2 % TFA and acetonitrile.

Amino acid analysis

The amino acid compositions of the desalted and hydrolyzed peptides were determined by precolumn derivatization with ortho-phthalaldehyde (OPA) and mercaptoethanol. The procedure of Umagat et al. (1982) was modified and optimized for the routine analysis of 50 pmol samples.

Sequence analysis

The pure peptides were manually sequenced following the 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC) method of Wittmann-Liebold and Kimura (1984).
The C-termini were enzymatically sequenced using carboxy-peptidase Y (Sigma, F.R.G.) as descibed by Allen (1981).

Biological activity and RIA

Corticotropic activity was tested by the dose dependent secretion of corticosteron from isolated rat adrenal cells (Sayers et al. 1971)
Radioimmunoassays using 125I-labelled tracers were performed for systems specific for α-MSH, CLIP and the middle portion of the ACTH-molecule.

RESULTS AND CONCLUSIONS

The prepurified extract of porcine pituitaries was subjected to reversed phase HPLC in a number of successive HPLC-systems with increasing separation power. The HPLC-systems were monitored by region specific ACTH-radioimmunoassays and by the corticotropic activity tested with isolated rat adrenal cells. Finally we obtained fractions F1 to F6, all showing a strong crossreactivity to ACTH-antisera directed against the middle portion of ACTH.
F1 to F6 were proven to be pure by OPA amino acid analysis . They were digested by trypsin, staphylococcus

aureus protease, proline specific endopeptidase and mouse submaxillary protease. The resulting peptide fragments were separated by means of HPLC and subjected to amino acid analysis and manual microsequencing.

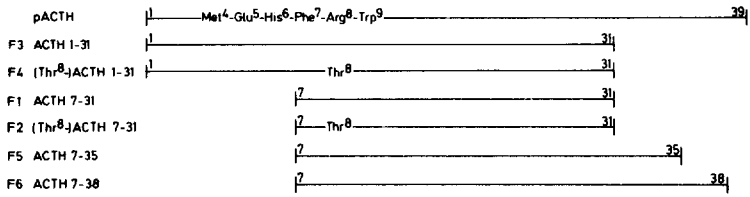

FIGURE 1. Schematic representation of the amino acid sequences of pACTH 1-39 and its variants F1 to F6.

The results are shown in figure 1. The amino acid sequence of F1 relates to ACTH 7-31 with the uncertainty of Asx in ACTH position 29. F3, F5 and F6 proved to be identical to ACTH 1-31, ACTH 7-35 and ACTH 7-38, respectively. The latter variant has been already described by Ekman et al. (1984).
F2 and F4 are ACTH-fragments with close sequence homologies but differ from the above mentioned variants by an exchange of Thr for Arg8 , thus resembling (Thr8-)ACTH 7-31 and (Thr8-)ACTH 1-31.
Our peptides F1 to F6 exhibited a corticotropic activity of about 2 to 25 % compared to porcine ACTH 1-39.
These by now unknown ACTH-variants could be generated from ACTH by truncating it C-terminally in an unspecific manner. In contrast the N-terminal cleavage site between His6 and Phe7 seems to be very specific since no other N-terminally truncated ACTH-variants have been isolated (Ekman et al. 1984). To our knowledge such a specifity has not been attributed to one of the known proteases.
The occurrence of the two (Thr8-)ACTH-variants points to the existence of a gene differing from the known POMC-gene.
Both genes could occur together in one animal or in a subpopulation of animals lacking the ordinary POMC-gene and having instead the gene for the ACTH-variants.
Oates and Herbert (1984) and Gossard et al. (1986) found only one porcine POMC-gene. Boileau et al. (1983) isolated two POMC mRNA species which coded for two different N-terminal POMC-sequences but they did not show differences in the ACTH-coding region.
The mutation at position 8 of the ACTH molecule might be of functional relevance because the partial sequence about it is crucial for the biological activity of a number of POMC derived peptides (ACTH, α-MSH, β-MSH, γ-MSH).

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A MEMBRANE BOUND SUBSTANCE P DEGRADING ENDOPEPTIDASE FROM RAT BRAIN

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ABSTRACT

From rat brain, a membrane bound substance P-degrading endopeptidase (SPE) was purified 1580 fold to near homogeneity. After extraction with 10mM CHAPS, the enzyme preparation was subjected to ion exchange chromatography on DEAE-cellulose, adsorption chromatography on hydroxyapatite, gelfiltration through Ultrogel AcA 44 and FPLC on Mono Q. This enzyme of 70000 molecular weight is optimally active at pH 7.5. Metal chelators(EDTA and EGTA) and sulfhydryl modifying reagents (N-ethylmaleimide and p-chloromercuriphenylsulfonic acid) are strongly inhibitory while the serine-protease inhibitor diisopropylfluorophosphate does not effect the enzyme activity. The enzyme is strongly inhibited by bacitracin but not by phosphoramidon and captopril. Degradation of substance P is strongly inhibited by neurotensin, somatostatin, ACTH 1-39, and less effectively by LHRH but not by Leucine-enkephalin. Substance P is preferentially hydrolyzed at the Gln^6-Phe^7 peptide bond but fragmentation at the Pro^4-Gln^5, Gln^5-Gln^6,Phe^7-Phe^8 and Gly^9-Leu^{10} bonds was also observed.

INTRODUCTION

The mechanism involved in the inactivation of the putative neurotransmitter substance P are unknown. A number of peptidases capable of degrading substance P have been identified (Akopyan et al. 1979, Chrètien et al. 1980, Leeet al. 1983, Matsas etal. 1983, Skidgelet al. 1984, Yokosawaetal. 1983), but their functions for the inactivation of synaptically released substance P remain unclear. We previously reported that substance P is hydrolyzed by a bacitracin sensitive enzyme localized on neuronal cells in primary culture (Horsthemke etal. 19841 and now report on the purification of a membrane bound and bacitracin sensitive substance P degrading endopeptidase from rat brain.

MATERIALS AND METHODS

Enzyme assay: The enzyme solution was diluted with buffer A (25mM K-diethylmalonate, pH 7.5, containing 0.04% NaN_3) and supplemented with 100pM diisopropylfluorophosphate (DFP) and 100µM bestatin (final volume=19µl). After preincubation at 35°C for 15 minutes the reaction was started by addition of 0.1µCi (^{3}H)-Phe^8-substance P (1µl;24.5Ci/mmol). At given time intervals 10µl of the reaction mixture was spotted on silica gel plates (Merck, F.R.G.). The plates were developed in ethyl acetate:pyridine:acetic acid:H_2O (125:100:20:60). Theamount of degraded substance P was determined as described (Horsthemke etal. 1984).

Enzyme purification: All steps were performed at 4°C. Frozen rat brains (100g) were thawed and homogenized in 380ml buffer B (5mM $NaPO_4$, pH7.5 containing 1mM DTE and 0.04%NaN_3). The homogenate was centrifuged for 1h at 100000g. The resulting pellet was rehomogenized in buffer B (380ml) and washed again for additional 5 times. The last homogenate was adjusted to 10mM CHAPS (Sigma) and stirred for 1h. The membrane extract obtained by centrifugation at 100000g for 1h was then fractionated by ion exchange chromatography on DE-52 cellulose (equilibrated in buffer B) using a linear NaCl gradient (0-250mM). The substance P degrading activity eluting at 4.5 to 8.5 mSi conductivity was pooled and applied to a hydroxyapatite column, equilibrated in buffer B. After washing with 50mM $NaPO_4$, two substance P degrading activities were desorbed in a linear gradient from 50 to 180mM $NaPO_4$. The enzyme eluting at higher $NaPO_4$-concentrations (6-9mSi conductivity) was pooled, concentrated by ultrafiltration (Amicon PM 10 membrane) and subjected to gel filtration through Ultrogel AcA 44. The substance P degrading endopeptidase eluting with a molecular weight of approx. 70000 was clearly separated from another substance P degrading activity of low molecular weight and was further purified over FPLC on Mono Q (Pharmacia) . The enzyme eluted as a single peak at approx. 120mM NaCl. After rechromatography on Mono Q this enzyme preparation was used for all further investigations.

RESULTS AND DISCUSSION

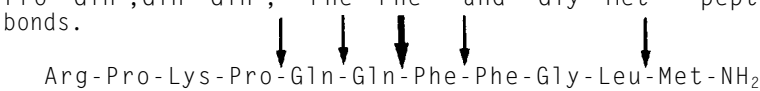
Although the enzyme assay contained 100pM DFP to inhibit the degradation of substance P by dipeptidyl-aminopeptidase IV and other serine-proteases, several substance P degrading activities could be separated by the purification procedure described. By this procedure the substance P degrading endopetidase was purified 1580 fold to yield an enzymatically homogenous-enzyme preparation.

The SPE was found to be strongly inhibited by metal-chelating compounds such as EDTA and EGTA and also by the -SH reactive reagents N-ethylmaleimide (NEM) or p-chloromercuriphenylsulfonic acid (pCMBS) but not by DFP, an inhibitor of serine-proteases (tab.1). SPE is strongly inhibited by bacitracin, a general inhibitor of various peptidases which also inhibits the substance P degrading activity associated with neuronal cells in primary culture (Horsthemke et al. 1984). These results suggest that SPE is different from the substance P degrading activity described by Leeet al. (1981) which is not inhibited by 1mM Bacitracin. SPE is obviously also different from other peptidases known to hydrolyze substance P and other peptides (e.g. Leu-Enkephalin), namely angiotensin converting enzyme and endopeptidase 24.11, since it is not inhibited by captopril and also not by phosphoramidon, the known inhibitors of these enzymes.

Test substance	concentration	%inhibition
EDTA	1mM	93
EGTA	1mM	70
NEM	100µM	96
pCMBS	100µM	100
DFP	100µM	0
Captopril	10µM	0
Phosphoramidon	10µM	0
Bacitracin	100µM	72
Substance P	100µM	83
Neurotensin	100µM	92
Somatostatin	100µM	85
ACTH 1-39	100µM	81
LHRH	100µM	24
Leu-enkephalin	100µM	0

TABLE 1.
Test substances (2µl, 10-fold concentrated in buffer A) were mixed with 17µl of buffer A containing 1µl enzyme. Except when peptides were tested, the mixture was preincubated for 15 min at 35°C. The reaction was started by adding 0.1µCi (1µl) (^{3}H)-substance P and stopped after incubation for 15 min at 35°C by spotting 1µl aliquots on silica gel plates. The amount of substance P degraded was determined as described under "materials and methods".

In agreement with these results, the degradation of (^{3}H)-substance P was not effected by addition of unlabeled Leu-enkephalin. In contrast, the degradation of the radiolabeled tracer was strongly inhibited not only by unlabeled substance P but also by neurotensin, somatostatin and ACTH 1-39 while LHRH was considerably less effective. These results strongly indicate that SPE is not a substance P-specific enzyme but a more general peptidase which also hydrolyzes other peptides by recognizing only certain structural elements of their substrates. This notion is supported by the observation that SPE catalyzes the hydrolysis of substance P at various peptide bonds. Identification of the enzymatically formed fragments by HPLC on a µBondapak C_{18}-column and amino acid analysis clearly demonstrated that substance P is preferentially hydrolyzed by SPE at the Gln^6-Phe^7 bond additionally also at Pro^4-Gln^5, Gln^5-Gln^6, Phe^7-Phe^8 and Gly^9-Met^{10} peptide bonds.



The present studies clearly demonstrated that SPE is distinctly different from the substance P degrading activities described so far and furthermore suggest that this bacitracin-sensitive endopeptidase is a good candidate likely to be important in the synaptic inactivation of substance P and/or other neuropeptides. However, since the physiological function of neuropeptide degrading enzymes appears to be primarily determined by their specific localisation, only immunocytochemical investigations at the electron-microscope level will finally answer the question as to the physiological function of this enzyme.

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IN SITU HYBRIDIZATION VERSUS NORTHERN ANALYSIS:

WORKING TOWARDS THE CORRELATION OF TWO QUANTITATIVE TECHNIQUES FOR OPIOID AND VASOPRESSIN mRNAs IN THE RAT HYPOTHALAMUS AND PITUITARY

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ABSTRACT

Several physical and technical aspects of in situ hybridization are examined to help ascertain to what extent the total specific mRNA population of interest in a tissue section is actually detected using complementary RNA probes. Two experimental paradigms, Rot curve hybridization analysis and varying the tissue section thickness, indicate that complementary RNA (cRNA) probe hybridization to a specific mRNA in a tissue section can be made saturable, but remains so only for those mRNAs within a limited distance from the surface of the section. Direct comparison with Northern analysis, however, shows that in situ hybridization still gives comparable relative quantitative results for mRNA levels changing due to physiological stimulation.

INTRODUCTION

The application of in situ hybridization for the autoradiographic identification of cellular mRNAs has gained general acceptance as a useful tool in studies of the anatomical localization of specific gene products. If this qualitative information could be extended to either the absolute or relative quantitation of specific mRNA transcripts, then in situ hybridization could be an even more useful tool for the regulation of gene expression in tissues possessing extreme cellular heterogeneity, such as the brain. Towards this end, several technical aspects of in situ hybridization, including probe hybridization kinetics, tissue section thickness, probe length and photographic emulsion preparation are being further characterized in hopes that such an understanding will aid in the application of in situ hybridization as a quantitative method. In this brief manuscript, we will discuss the application of [32P]-labeled cRNA probes, generated with the pSP6 vector system (Promega Biotec), for in situ hybridization studies of pro-opiomelanocortin (POMC) mRNA in the intermediate lobe of the rat pituitary, or a [32P]-labeled synthetic oligonucleotide complementary to rat vasopressin mRNA in the rat magnocellular hypothalamus.

MATERIALS AND METHODS

Many of the in situ hybridization methods presented in this manuscript have been extensively described elsewhere (Brahic and Haase 1978; Cox et al. 1984; Lewis et al. 1985; Sherman et al. in

press; Watson et al. in press; Watson et al. 1985). Northern gel analysis was conducted as previously described by Sherman et al. (1986).

RESULTS AND DISCUSSION

The kinetics of cRNA hybridization to mRNA immobilized in a tissue section, as is the case with in situ hybridization, more closely approximates the situation existing with Northern or dot-blot analyses than with classical solution-phase hybridization. The laws of nucleic acid hybridization kinetics tell us that the rate of duplex formation of excess cRNA probe to its complementary mRNA in an immobile phase should follow pseudo first-order kinetics as described by equation 1 (Young and Anderson 1985):

$$H = D_o \ (1 - e^{-kRoT}) \qquad \text{Equation 1}$$

where H is the amount of duplex RNA formed, [Do] is the initial amount of mRNA in the immobile phase, [Ro] is the initial amount of cRNA probe in the mobile phase, T is time and k is a constant possessing the units of 1/(sec-moles/liter). Practical application of this equation takes the form of the so-called Rot curve, where percent or amount of hybridization is plotted against the product of probe concentration and time of hybridization (Ro x t = Rot). That this can be shown to hold approximately true for in situ hybridization is shown in figure 1, in which varying concentrations of a mouse POMC cRNA probe are hybridized for varying lengths of time against rat POMC mRNA in the intermediate lobe of the pituitary.

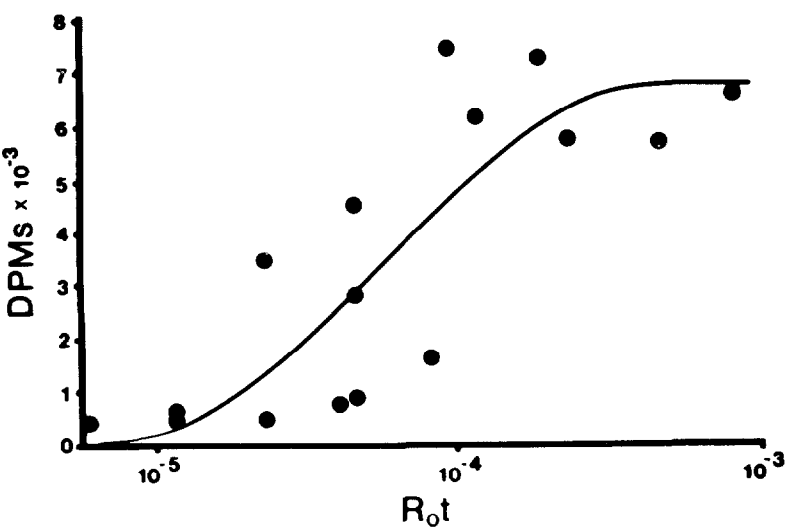

Figure 1. Rot curve for POMC cRNA hybridization to rat pituitary intermediate lobe. To each 10 micron section was added 250,000 to 5,000,000 dpms of [32P]-labeled cRNA probe (800 nucleotides). Hybridizations were conducted for 2 to 7 days.

In its simplest terms, figure 1 demonstrates that the extent of [32P]-cRNA:mRNA duplex formed increases with time or [32P]-cRNA concentration, and that with sufficient amount of either or both of these, the hybridization is saturable.

While it is good to know that in situ hybridization follows orderly kinetics, and is, to a limited extent, predictable, the situation is more complicated than it appears. It is more accurate to conclude from figure 1 that the POMC mRNA accessible by the cRNA probe is saturable; for recent preliminary experiments suggest that only the POMC mRNA localized in the top 2-4 microns of the tissue section is available for hybridization. Probe hybridization to pituitary sections ranging in thickness from 4.5 to 20 microns resulted in specific signals all lying within the same (relatively large) standard error. Thicker sections clearly did not give appreciably greater signal than much thinner sections. That this results from an effective limit of probe penetrance is a likely explanation.

Although in situ hybridization, as of this stage, remains non-quantitative in absolute terms, it still may be useful for relative quantitation studies where many uncharacterized sampling problems essentially cancel each other out of the results. Even Northern gel analysis remains at this level, since neither the efficiencies of RNA transfer from gel to membrane nor the hybridization to these immobilized RNAs are quantitative.

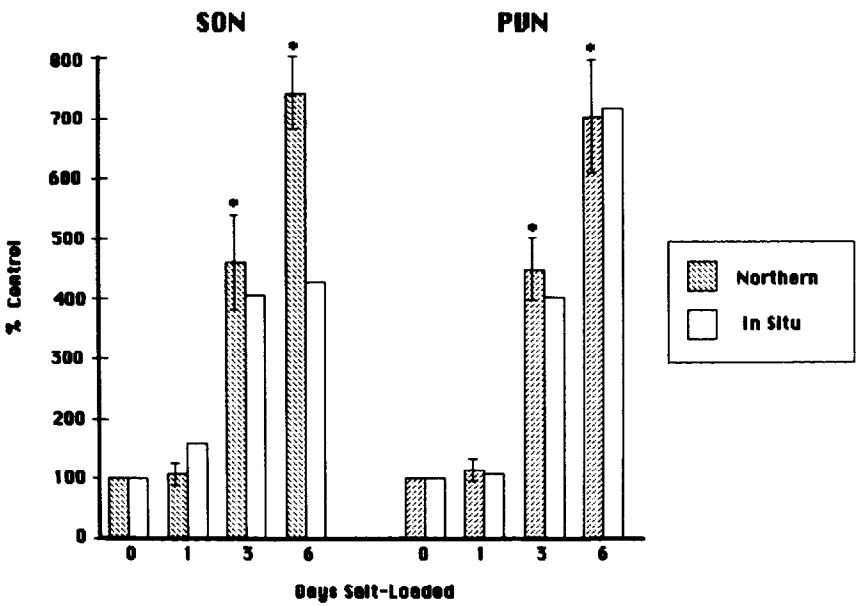

Figure 2. Correlation between Northern and in situ hybridiza tion quantitation of salt-loading induced increases in vasopressin mRNA in the supraoptic and paraventricular nuclei. The Northern gel data is published in Sherman et al. (1986). The in situ data rep resents average values from 2-4 sections for each nuclei. *$p \leq 0.005$.

The analysis is effective, nonetheless, because each sample suffers the same disadvantages experienced by all samples. This situation may, in some situations, be true for in situ hybridization analysis also. Figure 2 depicts the results of an experiment comparing the relative quantitation of vasopressin mRNA in hypothalamic magnocellular nuclei by either Northern gel or in situ hybridization using the same synthetic oligonucleotide probe (Sherman et al. 1986). The induction of cytoplasmic vasopressin mRNA occurring with chronic salt-loading, as detected by Northern gel analysis, is similarly detected by in situ hybridization. Both Northern hybridization bands and in situ positive nuclei were quantitated with a digital image analysis system using [32P] autoradiographic standards. At a first approximation, therefore, it appears the two results give comparable results. A similar comparison was made in the quantitation of POMC mRNA in the intermediate lobe of the pituitary during chronic haloperidol treatment (Kelsey et al. 1986). Clearly, however, there remains the question of tissue section sampling as a source of great error with in situ hybridization, which is not a problem with Northern analysis.

In conclusion, we have shown that in situ hybridization follows orderly hybridization kinetics, and can be a useful tool for the relative quantitation of cellular mRNAs in some circumstances. We feel, that with additional characterization and optimization (including the development of good autoradiographic standards), the capability of accurate, absolute, quantitation exists.

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IMMUNOHISTOCHEMICAL EVIDENCE FOR SUBPOPULATIONS OF DYNORPHINERGIC NEURONS

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ABSTRACT

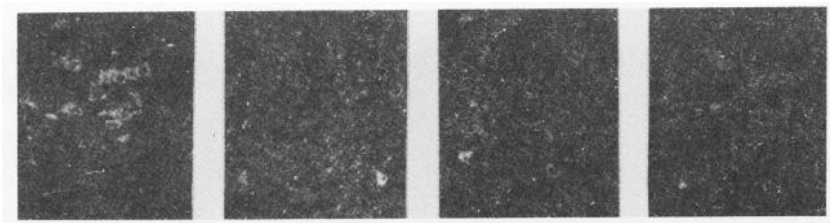
Biochemical studies have shown that the dynorphin precursor can generate at least seven opioid peptides. We report here a study of dynorphin products analyzed on adjacent semithin sections of rat brain, showing evidence for differential peptide localization among magnocellular neurons. In the oxytocinergic nucleus of the anterior commissure, staining was strong for dynorphin B but very weak for other prodynorphin markers; in the supraoptic nucleus, a discrete subset of dynorphin neurons were positive for leu-enkephalin.

INTRODUCTION

Several recent studies have suggested regional heterogeneity in the immunohistochemical localization of products from the POMC (Bloch et al. 1979; McGinty and Bloom 1983) and proenkephalin precursors (Williams and Dockray 1983.) Heterogeneity of hypothalamic magnocellular neurons has been reported for oxytocin/CRF expression (Sawchenko et al. 1984) and for vasopressin in the supraoptic nucleus (Richards et al. 1985.) When studied by RIA, the processing of the dynorphin precursor varies markedly among brain regions (Weber et al. 1982b); however, immunohistochemical heterogeneity has not been reported. Therefore, we have examined hypothalamic magnocellular dynorphin neurons for differential immunohistochemical staining of prodynorphin products.

MATERIALS AND METHODS

Male Sprague-Dawley rats were colchicine-treated (50 ug icv) 48 hrs before perfusion with 4% paraformaldehyde, and brains were immersed 24 hrs in 20% sucrose/3% PEG MW 400 before freezing in Cl_2F_2 Me (chilled in a liquid nitrogen bath). Serial sections were cut at 1-2 u in a -25° C cryostat. Sections were stained as previously described at 1:400-1:800 (Weber and Barchas 1983) and visualized with fluoroscein-labeled second antibodies. Antisera were raised in our laboratory and characterized previously: dynorphin A (Dyn A R2-4), Dyn A(1-8) (R2-2), Dyn B (R2-4), alpha-neo-endorphin (a-NEp R2-4), leu-enkephalin (L-Enk R1-3) and met-enkephalin-arg-gly-leu (MERGL R2-2). In each case staining was blocked by preincubation with 10-20 uM of the homologous peptide, but not by the other peptides.



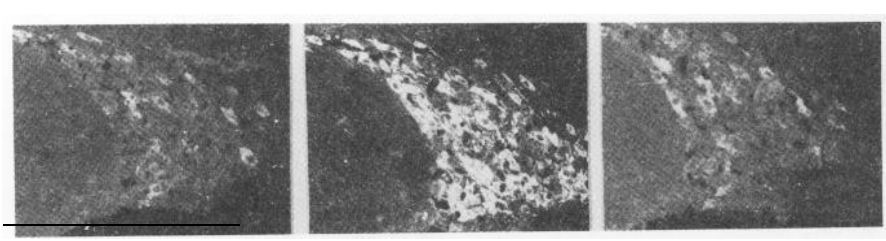
Dyn B Dyn A Dyn A(1-8) a - NEp

Figure 1. Serial sections, nucleus of the anterior commissure.

RESULTS AND DISCUSSION

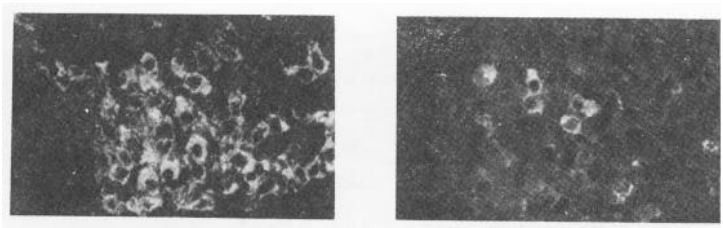
We have previously colocalized oxytocin and Dyn B in the nucleus of the anterior commissure (ACN; Quinn and Weber 1985.) As shown in figure 1, there was only weak staining in these cells for Dyn A, Dyn A(1-8), and a-NEp. Blocking controls suggest relative C-terminal specificity for these antisera. McGinty (1985) has reported cortical neurons staining with Dyn B(R2-4); after pretreatment with cholinesterase, other Dyn antisera produced staining in her preparations (J. McGinty pers. comm.) Dyn B (R2-4) may thus recognize its epitope in perikarya where other Dyn products are uncleaved.

In the paraventricular nucleus (PVN), the four Dyn antisera produced staining differing somewhat in intensity but consistent across PVN magnocellular neurons for each antiserum. In contrast, in the supraoptic nucleus (SON), isolated groups of neurons had sharply reduced staining for a-NEp, despite consistent staining with the other antisera (figure 2.) The same weak/strong aNEp cells could be followed on several adjacent sections, and this finding was similar in all animals studied. This result could reflect either differential processing of a-NEp among SON neurons, or possibly, different absolute precursor expression, to which this antisera may have been more sensitive. In support of the former hypothesis, dilution of Dyn A(1-8) antiserum to extinction (1:6400) never produced a pattern similar to a-NEp (data not shown.)



a - NEp Dyn A(1-8) a - NEp

Figure 2. Serial sections, supraoptic nucleus



Dyn A (1-8) Leu - Enk

Figure 3. Adjacent sections, supraoptic nucleus.

In the SON, isolated Dyn-positive neurons stained brightly for Leu-Enk, against a background of other Dyn neurons with no detectable Leu-Enk staining (figure 3); the majority of SON Dyn occurs in vasopressin neurons (Watson et al. 1982a; our obs.) Consistent with earlier work (Weber et al. 1982a; Watson et al. 1982b; Williams and Dockray 1983) we saw no MERGL staining in this study in the SON. Production of Leu-Enk from Dyn has been shown by RIA/lesion studies by Zamir et al. (1984.) We saw only scattered Leu-Enk-positive neurons in this nucleus, and in only about half of these colchicine-treated animals. However, in noncolchicine-treated rats, Martin and Voigt (1982) have reported a subset of Leu-Enk positive terminals in the vasopressin fibers of the neurohypophysis, while all vasopressin terminals were Leu-Enk positive after trypsin pretreatment. Our results further support a process for Leu-Enk production in a specific subset of the vasopressin/dynorphin pathway.

Heterogeneity of neuropeptide expression in the magnocellular system has been documented several times. Oxytocin neurons express CRF in the ACN, sometimes in the PVN, and occasionally in the SON (Sawchenko et al. 1984.) All ACN, but only rare SON oxytocin neurons contain Dyn (Quinn and Weber 1985.) Interestingly, in the mapping of several antisera to regions of the brainspecific precursor 1B236 Bloom et al. (1985) reported similar staining in almost all brain regions except the magnocellular system, where only the C-terminal immunogen (P7) was reactive; this finding resembles our observation for Dyn in the ACN. McCabe et al. (1986) found evidence of SON subgroups with differential vasopressin mRNA response, evaluated <u>in situ</u> under dehydration stress. Opioid peptides are thought of as co-transmitters with potential modulatory effects on terminals at sites of release (Pittman et. al. 1983.) Possibly, the subgroups reported here allow release of oxytocin/vasopressin with alternate complements of opioids, if these subgroups have distinct afferent pathways such as for norepinephrine (Sladek and Zimmerman 1982.) Alternatively, these opioid subgroups could reflect functional groups of oxytocin/vasopressin neurons selectively colocalized with yet-undescribed neuropeptides.

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CO-EXISTENCE OF PRODYNORPHIN - OPIOID PEPTIDES AND SUBSTANCE P IN PRIMARY SENSORY AFFERENTS OF GUINEA-PIGS

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ABSTRACT

Light microscopic immunoenzymatic and immunofluorescence histochemistry revealed co-existence of opiofd peptfdes with substance P in primary sensory neurons of all segmental dorsal root and trigainal ganglia of guinea-pig. Sensory opioid peptides appeared to be exclusively processed from prodynorphin and include [leu]enkephalin, neoendorphlns, and dynorphin A. We suggest the presence of presynaptfc opfoid autoreceptors in prlmary sensory afferences.

INTRODUCTION

It is a widespread concept that enkephalins in terminals of the dorsal horn presynaptically inhibit the release of substance P (SP) from spinal endings of primary sensory afferences although the neuroanatomical evidence for such a synaptic circuitry is missing (Basbaum and Fields 1984; Jessel 1983; Kosterlitz and NcKnight 1981). It was concluded that there is no primary sensory origin of spinal opfoid peptfdes (Botticelli et al. 1981; Vincent et al. 1984). Contrary to this view are immunohistochemical investigations with cultured mouse dorsal root ganglia demonstrating a few dynorphin-immunoreactive (ir) ganglfonic cells (Sweetnam et al. 1982). Also in neonatal rats very few enkephalfn ir small ganglfonic cells were found (Senba et al. 1982). First morphological evidence for a general opfofdergic primary sensory pathway in adult animals was obtained in an immunohistochemical study with guinea-pigs (Weihe et al. 1985a). Measurements by radioimmunoassay indicated the presence of low amounts of ir dynorphin and ir α-neoendorphin in human spinal ganglia (Przewlocki 1983). The main focus of this study was to investigate the posslble co-existence of opioid peptides, apparently exclusively derived from prodynorphin (Weihe et al. 1985a), with substance P in primary sensory afferences.

MATERIALS AND METHODS

Six male guinea-pigs of 200-250 g were deeply anaesthetized with Nembutal (i.p.) and fixed by perfusing 500 ml Bouin's solution retrogradcly through the distal abdominal aorta. Both trigeminal ganglia, the entire spinal cord and the medulla, the segmental cervical, thoracfc and lumbosacral ganglia, peripheral somatic nerves, somatic (e.g. skin) and visceral (e.g. heart) tissues were dehydrated in propan-2-ol, and embedded in paraplast. 4-6 µ thick serial sections were Incubated with the primary antisera at room temperature. Several rabbit polyclonal primary antisera directed against a variety of proenkephrlin-opiofd peptides ([Met]enkephalin,

heptapeptide, octapeptide, BAM 12P, metorphamide), against various prodynorphin-opioid peptides i.e. dynorphln A (1-8), dynorphin A (1-17), dynorphln B, α/ß-neoendorphin, and against [Leu]enkephalin were used as previously described (Weihe et al. 1985a). Additional polyclonal antisera were against dynorphln A (1-179, α-neoendorphin (Millan et al. 19869, amidorphin (Seizinger et al. 19859. A monoclonal antlbody against enkephalln which was found to stain the pentapeptides non-differentially (Cuello et al. 19849 but not dynorphln and neoendorphins or larger molecular forms of proenkephalin- or prodynorphin-opiolid peptides was also used. SP was immunoreacted with commercial monoclonal antlbody NCl/34HL. Specifities of antisera were tested by preincubating them at 37°C for 2 hours or more with 100 nmol homologous or heterologous peptides per ml working dilutions. Biotlnylated species-specific secondary antisera and Streptavidin- peroxldase-complexes were used in 1:50 respectively 1:200 dilutions. The diaminobentidine reaction was as described (Weihe et al. 1985a). Co-existence and co-distribution of ir material was preferentially determined using paired consecutive sections alternately stained immunoenzymatically with the antibody against SP vs. the various antisera against different opioid peptides. In order to assess co-ex istence on Identical sections the monoclonal SP antlbody and the different polyclonal and monoclonal antisera against opioid peptldes were applied slmultaneously and visualized by consecutive immunofluorescence double-staining using either Fluorescein-Streptavidin or Texas-Red-Streptavidin.

RESULTS AND DISCUSSION

Out of all opioid antisera Investigated that against [Leu]enkephalin produced the strongest immunoreactions in numerous small and some medium-sized dorsal root and trigeminal ganglionic cells and their processes even In dilutions up to 1:80000. Our novel preabsorption studies Indicated that It was less specific In immunohistochemistry than it could be expected from previous radioimmunoassay data (Weber et al. 19839. It crossreacted completely with C-terminal extenstons of [Leu]enkephalin including the neoendorphfns and with dynorphln A (1-13) and (1-17). There was less crossreaction with [Met]enkephalin, the hepta- and the octapeptide. BAM 12 P had a lower blocking potency than the shorter fragments. Contrary to the immunohistochemically relatively unspecific [leu]enkephalin antiserum those against dynorphln A (1-17) and α'-neoendorphln were highly specific and neither crossreacted with proenkephalin peptides nor with [Leu]enkephalin, dynorphln A (1-6), (1-7), (1-8), (1-13) but were fully preabsorbed with low concentrations (1-10 nmol) of homologous peptides. Equivocal results were obtained with higher molecular weight forms containing the dynorphin A (1-179 or neoendorphin sequences. In spite of the partial crossreactivity of the [Leu]enkephalin antiserum with [Met]enkephalin containing proenkephalin-sequencer we argue that the ir material In sensory ganglia visualized with this antiserum does not appear to be proenkephalln-derived because none of the numerous antisera against proenkaphalin-peptides revealed ir material In sensory ganglia. Since the monoclonal antibody against enkephalln was found to stain sensory gangllonlc cells and fibers we conclude that the pentapeptlde [Leu]enkephalin itself is processed which is in progress to be

substantiated by other methods as used for the identification of putative sensory opioid peptides in skin, heart and peripheral nerves (Weihe et al. 1983; 1985a,b).

Out of the antisera against prodynorphin opioid peptides we selected the most effective (against [leu]enkephalin, dynorphin A 1-17 and α-neoendorphin) for the evaluation of co-existence. Both, the immunoenzymatic and the immunofluorescence detection demonstrated co-existence of ir opioid peptides with ir SP in all segmental dorsal root and trigeminal ganglia. It appeared that a major population of small ganglionic cells which contained ir opioid peptides exhibited also SP immunoreactivity. Opioid-antisera did not crossreact with SP and the SP antibody was not preabsorbed with any of the opioid peptides. There was a substantial population of non-opioid but SP ir ganglionic cells. Preliminary morphometric data indicate that the cell diameters of the non-opioid SP ir cells are large. Assessment of the variations in the number of ir cells depending on the segmental level of ganglia or even on a non-random distribution pattern within individual ganglia (Price 1985) are needed for precise quantification of opioidergic SP-ergic primary sensory neurons. The co-existence of opioid- and SP-ir material in primary sensory ganglionic cells and their juxtaperikaryal processes was paralleled by co-distribution in small diameter fibers (C, Aδ) of dorsal horn, dorsal roots, Litsauer tract, peripheral somatic and visceral nerves, and of vascular and non-vascular nerves of many somatic (e.g. skin) and visceral (e.g. heart) tissues. Preliminary data indicate that the ir opioid material not unexpectedly was also co-existing with calcitonin gene related peptide (CGRP), a recently identified co-transmitter candidate of SP in primary sensory neurons (Skofitsch and Jacobowitz 19859. Further co-existence patterns of opioid and non-opioid peptides can be expected based on the current knowledge of the neurochemistry of primary sensory neurons (Jessel 1983).

We suggest that the well known presynaptic opioid receptors on spinal endings of primary sensory afferences are, at least in part, autoreceptors and not heteroreceptors as generally believed. Interestingly opioid receptors seem to be present also on perikarya of sensory ganglionic cells (Ninkovic and Hunt 1985; Werz and Macdonald 1984) which may be the target of opioidergic fibers occasionally observed in this study surrounding non-opioidergic sensory ganglionic cells. Primary sensory opioid peptides, to our opinion, are exclusively processed from prodynorphin and include [leu]enkephalin. All may inhibit the release of co-transmitters like SP or other peptides (e.g. CGRP) or non-peptide substances not only centrally but also peripherally (Dale's principle) by acting on mainly kappa, but also some delta, presynaptic autoreceptors, and perhaps, also on postsynaptic heteroreceptors. Experimental evidence for opioid receptors being functionally important in mechanisms of reflex vasodilation and extravasation has been obtained recently (Smith and Buchan 1984). The main function of primary sensory prodynorphin opioid peptides (Weihe 1985; Weihe et al. 1985a) may be antinociceptive as suggested for spinal segmental or ascending/descending opioid systems (Cruz and Basbaua 1985; Han and Xie 1984; Herman and Goldstein 1984; Jessel 1983; Kosterlitz and McKnight 1981; Millan et al. 1986). It will be interesting to see whether there are also trophic functions of sensory opioid peptides.

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DISTRIBUTION OF ENKEPHALIN-LIKE IMMUNOREACTIVITY IN THE FELINE HEART; ITS INTERRELATION WITH SUBSTANCE P- AND VASOACTIVE INTESTINAL POLYPEPTIDE-LIKE IMMUNOREACTIVE NERVE FIBERS

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ABSTRACT

Distribution of enkephalin (Enk)-like immunoreactivity in the feline heart was investigated by indirect immunofluorescence method. Enk-like immunoreactive (Enk-IR) nerve fibers and cells predominated in the atria but were rare in the ventricles. Enk-IR cells were mainly distributed near sinoatrial (SA) node and between aorta and pulmonary artery. These cells were usually grouped in clusters near blood vessels or cardiac ganglia. Some Enk-IR cells appeared to extend their processes to other Enk-IR cells, cardiac ganglia, blood vessels and rarely cardiac muscles in the atria. Furthermore, substance P (SP)- and vasoactive intestinal polypeptide (VIP)-IR nerve fibers were closely associated with some Enk-IR cells. Moreover, the distribution and morphological profiles of Enk-IR cells were very similar to those of catecholamine (CA)-containing cells.

INTRODUCTION

Recent radioimmunological and immunohistochemical studies have revealed the wide, but uneven distribution of several neuropeptides in the central and peripheral nervous system. In the heart, the occurrences of neuropeptides were reported by several investigators using radioimmunoassay or immunohistochemical method. Biochemical studies of Lang et al. (1983) detected the existence of methionine (Met)- and leucine (Leu)-Enk-like immunoreactivity in guinea pig heart. Weihe et al. (1983) characterized opioid peptides in guinea pig heart. Lundberg et al. (1983) reported that Enk-IR nerve fibers were localized in vagal nerve. Little is known, however, on the detailed distribution of Enk-like immunoreactivity in the heart. In this study, we elucidated the distribution of Enk-like immunoreactivity in the feline heart and examined the interrelation between Enk-IR cells and SP- or VIP-IR nerve fibers. Furthermore, we presented the possible coexistence of Enk and CA in the heart.

MATERIALS AND METHODS

Immunohistochemistry: Young cats, weighing 2.5-3.0 kg, were used in this study. Animals were anesthetized and perfused through the left

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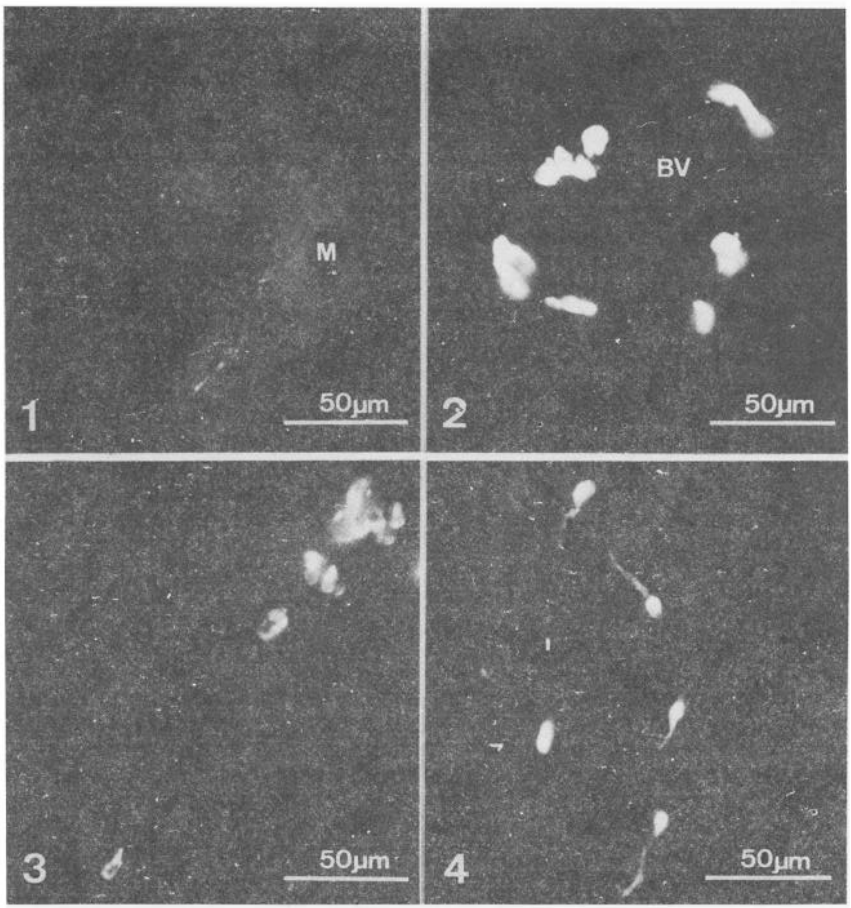
cardiac ventricle with ice-cold Zamboni fixative. Whole heart was carefully removed, separated cardiac atria under dissecting microscope and stored in the same fixative at 4°C overnight. Specimens were then frozen in isopentane cooled by the liquid nitrogen and serial frozen sections were cut at the thickness of 20-30 um with a cryostat. Indirect immunofluorescence method was applied to the sections. Sections were first incubated either with rabbit anti Met-Enk serum Ucb, diluted 1:1600), rabbit anti Leu-Enk serum (Ucb, 1:1600), monoclonal rat anti SP serum (Sera Lab, 1;1000) or rabbit anti VIP serum (INC, 1:1000) at room temperature overnight in a humid atomosphere. After rinsing several times, sections were then incubated in FITC-conjugated anti rabbit IgG (Miles, 1:1000), or FITC-conjugated anti rat IgG (Cappel, 1:1000) for 60 min at 37°C. For double immunostaining of Enk and SP, sections were first incubated with the mixture of rabbit anti Enk serum (final dilution 1:1600) and rat monoclonal anti SP serum (final dilution 1:1000) at room temperature overnight, rinsed several times and then incubated with the mixture of RITC-conjugated anti rabbit IgG (Cappel, final dilution 1:1000) and FITC-conjugated anti rat IgG (final dilution 1:1000). Sections were viewed with fluorescence microscope using appropriate exciting filter. After photography, sections were stained with hematoxylen-eosin to determine the structure of the tissue.

Catecholamine histochemistry: For demonstration of CA, glyoxylic acid-induced fluorescence method of Imai et al. (1982) was applied. Cats were killed by bleeding and hearts were removed immediately. Cardiac atria were cut into small pieces and fixed in FA-GLU-PA-GA-S solution (4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid, 2% glyoxylic acid and 15% sucrose in 0.1M phosphate buffer, pH 7.3) for 2 hours at 4°C. The specimens were then frozen and cut at the thickness of 20- 30 um with a cryostat. Sections were immersed in FA-GA-S solution (4% paraformaldehyde, 2% glyoxylic acid and 15% sucrose in 0.1M phosphate buffer, pH 7.3), air-dried for 60 min at 45°C, sealed with xylene and viewed with fluorescence microscope.

RESULTS

As the distribution of Met-Enk-IR and Leu-Enk-IR structures was very similar, we simply described as "Enk-IR." Enk-IR structures predominated in the atria but were rare in the ventricles. In the atria, many Enk-IR cells were localized near SA node and between aorta and pulmonary artery. Enk-IR cells were also distributed between coronary sinus and pulmonary vein and near inferior vena cava. Several thin Enk-IR nerve fibers with varicosities were observed in the atrial muscles (fig. 1), along the blood vessels and near the ganglionic cells. Enk-IR cells were distributed in clusters or, in some cases, in single cells. Many Enk-IR cells were localized at the walls of small blood vessels (fig. 2) and near the ganglia (fig. 3). Some Enk-IR cells were observed to extend their processes toward other Enk-IR cells, blood vessels, ganglionic cells and rarely cardiac muscles. Double immunostaining sections revealed that many SP-IR nerve fibers



FIGURES. 1-3: Immunofluorescence micrographs of Enk-like immunoreactivity.

FIGURE 1: Thin varicose Met-Enk-IR nerve fibers are observed in the atrial muscles (M) near SA node.

FIGURE 2: Several Leu-Enk-IR cells are localized at the walls of small blood vessels (BV) between aorta and pulmonary artery. **FIGURE 3:** Met-Enk-IR cells are present over the ganglion near SA node.

FIGURE 4: Histofluorescence micrograph between aorta and pulmonary artery. Several CA-containing cells are observed near the blood vessels. Some CA-containing cells extend their processes.

with varicosities were observed around the Enk-IR cells. Adjacent sections incubated with anti VIP serum showed that many varicose VIP-IR nerve fibers formed dense network over the Enk-IR cells. The distribution and morphological profiles of CA-containing cells were very similar to those of Enk-IR cells (fig. 4). Moreover, CA-containing nerve fibers were observed in close association with some CA-containing cells.

DISCUSSION

Our study demonstrated the detailed distribution of Enk-IR structures in the feline heart. Enk-IR nerve fibers with varicosities were present in cardiac muscles, at the walls of blood vessels and near the ganglionic cells. Some Enk-IR cells were observed to extend their processes to the ganglionic cells, blood vessels and rarely cardiac muscles. These findings suggest that Enk may directly control the cardiac functions. Recently Laurent et al; (1985) reported that Enk had a direct positive inotropic effect on cultured cardiac myocytes. Our present findings support their results. Moreover, our immuno-histochemical evidences that SP- and VIP-IR nerve fibers were closely associated with Enk-IR cells suggest that SP and VIP may regulate the effects of Enk in the heart. It was reported that Enk coexisted with CA in the chromaffin cells of adrenal gland and Enk might regulate the release of CA from the chromaffin cells. Although the coexistence of Enk and CA was not investigated in this study, it was shown that the distribution and morphological profiles of CA-containing cells were very similar to those of Enk-IR cells. Thus, our observation suggests that in the heart Enk-IR cells may also contain CA and regulate the release of CA.

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IMMUNOHISTOCHEMICAL AND SUBCELLULAR STUDIES OF AMINOPEPTIDASE M LOCALIZATION IN RAT BRAIN : MICROVESSELS AND SYNAPTIC MEMBRANES

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ABSTRACT

A predominantly microvascular localization of aminopeptidase M (APM) was established both by immunohistochemistry and biochemical studies of isolated microvessels. In addition a high relative specific activity of APM was evidenced in fractions enriched in synaptic membranes.

INTRODUCTION

APM (EC 3.4.11.2) which is sensitive to bestatin but relatively insensitive to puromycin has recently been identified in rat cerebral membranes (Schwartz et al. 1984 ; Gros et al. 1985). Its major role in the inactivation of endogenous enkephalins released from brain slices was demonstrated by the protecting effects of several inhibitors including antibodies directed against the active site of APM (Giros et al. 1986). Since the K_M values of enkephalins for APM and another cerebral aminopeptidase more sensitive to puromycin do not greatly differ, the preferential role of APM is likely to reflect topological rather than catalytic differences. We have therefore studied the localisation of APM in rat brain by immunohistochemical and subcellular fractionation methods.

MATERIAIS AND METHODS

Polyclonal antibodies (Ab) directed against an homogeneous preparation of rat kidney APM were obtained in rabbits (Gros et al. 1985).
Monoclonal Ab against "enkephalinase" (EC 3.4.24.11) were obtained after injecting to mice a preparation of rat kidney brush borders. The recognition of "enkephalinase" was assessed by immunoprecipitation of the enzyme activity (in preparation).
Immunolocalizations were established by incubating unfixed frontal sections of rat brain (or isolated microvessels) with either polyclonal anti APM Ab followed by antirabbit immunoglobulins (labelled with [^{125}I] or peroxidase) or [^{125}I] monoclonal anti-enkephelinase Ab.
Subcellular fractions were prepared and named according to Gray and Whittaker (1962).
Aminopeptidase activity was measured using 40 nM [^{3}H] (Met^{5})enkephalin as substrate. Incubations were for 10 min at 37°C in the presence of 1 µM Thiorphan and 20 µM puromycin, [^{3}H]Tyr being isolated by polystyrene bead column chromatography. APM activity was evaluated as the difference between values obtained in the presence and absence of APM antibodies.

RESULTS AND DISCUSSION

Immunostaining of rat brain sections (figure 1) indicates a rather uniform localization of APM within the brain parenchyma which contrasts with the highly heterogeneous localization of enkephalinase, the other major enkephalin-degrading peptidase.
In agreement, assay of APM activity in homogenates from several brain regions revealed a rather uniform distribution of the enzyme activity which represented approximately 1 % of the total aminopeptidase activity (not shown).

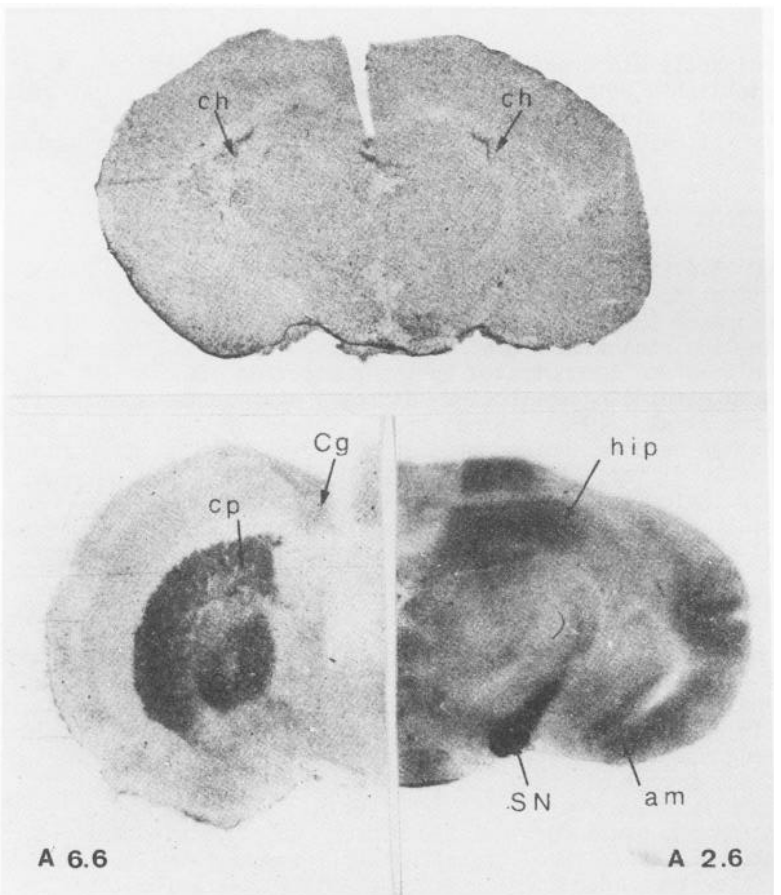

FIGURE 1: Autoradiography of APM (upper picture) and enkephalinase (lower pictures) immunoreactivities in frontaI sections of rat brain.
The [^{125}I] -Labelled enkephalinase Ab was obtained as described (H. Pollard, P. Ronco, P. Verroust and J-C. Schwartz submitted).
am : amygdala ; Cg : cingulate cortex ; Ch : choroid plexus ; cp : caudate putamen ; GP : globus pallidus ; hip : hippocampus ; SN : subtantia nigra

However a strong APM immunoreactivity was detected in the walls of vessels in all studied cerebral areas as well as within choroid plexuses such as those of the lateral ventricles (figures 1 and 2).

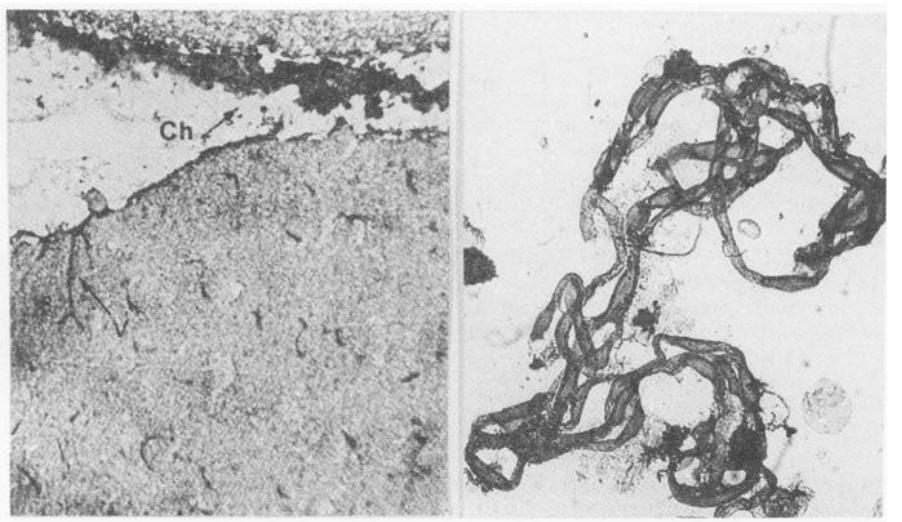

FIGURE 2: Immunoperoxidase staining of APM in a frontal section of rat brain (left) and in isolated microvessels (right).
Ch : choroid plexus ; v : vessel

The microvascular localisation of APM was confirmed after isolation of cortical microvessels by a sieving procedure. On this relatively pure fraction a dense immunostaining of all vascular elements was observed (figure 2). After solubilization of the isolated microvessels with Triton X-ICC, APM activity was clearly identified by establishing the potencies of various inhibitors.
APM antibodies inhibited about 80 % of the eminopeptidase activity of this fraction at a dilution (1/50,000) similar to that required to inhibit purified APM. Also the IC_{50} values of bestatin (2 µM), amastatin (5 µM) and puromycin (78 µM) were similar on the two preparations.
This major microvascular localization of APM suggests that the enzyme may participate in the inactivation of neuropeptides regulating the permeability and tone of cerebral vessels. Together with its presence in choroid plexuses, it also suggests that it participates in the enzymatic "blood-brain barrier" which prevents the access of circulating regulatory peptides into the CSF and brain extracellular fluids.

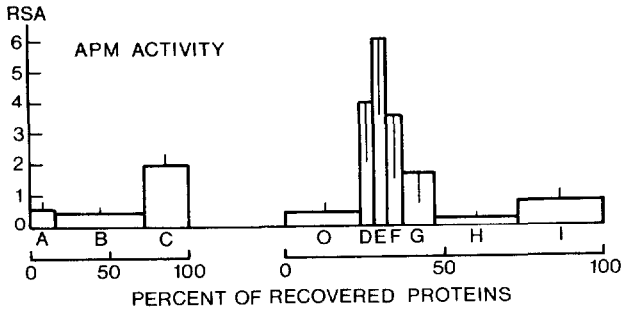

FIGURE 3: Subcellular distribution of APM into fractions from rat cerebral cortex.
Fractions were obtained after two different density gradient centrifugatiohs end designated according to Gray and Whittaker (1962). The relative specific activity (RSA) is the ratio percent of enzyme activity over percent of recovered proteins.

In primary subcellular fractions from cortical homogenates, a major portion (78 %) of APM was found to sediment with the heavy P_1 fraction containing microvessels whereas 7 % and 10 % sedimented with the P_2 and P_3 fractions respectively and no significant APM activity would be detected in soluble form. However upon density gradient analysis of the shocked synaptosomal fraction a clear enrichment (by about 5-fold as compared to the homogenate) was found in those subfractions containing synaptic membranes (figure 3). However it is difficult to exclude a contamination of these fractions by elements of the predominant vascular pool in brain.
In conclusion this study indicates a dual localization of APM in rat brain. The synaptic localization would be more consistent than the microvascular one with the major participation of APM in neuronal enkephalin inactivation.

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GAMMA-3-MSH AND B-ENDORPHIN IN MONKEY PITUITARY

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ABSTRACT

The content and extent of processing of gamma-3-MSH and B-endorphin were determined in the anterior (AL) and intermediate (IL) lobes of three monkey pituitaries. The peptides existed in equimolar amounts in each lobe. The processing of gamma-3-MSH paralleled that of B-endorphin in each lobe; the primary products in AL were B-lipotropin and a 9K form of gamma MSH, whereas in IL the predominant products were N-acetyl-B-endorphin and gamma-3-MSH.

INRODUCTION

Gamma-3-melanocyte stimulating hormone (MSH) is a 27-residue peptide found in the N-terminal region of proopiomelanocortin (POMC), containing the MSH core sequence His-Phe-Arg-Trp (Nakanishi et al. 1979). There are two major cleavage sites in the N-terminal domain of POMC, which potentially generate the extreme N-terminal peptide, gamma-3-MSH, and joining peptide, which is the segment found between gamma MSH and ACTH. Gamma-3-MSH thus can be found associated with joining peptide (molecular weight 8-10K, depending on the extent of glycosylation), the N-terminal fragment of POMC (molecular weight 10-12K), or alone (molecular weight 3-4K). Bovine and human (but not rat) gamma-3 MSH have an additional cleavage site that potentially generates the C-amidated II-residue peptide, gamma-1-MSH (Ling et al. 1979).

Gamma-3-MSH has been shown to exist in several forms in bovine (Tanaka et al. 1980; Shibasaki et al. 1980a, 1980b) and rat (Pedersen et al. 1982) AL and IL, as well as being present in whole human pituitary extract (Tanaka et al. 1980). To date, however, the nature of gamma-3-MSH in monkey pituitary has not been studied. In the present investigation we examined the content and degree of processing of gamma-3-MSH in monkey AL and IL, and compared these to the corresponding parameters for B-endorphin.

MATERIALS AND METHODS

Pituitaries

Three post-menopausal Rhesus monkeys (Macaca mulatta) were anesthetized and sacrificed. The pituitaries were immediately removed, placed on wet ice, and dissected into the AL and the IL. The IL was trimmed as closely as possible, but due to the anatomy of monkey pituitary, a clear separation between anterior/posterior and intermediate pituitary was not feasible, hence the IL had contaminating fragments of both anterior and posterior lobes. After dissection, the pituitaries were frozen on dry ice and stored at -70°C.

Extraction

The peptides were extracted from the pituitaries into acetone/0.1N HCl (3:1). The extract was dried by lyophilization, and the residue resuspended in 1.0% formic acid. For column chromatography, the formic acid extract was used. For radioimmunoassay, aliquots of formic acid extracts were dried and resuspended in the appropriate assay buffer.

Radioimmunoassays

B-endorphin and N-acetylated B-endorphin were assayed by previously established radioimmunoassays (Cahill et al. 1983a, Akil et al. 1981a). Gamma-3-MSH was measured using an assay recently developed. Samples were diluted in a 50 mM sodium phosphate buffer, pH 7.6 containing 0.12M NaCl, 0.004M EDTA, 0.016% NaN_3, 1.6% normal rabbit serum, and 0.1% polylysine. The assay was run at disequilibrium conditions for 72 hours at 4°C; the antiserum, raised in rabbits using synthetic bovine gamma-3-MSH conjugated to bovine serum albumin with bis-diazatized benzidine as an antigen, was used at a final dilution of 1/25000. The radioactive trace was human gamma-3-MSH, iodinated by the chloramine T method (Hunter and Greenwood, 1962). Separation of free from bound material was by the second antibody method, using sheep-anti-rabbit gamma-globulin. This assay has an intrassay CV of 9.2%, and an interassay CV of 5.0%. It has a useable range of 6-300 fmoles/tube, with an IC of 40 fmoles/tube. On a molar basis, its crossreactivity profile is: gamma-3-MSH = 1; Lys-gamma-1-MSH, $<10^{-3}$; gamma-1-MSH and gamma-2-MSH, $<10^{-4}$; a-MSH, human B-MSH, human B-endorphin, and ACTH, $<10^{-6}$.

Column Chromatography

Samples were applied to a sephadex G-50 column (1.5 x 90 cm) that had been precalibrated with molecular weight standards. Aliquots of each fraction were dried, resuspended in the appropriate buffer, and assayed for B-endorphin and gamma-3-MSH immunoreactivity. The fractions with immunoreactivity in the void volume of this column were pooled, and rechromotagraphed on a sephadex G-100 column (1.5 x 90 cm) to separate larger-sized fragments. The percentage of the total immunoreactivity found in each peak was calculated for each peptide.

RESULTS AND DISCUSSION

As shown in table 1, the immunoreactive gamma-3-MSH and B-endorphin concentrations were not significantly different fcr each pituitary lobe. In addition, much of the B-endorphin immunoreactivity in intermediate lobe was N-acetylated, whereas very little was in anterior lobe, similar to previous findings in rat pituitary (Akil et al. 1981b).

TABLE 1. Gamma-3-MSH and B-endorphin Content of Monkey Pituitary Lobes

Lobe	Peptide Content (pmoles/mg tissue)		
	gamma-3-MSH	B-endorphin	N-acetyl-B-endorphin
anterior	120 ± 33	91 ± 26	5 ± 3
intermediate	69 ± 20	98 ± 46	62 ± 52

The size distribution for B-endorphin and gamma-3-MSH immunoreactivity in each lobe are shown in table 2.

TABLE 2. Percent of Total B-endorphin and gamma-3-MSH Found In Each Peak

	PEAK			
	I (3-5K)	II (9-11K)	III (15-20K)	IV (Void)
Anterior lobe				
gamma-3-MSH	22 ± 10%	60 ± 7%	18 ± 3%	1 ± 1%
B-endorphin	37 ± 15%	54 ± 17%	8 ± 3%	1 ± 1%
Intermediate lobe				
gamma-3-MSH	48 ± 12%	35 ± 6%	16 ± 11%	1 ± 1%
B-endorphin	65 ± 15%	29 ± 14%	5 ± 3%	2 ± 1%

Peak I corresponds to 3-5 kdaltons, representing B-endorphin or gamma-3-MSH. Peak II is 9-11 kdaltons, and contains B-lipotropin or (tentatively) glycosylated gamma-3-MSH plus joining peptide. Peak III is 15-20 kdaltons, and in the case of gamma-3-MSH immunoreactivity probably represents the entire 16K N-terminal POMC fragment; the remaining 15K C-terminal piece may be the peak measured by the B-endorphin assay. Peak IV is the void volume of the G-100 column, presumably representing intact POMC. The processing for each peptide is quite similar, with a tendency toward the presence of smaller fragments in both lobes. The AL manifests predominately B-lipotropin-sized material when B-endorphin-immunoreactivity is measured, although a significant portion of the immunoreactivity is B-Endorphin-sized, as has been previously reported (Cahill et al. 1983b). Gamma-3-MSH-immunoreactivity in AL is found in a similar sized fraction which is tentatively gamma-3-MSH extended with joining peptide. The IL shows a pattern of smaller, more processed material

(B-endorphin or gamma-3-MSH-sized), and the B-endorphin is N-acetylated.

These findings are in agreement with previous studies on lower animals. Pedersen et al. (1982) demonstrated that gamma-3-MSH exists in 6K and 11K forms in rat AL and IL; the smaller form predominates in IL, whereas the larger form is more predominant in AL. Similar findings were reported by Shibasaki et al. (1980a) from their studies on bovine AL and IL. Fractions of 15K, 8K, and 4.5K daltons have been shown to have gamma-3-MSH immunoreactivity in bovine whole pituitary, but in humans the predominant form of gamma-3-MSH immunoreactivity is 9K daltons (Tanaka et al. 1980). The presence of gamma-3-MSH immunoreactivity in 16K, 9K, and 4K fractions observed in monkey pituitary is consistent with these previous reports in other species.

In summary, we have shown that gamma-3-MSH immunoreactivity is detectable in monkey AL and IL, is present in equimolar amounts with B-endorphin, and is processed into primarily 4K and 9K fragments, paralleling the processing of B-endorphin. The smaller fragment predominates in IL, while the 9K fraction is the primary product in AL, similar to the way B-endorphin is handled by the pituitary lobes. We are currently developing an assay for gamma-1-MSH to determine if gamma-MSH in monkey pituitary is processed to this smaller form.

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MULTIPLE OPIOID RECEPTOR SUBTYPES IN THE PITUITARY-ADRENAL AXIS: A CROSS-SPECIES STUDY.

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ABSTRACT

This study examines the multiple opioid receptor subtypes present in the pituitary-adrenal axis of several species using in vitro autoradiography. The monkey neural lobe was found to contain all three subtypes with a preponderance of kappa sites, while the rat and bovine neurohypophyses contained only kappa sites. In the adrenal, opioid receptor sites were found exclusively in the medulla and varied with species. Monkey adrenal contained predominantly delta sites with a relatively small number of kappa sites present, while bovine adrenal medulla had predominantly kappa sites and only a relatively small proportion of delta receptor sites. No specific opioid receptor binding could be detected in the rat adrenal. These results emphasize the importance of species-differences in understanding the role of opioids in neuroendocrine regulation.

INTRODUCTION

Several lines of evidence suggest that the endogenous opioid systems may modulate the release of pituitary hormones. Acute administration of mu opiates, for instance, has been reported to increase the release of ACTH, growth hormone, and prolactin while decreasing the release of luetinizing hormone and thyroid stimulating hormone (Martin et al. 1975, Bruni et al. 1977, Cicero et al. 1977). Kappa agonists, on the other hand, have been reported to antagonize the antidiuretic action of vasopressin (Leander, 1983) and modulate the release of oxytocin (Bicknell et al. 1985, Samson et al. 1985). While the neuroendocrine effects of the endogenous opioids are well established, the opioid receptor subtypes mediating these actions and their anatomical localization is not clear. To clarify this issue futher, this study has examined the opioid receptor subtypes found in the pituitary-adrenal axis across several species using in vitro receptor autoradiography.

MATERIALS AND METHODS

Pituitaries and adrenals of rats, monkeys, and cows were frozen, sectioned on a Bright cryostat (25 um) and thaw-mounted on precleaned and subbed microscope slides. The tissue sections were then dried

overnight at 4°C under reduced pressure in a glass desiccator and stored in a -80°C freezer. Immediately prior to using the tissue, the slide-mounted sections were gradually brought up to room temperature and incubated with various opioid ligands in 50 mM Tris buffer (pH 7.5 at 25°C). The tritiated opioids used were DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol, 57.5 Ci/mmol 20.0 - 0.6 nM) to label mu sites, DPDPE (D-Pen^{2}, D-Pen^{5}-enkephalin, 29 Ci/mmol, 30.0-0.9 nM) to label delta sites, and (-)bremazocine (41.4 Ci/mmol, 10.0 - 0.3 nM) to label kappa sites. As bremazocine does not selectively bind to kappa receptors, all [^{3}H]bremazocine binding was conducted in the presence of IGO nM DAGO and DPDPE in order to block the mu and delta sites. The slides were incubated for 60 minutes, drained, and washed in four consecutive 250 ml, 50 mM Tris washes (pH 7.6, 4°C). Slides incubated with [^{3}H]DAG0 and [^{3}H]DPDPE were given four 30 second washes, while those incubated with [^{3}H]bremazocine were given four 4 minute washes. Following the Tris washes, all slides were rinsed (2 seconds) in a 250 ml wash of distilled water (4°C) and quickly dried with a portable hair dryer set to cool. Nonspecific binding was evaluated by treating a parallel set of slides with the same concentration of [^{3}H]ligand with a 1 uM final concentration of unlabelled competitor: levorphanol to displace [^{3}H]DAG0, DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) to displace [^{3}H]DPDPE, and UM 1071 (MR 2034) to displace [^{3}H]bremazocine. Slides were then arranged in X-ray cassettes and apposed to tritium-sensitive LKB Ultrofilm for 179 days.

RESULTS AND DISCUSSION

Marked species differences were observed in the distribution of the opioid receptor subtypes in the pituitary and adrenal. In the pituitary, opioid receptor binding was found only in the neural lobe, but the subtypes present varied with species (table 1).

TABLE 1. MULTIPLE OPIOID RECEPTOR SUBTYPES IN PITUITARY AND ADRENAL

I. Pituitary (Neural Lobe)

	Rat	Monkey	Bovine
Kappa	++	+++	+++
M u	---	+	---
Delta	---	++	---

II. Adrenal

	Rat	Monkey	Bovine
Kappa	---	+	+++
M u	---	---	+
Delta	---	+++	+

(+++ = dense, ++ = moderate, and + = light labelling)

More specifically, the monkey neurohypophysis contained all three opioid receptor subtypes with a preponderance of kappa sites, while the rat and bovine pituitaries contained only kappa receptor sites. The lack of opioid receptors in the anterior lobe would suggest that the endogenous opioids do not act directly on the pituitary to moderate hormonal release, but most likely have their effects on the hypothalamus itself. While debate in the literature has centered around whether the opioid receptors present in the neural lobe are on pituicytes (Lightman et al. 1983, Bunn et al. 1985) or nerve terminals (Falke and Martin 1985), their localization within the neurohypophysis provides an anatomical basis for the modulation of neural lobe hormones, such as, vasopressin and oxytocin.

In the adrenal gland, both kappa and delta receptors were observed in the monkey and bovine medulla (table 1). The animals differed, however, in the relative proportions of these sites in the medulla. In the bovine adrenal, kappa sites were prominent with a relatively small proportion of mu and delta sites present, while in the monkey, delta sites were predominant, with a small proportion of kappa sites present. In contrast, no opioid receptor binding was observed in the rat adrenal owing, most likely, to the relative paucity of opioid receptor sites in this tissue. Illustrative examples of opioid receptor autoradiography in the monkey pituitary and adrenal are present below in figure 1.

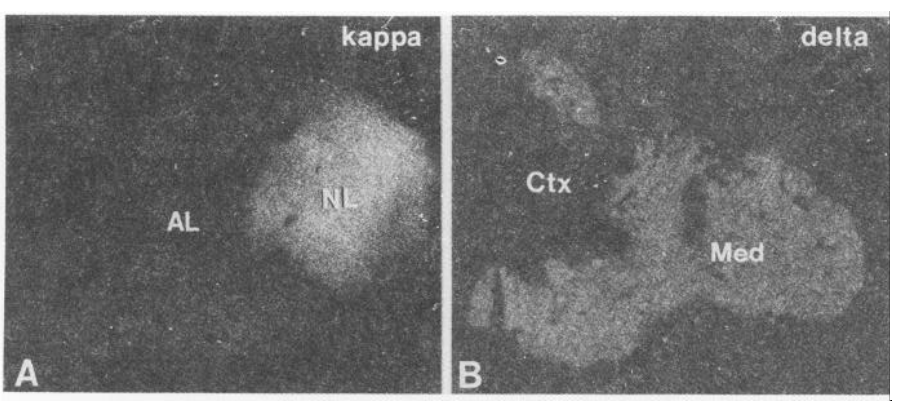

FIGURE 1. Darkfield autoradiograms of opioid receptors in the monkey pituitary (A) and adrenal (B). Note the labelling of kappa sites in the neural lobe of the pituitary and the delta sites in the adrenal medulla. Abbreviations include AL = anterior lobe, Ctx = cortex, Med = medulla and NL = neural lobe.

These results emphasize the importance of considering species-differences in understanding the role of opioid receptors in neuroendocrine regulation. In the adrenal, for instance, where the predominant receptor subtype varies with species, different opioid receptor subtypes may serve the same function in different animals. If catecholamine (CA) release is the functional endpoint, kappa receptors may be important in modulating CA release in bovine adrenal medulla (Dumont and Lemaire 1985), while delta receptors may mediate these actions in the monkey adrenal.

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AUTORADIOGRAPHIC LOCALIZATION OF OPIOID RECEPTOR TYPES IN THE RAT SMALL INTESTINE

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ABSTRACT

The selective μ and δ ligands [^{3}H]DAGO and [^{3}H]DPDPE have been used to investigate the distribution of specific opioid subtypes in the rat small intestine by in vitro autoradiography. There was a greater density of [^{3}H]DPDPE binding at regions of the villi and crypts than [^{3}H]DAGO binding. These results suggest that the opioid receptors located in these regions are predominantly of the δ subtype.

INTRODUCTION

It has been suggested that opioid peptides are important in regulating the transport of water and electrolytes across the rat small intestine (Warhurst et al. 1983). Recently, using in vitro autoradiography, we have demonstrated dense [^{3}H]naloxone and [^{3}H] dihydromorphine binding in proximal and distal regions of the rat small intestine (Dashwood et al. 1985). In the present autoradiographic study we have used [^{3}H]DAGO and [^{3}H]DPDPE in order to determine which opioid receptor subtype is most prominent in the villi and crypt regions of the small intestine of the rat.

MATERIALS AND METHODS

Radioligand binding studies

Sprague Dawley rats (male, 150-200 g) were obtained from Charles River. All radiolabelled ligands were obtained from Amersham. DAGO, (D-Ala^2-Mephe^4-Glyol^5)-enkephalin and DPDPE, (D-Peu^2,D-Peu^5)-enkephalin were obtained from Cambridge Research Biochemicals (UK). Rats were stunned and decapitated and the whole brain, minus cerebellum, was removed. Tissue was homogenised (Polytron Setting 5, 20 sec) in 50 vols Tris-HCl buffer (50 mM pH 7.7 at 25°C) and centrifuged for 20 min at 40,000 g. The resultant pellet was resuspended in 100 vols Tris buffer, incubated at 37°C for 45 min and centrifuged. The final pellet was suspended in 100 vols Tris buffer and used immediately. The membrane suspension was incubated for 45 min at 25°C in the presence of [^{3}H]ligand and either drug or buffer. The reaction was terminated by filtration under reduced pressure through Whatman GF/B filters and washed with buffer. Specific binding was that component displaced by 10 μM diprenorphine. Displacement data were analysed using the ALLFIT computer programme (DeLean et al. 1978). Saturation data were analysed using the Ligand programme (Munson and Rodbard, 1980).

In vitro autoradiography

Serial 20 µM longitudinal frozen sections of the rat proximal small intestine were taken and thaw mounted onto gelatinised microscope slides. These sections were then incubated in 4 nM [^{3}H]naloxone, [^{3}H]DPDPE and [^{3}H]DAGO (Amersham). Alternate sections were incubated in the presence of 1 µM unlabelled naloxone in order to determine the degree of binding to non-specific sites. The incubation conditions used were those described by Gillan and Kosterlitz (1982). After washing in buffer and drying, the sections were placed in 24 x 30 cm X-ray cassettes and apposed to LKB [^{3}H] Ultrofilm for 4-6 weeks at 4°C. Tritium standard scales (American Radiolabelled Chemicals Inc.) were included in each cassette. At the end of the exposure period the film was processed and areas of high [^{3}H]ligand binding were evident as accumulation of silver grains on the film. Photographs of autoradiographs were taken where appropriate and preliminary densitometric measurements were carried out on a Cambridge Quantimet 970 image analysis system.

RESULTS

Radioligand binding studies

Saturation analysis of [^{3}H]opioid binding revealed that both DAGO and DPDPE bound to one class of site with K_Ds of 1.7 ± 0.5 (n=4) and 1.6 ± 0.6 (n=0.4) nM respectively. Unlabelled DPDPE was a potent and selective inhibitor of [^{3}H]DPDPE. Whereas, as expected, DAGO potently and selectively interacted with the [^{3}H]DAGO binding site. Naloxone interacted with both sites but with some selectivity for the [^{3}H]DAGO binding site. Data on the δ selective antagonist ICI 174,864 is shown for comparison (Table 1). Thus, it can be concluded that, under the conditions used in the autoradiographic studies, DPDPE will label exclusively δ sites whereas DAGO will label exclusively µ sites. Naloxone, at the concentration used, although labelling a mixture of µ and δ sites will interact primarily with the µ population.

	K_I (nM)	
	µ site = [^{3}H]DAGO	δ site = [^{3}H]DPDPE
DPDPE	780 ± 40 (n=4)	1.3 ± 0.4 (n=5)
DAGO	1.7 ± 0.3 (n=4)	570 ± 80 (n=4)
Naloxone	1.8 ± 0.4 (n=3)	14.3 ± 0.5 (n=3)
ICI 174,864	> 10,000 (n=4)	62 ± 14 (n=4)

TABLE 1: Inhibition of [^{3}H]opioid binding

Autoradiography

On development of the autoradiographs there was a striking degree of [^{3}H]naloxone binding to the villi and crypts as reported previously (Dashwood et al. 1985). From the raw autoradiographic data the degree of [^{3}H]DPDPE binding to the intestine was far greater than [^{3}H]DAGO binding indicating that a higher population of δ opioid

receptors are present than μ receptors. In all cases [^{3}H]ligand was displaced when incubated with 1 μM naloxone (see Figure 1). Preliminary densitometric analysis was carried out whereby measurements of the grey levels of the autoradiographs were compared to densities of the tritium standard scales included with each cassette. These measurements showed that the rank order of opiate ligand binding to villi and crypt regions of the rat small intestine was [^{3}H]DPDPE > [^{3}H]naloxone > [^{3}H]DAGO where:

DPDPE = 225 fmol/mg protein
naloxone = 65 fmol/mg protein
DAGO = <34 fmol/mg protein

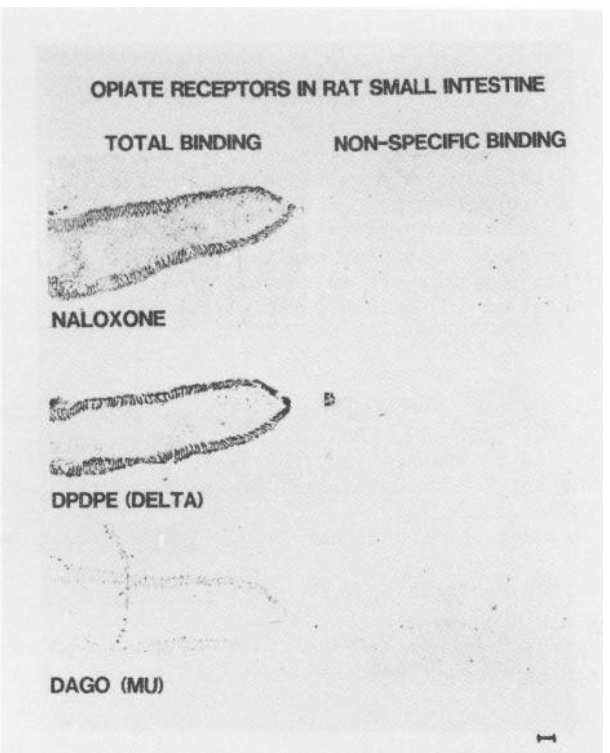

FIGURE 1. Binding of [^{3}H]naloxone, [^{3}H]DPDPE and [^{3}H]DAGO to longitudinal sections of rat small intestine (left). All [^{3}H]ligands were displaced when incubated in the presence of 1 μM unlabelled naloxone (right). Scale bar = 500 μm.

DISCUSSION

Opiates are known to exert profound effects on fluid and electrolyte transport across the epithelium of the small intestine (Dobbins et al. 1980; Hughes et al. 1982; Turnberg 1983). The possible location of the opioid receptors involved in the regulation of fluid transport has emerged from the demonstration of dense [^{3}H]naloxone and [^{3}H]dihydromorphine binding to the villi and crypt regions of the rat intestinal mucosa using an in vitro autoradiographic technique (Dashwood et al. 1985).

In the present study we have used [^{3}H]DAGO and [^{3}H]DPDPE, specific ligands for the μ and δ opioid receptors respectively in order to determine which subtypes are located in the villi and crypt regions of the rat small intestine. The results indicate a greater binding of [^{3}H]DPDPE (at least eight times greater) to the villi and crypts compared to [^{3}H]DAGO binding. These data are consistent with a previous study which suggested that the effects of opiates on intestinal fluid movement are mediated by the specific δ opiate receptor (Kachur et al. 1980). Recently we have demonstrated a marked increase in opioid receptor density in the villi and crypts of rat small intestine with streptozotocin-induced diabetes mellitus when compared to normal rats (Dashwood and Thompson 1986). Although the functional significance of the identification of opioid receptors in the small intestine awaits clarification it is suggested tentatively that the intestinal opioid receptors are capable of adaptation in certain pathological conditions.

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MU OPIOID RECEPTOR BINDING SITES IN HUMAN BRAIN

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ABSTRACT

Our experiments focused on the examination of the distribution of mu opioid receptor binding sites in normal human brain using the highly selective ligand [^{3}H]DAG0, in both membrane binding assay and in vitro receptor autoradiography. Mu opioid binding sites are very discretely distributed in human brain with high densities of sites found in the posterior amygdala, caudate, putamen, hypothalamus and certain cortical areas. Moreover the autoradiographic distribution of [^{3}H]DAG0 binding sites clearly reveals the discrete lamination (layers I and III-IV) of mu sites in cortical areas.

INTRODUCTION

Various investigators have reported on the presence of opioid receptor binding sites in human brain. For example, the gross regional distribution of mu and delta receptor subtypes and the predominance of kappa receptors have been shown (Kuhar et al.1973; Bonnet et al. 1981; Pfeiffer et al.1982). However, the characterization of these various opioid receptor types has not been studied in detail. As a first series of experiments, we investigated the distribution of mu opioid receptors in normal human brain using a highly selective ligand [^{3}H]D-Ala^2, (Me)Phe^4, Gly-ol^5 enkephalin (DAGO; Peterson et al. 1983) in both membrane binding assays and in vitro receptor autoradiography.

MATERIALS AND METHODS

Membrane binding assays were performed using normal human brains obtained at autopsy, frozen on dry ice and then kept at -80°C until used (Quirion 1985). Subsequently, punches (500-750 mg) were taken from various areas, homogenized in 50 mM Tris.HCl buffer, pH 7.4 at 4°C using a Brickman polytron (setting 5, 20 s). The homogenate was then centrifuged for 15 min at 49,000 x g. The supernatant was discarded and the pellet resuspended in buffer and homogenized as above. This preparation was then incubated at 37°C for 40 min and centrifuged for 15 min at 49,000 x g. The final pellet was washed twice with buffer and resuspended in buffer to a final concentration of 0.8 - 1 mg protein/ml (Lowry et al.1951). For binding assays, 0.1 ml of membrane preparation was added to 0.85 ml of 50 mM Tris.HCl buffer, pH 7.4 at 25°C, with various concentrations of [^{3}H]DAGQ (44.7 Ci/mmol, New England Nuclear) ranging between 0.05 - 1 nM in 0.05 ml, to a total incubation volume of 1,0 ml. After 60 min, incubations were terminated by rapid filtration through Whatman GF/B filter strips followed by 3 x 5 ml rinses using cold incubation buffer. Filter strips were pre-soaked in 0.05% polyethyleinine to reduce binding to filters. Specific binding was calculated as the dif-

ference in radioactivity bound in presence and absence of 5 µM cyclazocine. Binding was quantitated by counting filters in 6.0 ml Scinti-Verse II (Fisher Scientific Ltd., Canada) scintillation cocktail.

For receptor autoradiography, human brain slide-mounted sections were prepared as described before (Quirion 1985). Sections were then incubated for 60 min at room temperature in 50 mM Tris.HCl, pH 7.4 containing 1 nM [^{3}H]DAG0. Specific binding was determined as above. At the end of incubation, slides were rinsed for 5 min in cold incubation buffer, dipped in cold distilled water to remove salts, dried and then juxtaposed tightly against tritium-sensitive film (Ultrofilm, LKB Instruments). After 12 weeks, films were developed as previously described (Quirion et al.1981).

RESULTS AND DISCUSSION

As shown in table 1, mu opioid binding sites are discretely distributed in human brain. More specifically, high densities of binding sites are found in the posterior amygdala, caudate, putamen, various thalamic and hypothalamic nuclei (table 1). Much lower densities are present in various brainstem nuclei including the pontine, hypoglossal and trigeminal nuclei (table 1). In the cortex, Brodmann's areas #30, 24, 23, 22, 21, 20, 17, 9, 6 and 4 (in descending order) are especially enriched in mu opioid binding sites (table 1).

Preliminary autoradiographic data support results obtained in membrane binding assay. As shown in figure 1, high densities of [^{3}H]DAG0 binding sites are seen in the caudate, putamen, ventral pallidum-substantia innominata area and cortical layers I and IV. Much lower densities are found in the globus pallidus, internal capsule and claustrum (figure 1).

The distribution of mu sites in human brain reported here using a highly selective ligand correlates fairly well with previous results obtained using less selective probes (Kuhar et al.1973; Pfeiffer et al.1982; Bonnet et al.1981). In all these studies, it was found that the amygdala, hypothalamus, thalamus, caudate and putamen were enriched in mu sites. Additionally, we provided preliminary data on the distribution of these sites in multiple cortical areas and brainstem.

The autoradiographic distribution of mu sites in human brain is also most interesting. As observed in rat brain (Goodman et al.1981; Quirion et al.19831, high densities of sites are found in caudate, putamen and certain cortical layers. However, it appears that the "patchy" distribution of mu sites in the striatum is less apparent in human brain. The possible significance of this observation remains to be established. We are currently studying the comparative distribution of other opioid binding sites (delta and kappa) in normal and pathological human brain.

Table 1. Distribution of [^{3}H]DAG0 binding sites in different regions of the human brain using membrane assay.

Region	K_d (nM)	B_{max} (fmol/mg protein)
Caudate	1.1	32.3
Posterior amygdala	1.0	53.8
Hippocampus	1.1	19.1
Hypothalamus	4.2	49.3
Putamen	2.2	25.2
Thalamus	1.1	30.5
Substantia nigra	2.2	11.1
Internal capsule	0.4	7.8
Cortex - Brodmann area		
9	0.4	35.2
44	0.6	26.2
24	0.9	36.1
8	0.2	24.7
6	0.5	37.5
22	0.4	32.3
20	0.5	46.7
24	0.2	25.3
4	1.3	39.9
23	0.9	78.9
31	0.3	27.1
21	0.5	42.6
17	1.0	25.6
30	0.6	30.0
39	0.6	28.9
19	0.3	12.6
18	0.4	11.4
Cerebellum	8.1	46.8
Pontine nucleus	1.0	23.6

Data represent mean of 2-3 experiments using 6 different concentrations of ligand. All data were analyzed by computerized linear regression analysis (Bio-Soft, Elsevier, Cambridge, England).

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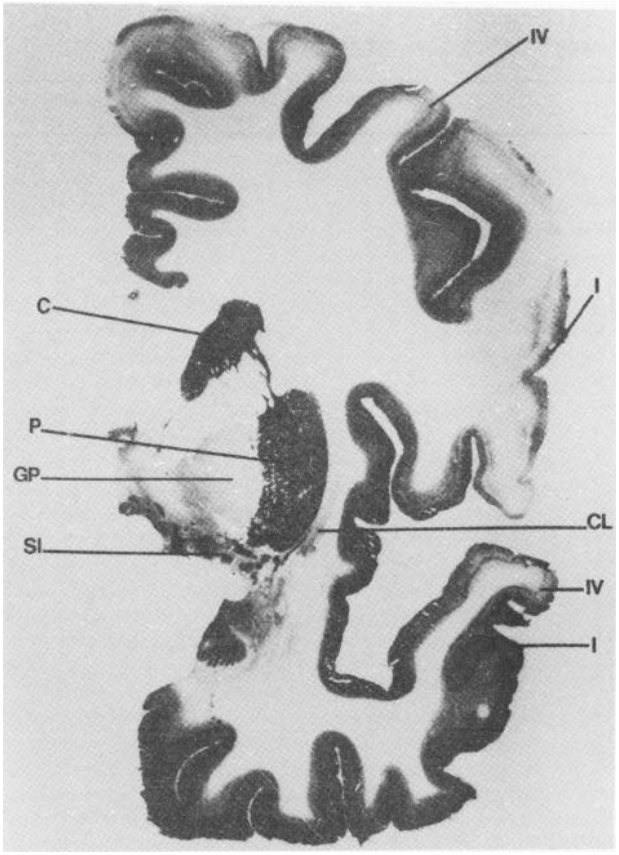

Figure 1. Photomicrograph of the autoradiographic distribution of [^{3}H]DAGO opioid binding sites in human brain. Abbreviations used: C, caudate; CL, claustrum; GP, globus pallidus; P, putamen; SI, substantia inominata, I and IV, layers I and IV of the cortex.

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BEHAVIORAL INTERACTION BETWEEN ENDOGENOUS OPIOIDS, CCK-8 AND DOPAMINERGIC SYSTEMS IN THE NUCLEUS ACCUMBENS OF RATS

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ABSTRACT

Injection of apomorphine into the nucleus accumbens of rats elicits hypermotility, presumably by activating postsynaptically located dopamine receptor systems. This behavioral response is inhibited by local treatment with CCK-8 or γ-endorphin. The action of both peptides is antagonized by intra-accumbal treatment with naloxone. These findings suggest that endogenous opioids are implicated in certain neuroleptic-like effects of CCK-8 and that in the nucleus accumbens a functional interaction exists between CCK-8, endogenous opioids and dopaminergic systems.

INTRODUCTION

The influence of peptides related to γ-endorphin and cholecystokinin (CCK) on behaviors of rodents suggest that these peptides induce effects that can also be elicited by neuroleptic drugs (De Wied et al. 1978; Van Ree et al. 1980; Zetler 1983; Van Ree et al. 1983). Clinical studies have revealed that treatment with these peptides results in an antipsychotic effect in a number of schizophrenic patients (Van Ree et al. 1986; Nair et al. 1986). Since brain dopamine has been implicated in the antipsychotic action of neuroleptics, extensive studies have been performed to investigate the interaction between these peptides and dopaminergic systems in the brain. One approach to study this interaction is to determine the behavioral effect of dopaminergic drugs micro-injected into restricted brain areas, and to investigate whether known or purported antipsychotic substances interfere with this behavioral effect. Following this approach and focussing on the nucleus accumbens area, that contains terminals of the dopaminergic mesolimbic pathway, we have found that relatively small doses of apomorphine induce hypomotility in rats. This behavioral response that may be mediated by presynaptically located dopamine receptor systems, can be blocked by local pretreatment with neuroleptic drugs (e.g. haloperidol and sulpiride) and peptides related to γ-endorphin and cholecystokinin (Van Ree et al. 1982, 1983, 1984). The active peptides in this respect are the opioid γ-endorphin and the non-opioid N^{α}-acetyl-γ-endorphin, des-Tyr^{1}-γ-endorphin and desenkephalin-γ-endorphin and both sulphated and desulphated CCK-8. When higher doses of apomorphine are injected into the nucleus accumbens, the rats show hypermotility, a behavioral response probably mediated by postsynaptically located dopamine receptor systems. This response is antagonized by pretreatment with γ-endorphin and sulphated CCK-8 (CCK-8-S) but not by the non-opioid γ-type endorphins and desulphated CCK-8 (CCK-8-DS) (Van Ree et al. 1983, 1984; Van Ree and Gaffori 1983). Since the effect of γ-endorphin can be blocked by

naloxone and is not mimicked by the non-opioid related peptides N^{α}-acetyl-γ-endorphin and des-Tyr^{1}-γ-endorphin), the effect is likely mediated by opioid receptor systems. Interestingly this action of γ-endorphin is unique in that other opioid peptides, like enkephalins and α- and β-endorphin, are not active in this respect (Van Ree and Gaffori 1983). Evidence has been presented that CCK-8-S counteracts opioid-induced analgesia (Faris et al. 1983), suggesting an interaction between CCK-8-S and endogenous opioids. The present experiments were designated to investigate whether opioid receptor systems are involved in the antagonizing action of CCK-8-S on the apomorphine-induced hypermotility following injection into the nucleus accumbens.

MATERIALS AND METHODS

Male Wistar rats weighing 130 - 140 g were equipped with a stainless steel cannula at each side of the brain. The cannulae were aimed at the nucleus accumbens (for details see Van Ree et al. 1982, 1983). At least 7 days after the operation, the rats were three times injected bilaterally with 1 µl. Groups of rats received placebo, CCK-8-S (10 ng) or γ-endorphin (10 ng), after 30 min placebo or naloxone (2 µg) and 10 min later placebo or apomorphine (10 µg). Twenty minutes after the last injection the rats were placed in a small open field, a perspex circular test cage, the bottom of which was divided into 4 equal sections. Motor activity was measured for 3 min by counting the number of sections explored. After experimentation the sites of injection were evaluated histologically. Data of rats outside the nucleus accumbens were discarded from the analysis. Group means and standard errors were calculated and the statistical significance was determined using ANOVA and subsequently Newman-Keuls analysis. The peptides were donated by Organon International B.V., Oss, The Netherlands and naloxone by Endo, New York, U.S.A. Drugs and peptides were dissolved in saline just prior to experimentation.

RESULTS AND DISCUSSION

Injection of apomorphine (10 µg) into the nucleus accumbens resulted in hypermotility (table I). This behavioral effect was completely antagonized by local pretreatment with either CCK-8-S or γ-endorphin. As already mentioned desulphated CCK-8 and non-opioid peptides related to γ-endorphin e.g. N^{α}-acetyl-γ-endorphin are inactive in this respect (Van Ree et al. 1983, 1984). Thus, small changes (i.e. desulphation and acetylation) in the peptide structure lead to inactivation with respect to this action of the peptides. Interestingly, both sulphated and desulphated CCK-8 and both the opioid and non-opioid γ-type endorphins interfere with another dopaminergic system in the nucleus accumbens, which can be activated by small doses of apomorphine (Van Ree et al. 1983, 1984). Desulphation and acetylation thus result in more selectively acting peptides in this case. Whether these enzymatic processes occur before or after the release of the peptides, thus intra- or extraneuronally, is unknown, although the present knowledge favors intraneuronal processing.

The opioid antagonist naloxone antagonized the inhibitory effect of both CCK-8-S and γ-endorphin on apomorphine-induced hypermotility (table I). Naloxone did not interfere with the apomorphine response per sé (Van Ree et al. 1984). The apomorphine-induced hypermotility

TABLE I

Effect of naloxone on the antagonistic action of CCK-8-S and γ- endorphin (γE) on apomorphine-induced hypermotility following injection into the nucleus accumbens

Treatment - 60'	Treatment - 30'	Treatment - 20'	Motor activity scores (mean ± SEM)	
placebo	placebo	placebo	15.2 ± 0.4	(28)
placebo	placebo	apomorphine 10 µg	23.1 ± 0.6*	(27)
CCK-8-S 10 ng	placebo	apomorphine 10 µg	15.5 ± 0.7^{+}	(23)
CCK-8-S 10 ng	naloxone 2 µg	apomorphine 10 µg	22.6 ± 1.7°	(10)
γE 10 ng	placebo	apomorphine 10 µg	14.8 ± 0.7^{+}	(14)
γE 10 ng	naloxone 2 µg	apomorphine 10 µg	23.9 ± 1.2°	(7)

( ) number of observations
ANOVA: F (5,103) = 32.48 (p < 0.001), Newman-Keuls:
* different from placebo, placebo, placebo (p < 0.05),
$^{+}$ different from placebo, placebo, apomorphine (p < 0.05),
° different from peptide, placebo, apomorphine (p < 0.05).

is thought to be mediated by activation of postsynaptically located dopamine receptor systems. Thus, CCK-8-S and γ- endorphin may act in this brain area as a dopamine antagonist. Indeed the dopamine antagonist haloperidol blocked the hypermotility induced by apomorphine following injections into the nucleus accumbens, but the effect of haloperidol (100 pg) was not affected by similar treatment with naloxone. It may therefore be postulated that activation of opioid receptor systems or release of endogenous opioids is involved in the effect of CCK-8-S on the apomorphine-induced hypermotility. Candidates in this respect are γ- endorphin receptors or γ-endorphin respectively, since γ- endorphin in contrast to other opioid peptides mimics this effect of CCK-8-S. Endogenous opioids may be involved in some, but not all interactions between CCK-8-S and dopaminergic systems in the nucleus accumbens. In fact, the CCK-8-S-induced inhibition of the hypomotility elicited by small doses of apomorphine is not affected by local treatment with naloxone. CCK-8-S may thus have multiple actions on the function of dopaminergic neurons in the nucleus accumbens, and in one or some of these actions endogenous opioids are concerned. The functional interaction between CCK-8, endogenous opioids and dopaminergic systems may contribute to further elucidate the antipsychotic action of certain endogenous peptides.

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SUPPRESSION OF SYMPATHETIC NERVE ACTIVITY BY METHIONINE ENKEPHALIN: ADDITIVE INTERACTION WITH CLONIDINE IN INTACT ANESTHETIZED RABBITS

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ABSTRACT

In order to study the role of methionine enkephalin (met-enkephalin) in cardiovascular system, interaction between clonidine and met-enkephalin on the rate of sympathetic nerve discharge was investigated. In anesthetized rabbits multiunit renal nerve activity (RNA) was monitored after the treatment of met-enkephalin alone or met-enkephalin plus clonidine pretreatment. Met-enkephalin suppressed RNA, and reduced heart rate and blood pressure. Treatment of met-enkephalin after clonidine (2 to 20 µg/kg) increased the duration of RNA suppression. The data suggests that the cardiodepressive action of met-enkephalin is mediated by the stimulation of central presynaptic adrenergic α_2 receptor as proposed for clonidine.

INTRODUCTION

Met-enkephalin has profound effects on the cardiovascular system in addition to diverse other physiological actions (Evans et al. 1986; Simon and Hiller 1978). Met-enkephalin decreased blood pressure and heart rate in parallel with the suppression of sympathetic nerve activity in anesthetized intact rabbits (Rhee et al. 1985), although the response to the peptide in conscious animal may be different (Yukimura et al. 1981). This cardiodepressive effects of the peptide was blocked by naloxone, but not by naloxone methobromide, which is a quaternary analog of naloxone. The cardiovascular action of the peptide was also partially blocked by phentolamine, a non-specific alpha-adrenergic receptor blocker (Eulie and Rhee 1984). It is understood that met-enkephalin effect is mediated by opioid receptors and alpha adrenergic system is involved in the hypotensive action of met-enkephalin, but the exact nature of interaction between the peptide and clonidine is not clear. Therefore, the main object of this research was to establish potential synergistic or additive interaction of the two drugs.

MATERIALS AND METHODS

In pentobarbital (30 mg/kg i.v.) anesthetized rabbits, the right femoral artery and vein were cannulated for the measurement of arterial pressure and subsequent delivery of drugs. The rabbits were allowed to respire spontaneously through a tracheostomy. Arterial blood pressure was monitored by a Statham P23 pressure transducer coupled to a pressure processor amplifier (Gould Instruments, Cleveland, OH, model 13-4615-52) which computes the systolic,

mean, diastolic, and pulse pressures. Heart rate (HR) was monitored by a Gould Biotec ECG amplifier (model 13-4615-65).

After the basic surgery, the animals were placed on their right side and the kidney was exposed retroperitoneally. A branch of the left renal nerve was carefully dissected from the surrounding connective tissue, cleaned and suspended on a bipolar electrode as reported Rhee et al. (1985). Multiunit renal nerve activity (RNA) was monitored, and integrated for a given time before analysis. All of the above parameters were recorded on a Gould 16-channel electrostatic recorder (model ES 1,000). Renal blood flow was determined electromagnetically.

Animals were given met-enkephalin (Sigma Chemical Co., St. Louis, MO) in bolus doses of 3, 10, and 30 µg/kg in ascending order with a 5-min interval between doses. After establishing the control dose-response curve of met-enkephalin, the same dose-response test was repeated 10 min after pretreatment with 1, 2, 5, 10, and 20 µg/kg of clonidine. The mean values for arterial pressure, HR and RNA were calculated and time for half-maximal recovery (T_{50}) of systolic blood pressure was also determined. Each datum point was analyzed by unpaired student t test and significant difference was considered as P is lesser than .05.

RESULTS AND DISCUSSION

In anesthetized rabbits an intravenous injection of met-enkephalin decreased systolic, mean and diastolic blood pressure in the peptide dose dependently, which confirms our previous reports (Rhee et al. 1985; Eulie and Rhee 1984). However, pulse pressure was slightly increased in many experiments as shown in fig. 1. Heart rate and renal nerve activity (RNA) were reduced by the peptide so that integrated RNA per min was also significantly reduced. Renal blood flow was reduced by the treatment of met-enkephalin from the control 26 ml/min for a kidney to 21 ml/min. It is not clear whether this effect on renal blood flow is secondary to its primary effect on blood pressure or its potential direct effect on renal blood vessel. Clonidine initially increased blood pressure which was followed by a gradual but a profound reduction in blood pressure and heart rate in the drug dose-dependent manner. Clonidine also suppressed RNA in a manner similar to met-enkephalin, which usually manifested at the time of initial increase in blood pressure by clonidine. Simultaneous administration of clonidine and met-enkephalin produced an additive action in duration of RNA suppression. Clonidine also increased the time that is required 50% recovery from the maximal reduction of blood pressure (T_{50}). But it was not always possible to see clonidine additively interact with met-enkephalin in the reduction of blood pressure or heart rate due to presumable interference with baroreflex and other physiological compensating mechanisms (fig. 2).

Antihypertensive effect of clonidine is known to be mediated by the stimulation of central α_2 adrenergic receptors which will decrease peripheral sympathetic outflow. The similarity between the action

of the two drugs, particularly at low doses of clonidine (e.g., under 5 μg/kg) suggests the mechanism of met-enkephalin on cardiovascular action indeed involves central stimulation of α_2 receptors. It is also supported by the fact that adrenergic blocker such as phentolamine antagonized cardiovascular effects of met-enkephalin instead of potentiating the effects (EuLie and Rhee 1984).

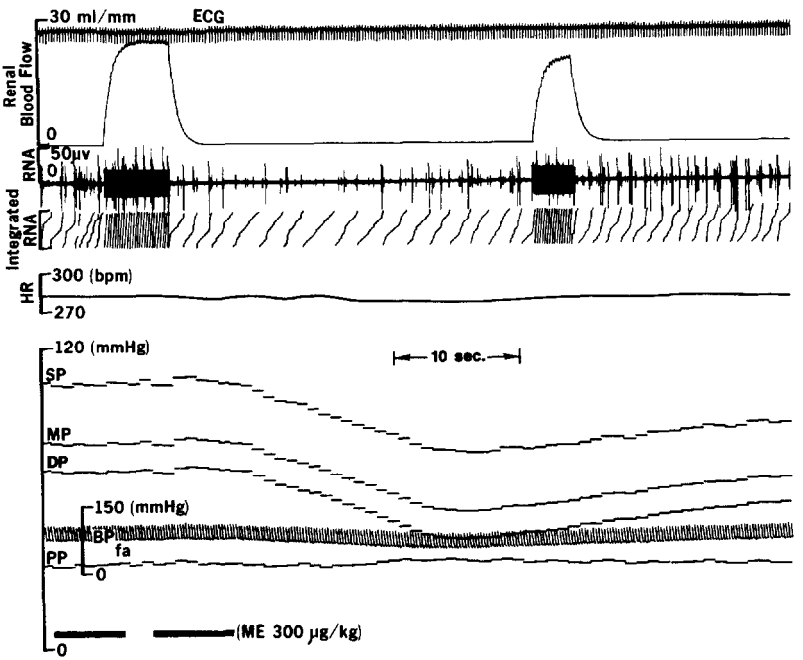

Figure 1. Effects of met-enkephalin (ME) on cardiac parameters in intact anesthetized rabbit. Animal was prepared as in "methods" and ME (300 μg/kg) was loaded at the first bar in a volume of .25 ml of saline and flushed with saline as shown at the second bar. RNA, SP, MP, DP, PP and BP_{fa} indicate renal nerve activity, systolic, mean, diastolic and pulse pressures, and femoral arterial blood pressure, respectively. Note that there is no unit in integration of RNA.

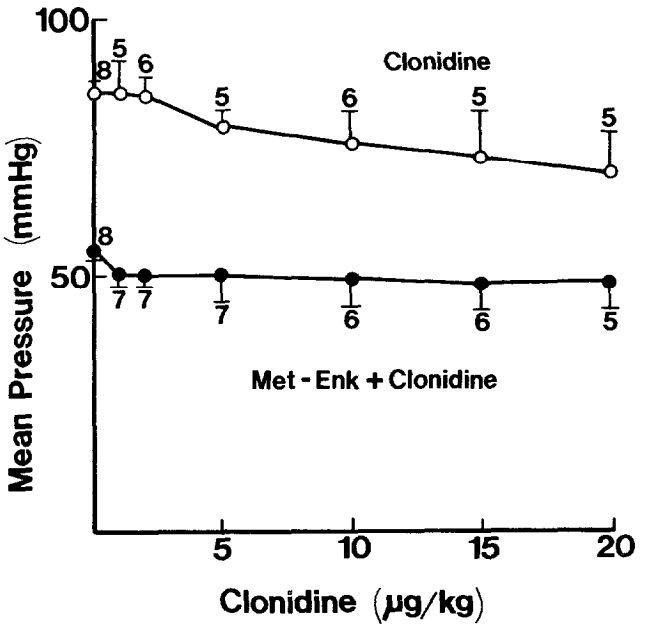

Figure 2. Interaction between clonidine and met-enkephalin on mean blood pressure in anesthetized rabbits. Animals were prepared as in fig. 1 and met-enkephalin was given 10 min after the treatment of clonidine. Vertical bars indicate S.E. and numbers indicate the number of animal used.

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BETA-ENDORPHIN MODULATES THE INHIBITORY ACTION OF CORTICOTROPIN-RELEASING FACTOR ON LUTEINIZINC HORMONE SECRETION

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ABSTRACT

The present study investigates the possible role of endogenous opioid peptides in mediating the inhibitory effect of corticotropin-releasing factor (CRF) on circulating Iuteinizing hormone (LH) . The centraI injection of an antiserum against β-endorphin (β-END) reversed CRF-induced LH decrease in castrated male rats, while antisera raised against DYN-A or M-Enk were inactive. β-END-(6-31) (2 nmole, icv), a β-END antagonist, and beta-funaltrexamine (4.8 nmole, icv), a mul opiate receptor antagonist, also reversed the effect of CRF on LH. Either kappa (Mr1456 and Mr2266) (10 mg/kg, ip), or delta (ICI 154,129) (10nmole, icv) opiate receptor antagonists did not significantly modify the'inhibitory effect of CRF on circulating LH levels. These results suggest that central β-END participates in the inhibitory action of CRF on LH secretion.

INTRODUCTION

Endogenous opioid peptides (EOP) exert a tonic inhibitory effect on luteinizing hormone (LH) secretion. They modulate the hypothalamic secretion of gonadotropin-releasing hormone (GnRH), and participate in the negative feedback action of gonadal steroids on hypothalamic-pituitary function (Cicero et al. 1979; Van Vugt et al. 1982). Recently, the central injection of corticotropin-releasing factor (CRF) has been shown to decrease plasma LH levels in castrated rats Rivier and Vale 1984; Ono et al. 1984). The action of CRF on LH secretion is independent from the stimulation of the pituitary-adrenal axis, and appears to be due to an effect of CRF on the mechanisms that control GnRH secretion (Rivier and Vale 1985). Interestingly, the coexistence of CRF and EOP has been described in the arcuate-ventromedial hypothalamus (Watson et al. 1982; Millan et al. 1986), thus suggesting a possible functional interaction in between these pathways into these brain areas, particularly rich of GnRH neurons. The aim of the present study was to evaluate whether the inhibitory action of CRF on LH secretion involves EOP activation. Passive immunoneutralization of the three most important brain EOP (beta-endorphin, β-END; dynorphin-A, DYN-A; methionine-enkephalin, M-Enk) and specific antagonists for the main opiate receptor subtypes (mu/epsilon, kappa, delta) were used to antagonize the action of CRF on circulating LH.

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MATERIALS AND METHODS

Male Sprague-Dawley rats (220-240 g) were used. One week before the bioassay, rats were castrated and a permanent cannula was implanted into lateral ventricle of the brain. The implantation of a jugular catheter was done 24 hours before the experiments. Ovine CRF (2 nmole) (Rivier et al. 1983) was dissolved in water and injected icv (5µl) In groups of 6-8 rats antiserum raised against β-hEND or DYN-A or M-Enk was injected icv (5 µI) two hours before the CRF administration. In another set of experiments the following opiate receptor antagonists were administered centrally: β-hEND(6-31), a β-END antagonist (2 nmole) (gift of Dr. C.H. Li, San Francisco, CA); beta-funaltrexamine (β-FNA) (4.8 nmole) (Res. Biochem. Inc., Wayland, MA), a long acting irreversible mu1 receptor antagonist; ICI 154,129 (10 nmole) (gift of Dr. P. Sacerdote, Bethesda, MD), a delta antagonist. Two different kappa opiate receptors antagonists were injected intraperitoneally: Mr1456 MS and Mr2266 BS (10 mg/kg BW) (gift of Drs. Plauth and Elich, Boerhinger Ingelheim, Ingelheim an Rhein, FRG). Blood samples were collected before and every 30 minutes following CRF injection. Plasma LH levels were measured by radioimmunoassay, using materials provided by NIADDK (Bethesda, MD). The assay sensitivity was 10 pg; the inter- and intrassay coefficients of variation were 6% and 3.5% respectively. Statistical analysis of the results was performed using the one-side multiple comparison test of Dunnett and the multiple range test of Duncan.

RESULTS AND DISCUSSION

The central injection of CRF significantly decreased plasma LH levels in castrated male rats from 6.8 + 0.7 ng/ml to 3.4 + 0.3 ng/ml (M + SEM) ($P < 0.01$). The inhibitory effect of CRF on LH secretion was measurable over a 60 minute period following the administration of the peptide (4.6 + 0.7 ng/ml, $P < 0.019$, reaching the nadir values after 180 minutes. The central injection of an antiserum raised against β-END completely reversed the inhibitory effect of CRF (fig. 1). In β-END immunoneutralized rats, plasma LH levels were in the same range as control rats. By contrast, in rats injected with DYN-A or M-Enk antiserum the inhibitory effect of CRF on LH was not significantly modified (fig. 1). These results showed that CRF interact centrally with β-END in modulating LH decrease. In support of this hypothesis, we have observed that the central injection of β-END(6-31) or β-FNA also reversed the decrease of LH levels induced by icv injection of CRF (fig. 1). However, in rats pretreated with β-FNA a slight CRF-induced LH decrease was still evident (fig. 1), thus suggesting a secondary role of mu1 receptor subtype regulating LH secretion. Kappa (Mr1452 or M-2266) and delta (ICI 154,129) opiate receptor antagonists did not significantly influence the LH decrease following CRF injection (fig. 1). These results suggest that CRF decreases plasma LH levels by stimulating the inhibitory action of β-END on GnRH neurons. Moreover, these results agree with the observation that β-hEND is the main EOP involved in the control of LH secretion. The use of a specific antiserum to β-END (Schultz et al. 1981; Forman et al. 1983) or of mu/epsilon opiate receptor antagonists (Petraglia et al. 1984; Panerai et al. 1985) have

indicated a major role of β-END pathway in tonically inhibiting LH secretion. These results may also explain recent reports showing that either a CRF antagonist (Rivier et al. 1986) or naloxone (Hulse et al. 1982; Brisky et al. 1984) completely reversed stress-induced LH decrease in rats. It is possible that stress activates hypothalamic CRF/β-MND pathways, leading to a decrease in GnRH as well as LH secretion. It is therefore possible that the antireproductive actions of stress activation of pituitary-adrenal axis may be explained not only by a pituitary (Suter and Schwartz 1985) or gonadal (Sapolsky 1985) action, but also by the central activation of CRF/β-END pathways. In conclusion these results show that CRF and β-END interact to modulate LH secretion.

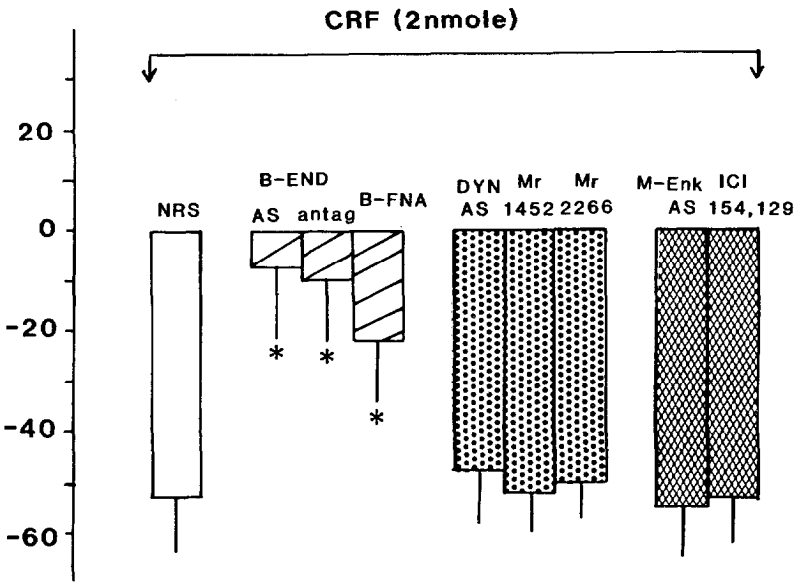

Figure 1: Effect of various antisera (AS) raised against β-END, DYN or M-Enk or of antagonists for the different opiate receptor subtypes on CRF-mediated LH inhibition. *P < 0,01 vs. control rats (NRS, normaI rabbit serum).

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LEUMORPHIN IS A POTENT INHIBITOR OF VASOPRESSIN SECRETION

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ABSTRACT

The effects of intracerebroventricular (i.c.v.) injection of leumorphin on the vasopressin secretion induced by angiotensin II or dehydration were studied in conscious unrestrained rats. The vasopressin secretion induced by angiotensin II (100 pmol) was significantly inhibited by the simultaneous injection of leumorphin (6 pmol-600 pmol) in a dose-dependent manner. In 72-hour water-deprived rats, the i.c.v. injection of leumorphin (60 pmol or 600 pmol) significantly suppressed the vasopressin secretion dose-dependently. These results suggest that leumorphin is involved in the regulation of the vasopressin secretion.

INTRODUCTION

Leumorphin is a proenkephalin B-derived 29-amino acid polypeptide containing leucine-enkephalin sequence at its N-terminus (Kakidani et al. 1982, Suda et al. 1983). Using a specific radioimmunoassay (RIA) for leumorphin coupled with high performance liquid chromatography, we demonstrated the presence of leumorphin in the pituitary and brain (Nakao et al. 1983, Suda et al. 1984) and parallel distribution of leumorphin and dynorphin in the brain (Suda et al. 1985). We also reported that leumorphin acts as an agonist at the κ-type opioid receptor (Suda et al. 1983) and that intracerebroventricular (i.c.v.) injection of leumorphin inhibits water drinking induced by water deprivation or angiotensin II (AII) in rats, suggesting its involvement in water and electrolyte balance (Imura et al. 1985, Imura et al. 1986). In addition, dynorphin derived from the same precursor as that of leumorphin, is known to co-exist with vasopressin (AVP) in magnocellular neurons in the supraoptic and paraventricular nuclei (Watson et al. 1982). In this study, to further elucidate the involvement of leumorphin in fluid balance, we have examined the effect of i.c.v. injection of leumorphin on the AVP secretion in conscious unrestrained rats.

MATERIALS AND METHODS

Peptides

Synthetic angiotensin II (AII) was purchased from Protein Research Foundation, Osaka, Japan. Human leumorphin was synthesized by a conventional solid phase method (Yamamoto et al. 1983). These peptides were dissolved in physiological saline solution for intracerebroventricular (i.c.v.) injection at a volume of 5 µl.

Experimental animals

Male Wistar rats, weighing 200-230 g, were housed in a temperature-controled room (25±1°C) with a 12-hour light-dark cycle (light on 7:00 A.M.). The rats were fed standard rat biscuits and given free access to food and water. They were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, Chicag, 50 mg/kg, intraperitoneal) and a stainless steel cannula was implanted stereotaxically into the left lateral ventricle as reported elsewhere (Ikeda and Matsushita 1980). Four days later, a jugular catheter was positioned into the superior vena cava under the pentobarbital anesthesia. The experiments were performed after a 3-day recovery period. At the end of experiments, 5 µl of 1 % Evans blue solution was injected through the cannula to confirm that the placement of the cannula was appropriate.

Effect of leumorphin on AII-stimulated AVP secretion

Rats were simultaneously injected i.c.v. with AI1 (100 pmol) and leumorphin (0, 6 pmol, 60 pmol or 600 pmol). Blood (250 µl) samples were taken from the jugular catheter four times, just before the injection and 1.5, 5 and 10 minutes after the injection.

Effect of leumorphin on dehydration-induced AVP secretion

After 72-hour water-deprivation, the effect of i.c.v. injection of leumorphin (0, 60 pmol or 600 pmol) on the AVP secretion was examined. Blood (250 µl) samples were drawn from the jugular catheter at 0, 1.5, 5 and 10 min. An equal volume of physiological saline (250 µl) was injected intravenously just after the blood sampling.

Radioimmunoassay (RIA)

Blood samples were collected into polypropylene tubes containing Na_2EDTA (1 mg/ml). Plasma was separated immediately and stored at

TABLE 1. Effect of i.c.v. injection of leumorphin on AVP secretion induced by AII

Treatment	Dose	No. of rats	Time after i.c.v. injection			
			before	1.5 min	5 min	10 min
AII	100 pmol	7	3.1 ±0.4	16.6†††±1.2	8.9†††±0.7	6.1†±0.5
Leumorphin§	6 pmol	4	4.7 ±0.7	13.3††±1.6*	6.0 ±1.2*	5.1 ±0.7
	60 pmol	6	3.3 ±0.4	6.8 ±1.2**	3.5 ±0.4**	3.4 ±0.4**
	600 pmol	7	3.4 ±0.4	4.0 ±0.6**	3.4 ±0.4**	3.4 ±0.4**

(pg/ml)

Values are means ± S E . § AII as given simultaneouly.
* P(0.05, ** P(0.01 vs AII (Duncan's multiple range test).
†P<0.05, ††P<0.01, †††P<0.001 vs before injection (paired t test).

-20°C. The plasma vasopressin concentration was measured by RIA (Yamada et al. 1986).

Statistical analysis

The data were analyzed by paired t test or Duncan's multiple range test following one way analysis of variance.

RESULTS AND DISCUSSION

I.c.v. injection of AII (100 pmol) stimulated the AVP secretion from the posterior pituitary in conscious unrestrained rats. This AVP secretion was significantly inhibited by the simultaneous i.c.v. injection of leumorphin (6 pmol-600 pmol) in a dose dependent manner (table 1). The plasma AVP concentration was markedly elevated in 72-hour water-deprived rats. As shown in table 2, the i.c.v. injection of leumorphin (60 pmol or 600 pmol) also suppressed the dehydration-induced AVP secretion dose-dependently.

The present study has demonstrated that leumorphin possesses a potent inhibitory action on the AVP secretion in conscious unrestrained rats. This observation is consistent with the inhibitory effects of i.c.v. administration of endogenous opioid peptides such as β-endorphin and enkephalins on the AVP secretion (Van Wimersma Greidanus et al. 1979, Summy-Long et al. 1981). In the present study, a relatively small dose of leumorphin (6 pmol) significantly inhibited the AVP secretion, when compared with those of β-endorphin and enkephalins (Van Wimersma Greidanus et al.1979, Summy-Long J.Y. et al. 1981). This potent inhibitory effect of leumorphin on the AVP secretion corresponds to our previous findings that leumorphin inhibits water drinking induced by i.c.v. injection of AII or water deprivation much more effectively than α-neo-endorphin or leucine-enkephalin (Imura et al. 1985, Imura et al. 1986).

The potent inhibitory effect of leumorphin on the AVP secretion together with its potent antidipsogenic action indicates that leumorphin plays a regulatory role in fluid homeostasis in the central nervous system.

TABLE 2. Effect of i.c.v. injection of leumorphin on AVP secretion induced by 72-hour water deprivation

Treatment	Dose	No. of rats	Time after i.c.v. injection			
			before	1.5 min	5 min	10 min
Leumorphin	0	5	13.8 ± 1.5	-	14.2 ± 2	-
	60 pmol	5	15.4 ± 2.0	14.8 ± 3.4	9.5 † ± 1.9*	7.9 †† ± 2.2
	600 pmol	5	14.0 ± 1.5	6.7†† ± 1.2	4.8†† ± 0.5**	5.7† ± 1.3

(pg/ml)

Values are means ± SE.
* P<0.05, ** P(0.01 vs Leumorphin 0 (Duncan's multiple range test).
† P<0.05, †† P<0.01 vs before injection (paired t test).

ACKNOWLEDGEMENTS

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THE EFFECT OF 17-ALPHA-ESTRADIOL, A POSSIBLE ENDOGENOUS OPIATE ANTAGONIST, ON D-ALA^2-MET^5-ENKEPHALINAMIDE-INDUCED BLOOD PRESSURE RESPONSES IN CONSCIOUS, UNRESTRAINED RATS

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ABSTRACT

D-ala^2-met^5-enkephalinamide (DAME) produced a dose-related increase in the mean arterial blood pressure of conscious, unrestrained rats. Intravenous injection of DAME (0.5, 1, 2, and 4 mg/kg) resulted in mean systemic arterial blood pressures of 138±2, 146±5, 141±4, 156±5 mmHg, respectively. 17-alpha-estradiol and its derivatives are known to be inactive in target tissues responsive to estrogenic hormones such as 17-beta-estradiol. However, LaBella et al. (1978) found after testing a large number of steroid hormones and their metabolites that only 17-alpha-estradiol significantly inhibited binding of ^{3}H-naloxone, an opiate antagonist, in rat-brain homogenates. The present study was designed to determine whether 17-alpha-estradiol could antagonize the cardiovascular responses elicited by intravenous injections of DAME. Intravenous infusion of 17-alpha-estradiol (1.5 mg/kg) every 2 hours for 24 hours (total infusion time was 2 minutes for each infusion) did not change the mean systemic arterial blood pressure (94±5 mmHg) compared to the blood pressure prior to infusion of 17-alpha-estradiol (99±7 mmHg). Intravenous infusion of 17-alpha-estradiol (1.5 mg/kg) 10 minutes prior to DAME (1 mg/kg, i.v.) resulted in a blood pressure of 106±9 mmHg, which is significantly less than the blood pressure of 146±5 mmHg seen with DAME (1 mg/kg, i.v.) alone. Intravenous injection of DAME (1 mg/kg) 8 hours after the last infusion of 17-alpha-estradiol produced an increase in mean systemic arterial blood pressure of 136±8 mmHg. These results indicate that 17-alpha-estradiol may function as an opiate antagonist.

INTRODUCTION

Pinsky et al. (1975) studied the role of endogenous sex steroids in morphine antinociception. They showed that morphine was ineffective in relieving the stress of mild pain in gonadectomized rats. However, adrenalectomy alone enhanced morphine antinociception. The authors suggested that mild pain may mobilize an endogenous steroid of gonadal origin which acts to reduce the nociception response and that the adrenals may release a substance that antagonizes the effects of both the antinociceptive steroid and morphine. LaBella et al. (1978) showed that a large number of steroids of the estrane, androstane, pregnane, and etianic-acid series, including their sulfate and glucuronide conjugates, were inactive in an opiate radioreceptor assay (displacement of ^{3}H-naloxone). In their experiments, only 17-alpha-OH estrogens showed significant affinity for displacement of ^{3}H-naloxone in rat-brain homogenates.

The present study investigated the possibility that the 17-alpha-estradiol (a potent competitor for binding of ^{3}H-naloxone and other opiate drugs--such as morphine, methionine-enkephalin, and leucine-enkephalin--to brain membranes or brain slices [see LaBella 1985]) may function as an opiate antagonist and may antagonize the cardiovascular responses elicited by d-ala^{2}-met^{5}-enkephalinamide in conscious, unrestrained rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 300-350 gm were used in the study. Under ether anesthesia, the femoral artery and vein were catheterized with PE-50 tubing in order that blood pressure might be measured and drugs injected. The PE-50 tubing was guided through subcutaneous fat on the back and was exited from the neck. A heparin solution (285 units/ml) was injected into the tubing to prevent blood clotting. The PE-50 tubing was then connected to a 21-gauge needle connected to a three-way stopcock fastened to a transducer, which in turn was connected to a Model 7 Grass Polygraph used to record blood pressure. 17-alpha-estradiol was dissolved in vehicle (propylene glycol/absolute ethanol/glacial acetic acid/ascorbic acid; 95/5/0.6/0.01; v/v/v/w). D-ala^{2}-met^{5}-enkephalinamide (DAME) was dissolved in 0.9% normal saline. Three groups of animals were used in these experiments.

(1) Rats were given intravenous injections of various doses of DAME (0.5, 1, 2, and 4 mg/kg).

(2) An intravenous infusion of 17-alpha-estradiol (1.5 mg/kg) was given every 2 hours for 24 hours (total infusion time, 2 minutes for each infusion). One hour after the last infu-sion, a bolus injection of 17-alpha-estradiol (1.5 mg/kg, i.v.) was given 10 minutes prior to DAME (1 mg/kg, i.v.). Eight hours later, the injection of DAME (1 mg/kg, i.v.) was repeated.

(3) An intravenous infusion of vehicle was given at the rate of 0.1 ml every 2 hours for 24 hours (total infusion time, 2 minutes per infusion). One hour after the last infusion, a bolus injection of vehicle (0.1 ml, i.v.) was given 10 minutes prior to DAME (1 mg/kg, i.v.). Eight hours later, the injection of DAME (1 mg/kg, i.v.) was repeated.

Statistical evaluation of the data was performed by ANOVA and by the Student Newman-Keul test for multiple comparisons of means (Zar 1974).

RESULTS AND DISCUSSION

Intravenous injection of d-ala^{2}-met^{5}-enkephalinamide (DAME, primarily a delta-receptor agonist) at 0.5, 1, 2, and 4 mg/kg caused a dose-related increase in mean arterial blood pressure (fig. 1) with a duration of 0.98±0.14, 2.25±0.42, 1.90±0.72, and 9.17±0.79 minutes, respectively. However, the heart exhibited bradycardia (165±15, 150±16, 146±14, 135±15). 17-alpha-estradiol

has been found not to have biological activity in target tissues responsive to estrogenic hormones, such as 17-beta-estradiol (Ruediger et al. 1983; Foy and Teyler 1983; Pert et al. 1973). It was also shown that in a family of steroids, only 17-alpha-estradiol was effective in displacing ^{3}H-naloxone in rat-brain homogenates (LaBella et al. 1978, 1985). This observation is consistent with the idea that 17-alpha-estradiol might have opioid antagonist properties. Intravenous infusion of 17-alpha-estradiol, 1.5 mg/kg (infused every 2 hours for 24 hours; total infusion time of 2 minutes for each infusion), did not significantly alter blood pressure and heart rate in the conscious rat (fig. 2). One hour after the last infusion of 17-alpha-estradiol (1.5 mg/kg, i.v.), a bolus injection of 17-alpha-estradiol (1.5 mg/kg, i.v.) was given 10 minutes prior to DAME (1 mg/kg, i.v.). The hypertension produced by DAME was significantly inhibited. Eight hours later, the injection of DAME (1 mg/kg, i.v.) produced a mean arterial blood pressure of 136±9 mmHg, which was significantly higher than the blood pressure response to DAME (106±9 mmHg) given 10 minutes after 17-alpha-estradiol (fig. 3). An interesting observation was that a bolus injection of 17-alpha-estradiol alone could significantly decrease the mean arterial blood pressure response (78±5 mmHg) with a duration of 6.00±0.41 minutes. This is in contrast to the lack of effect of the same dose of 17-alpha-estradiol infused over two minutes. Moreover, 17-alpha-estradiol produced sedation after the infusion of the steroid or after a bolus injection. It has been reported that the gonadal steroid progesterone and the mineralocorticoid deoxycorticosterone, as well as several of their metabolites, caused sedation and anesthesia in the rat (Atkinson et al. 1965). Recently, Majewska et al. (1986) found that reduced metabolites of progesterone and deoxycorticosterone are potent modulators of the GABA-receptor complex in crude synapto-somal membranes from rat brain and that these metabolites interact with the receptor complex in a manner similar to that of the barbiturates. In our results, 17-alpha-estradiol antagonized the hypertension resulting from intravenous injections of DAME, suggesting that 17-alpha-estradiol may function as an endogenous opioid antagonist.

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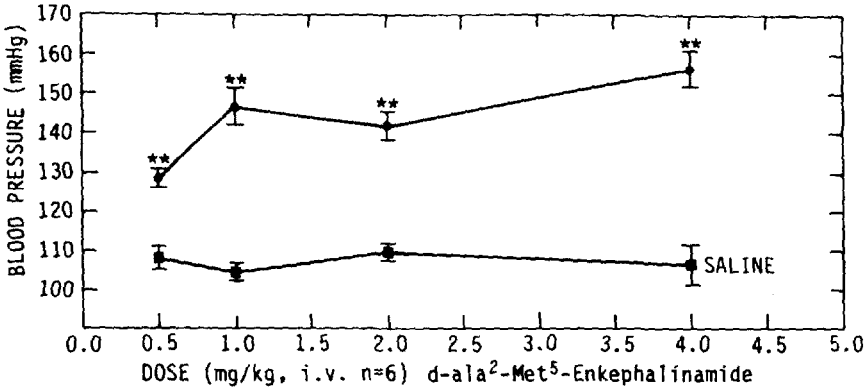

FIGURE 1. The effect of intravenous injection of d-ala^2-met^5-enkephalinamide (DAME) on the blood pressure of conscious, unrestrained rats.
**P < 0.01 compared to the saline injection. n-=-number of animals.

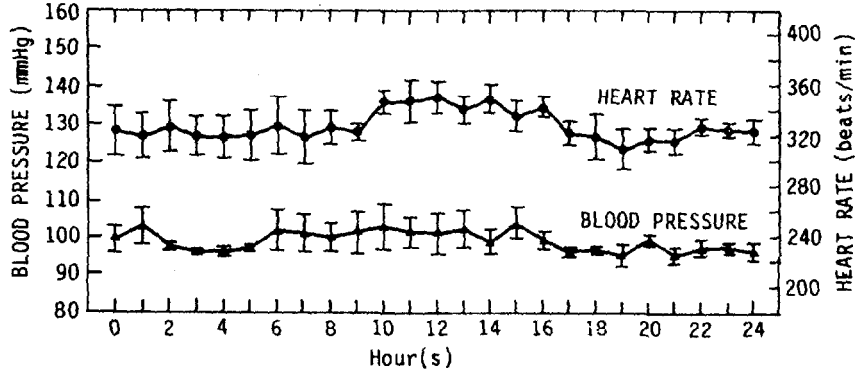

FIGURE 2. The effect of intravenous infusion of 17-alpha-estradiol. 1.5mg/kg (infused every 2 hours for 24 hours; total infusion time, 2 min. for each infusion) on the blood pressure and heart rate response of conscious. unrestrained rats. There was no significant difference in the blood pressure and heart rate during the 24-hours.

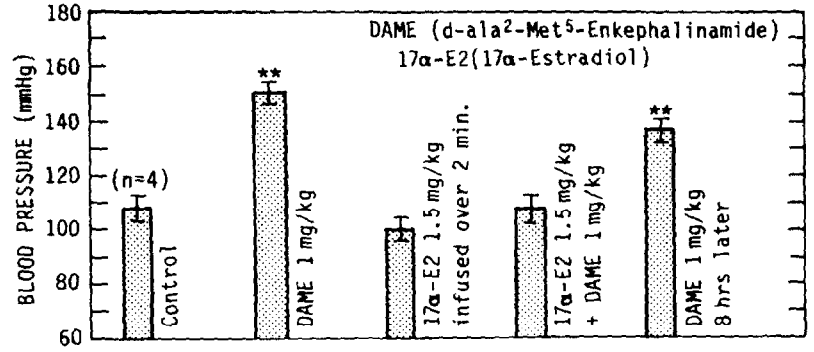

FIGURE 3. The effect of intravenous infusion of 17-alpha-estradiol, 1.5mg/kg (infused every 2 hours for 24 hours; total infusion time. 2 min. for each Infusion) on the mean blood pressure response to DAME (1mg/kg. i.v.) of conscious, unrestrained rats.
**P < 0.01 compared to control. n = number of animals.

STRESS-INDUCED-ANTINOCICEPTION AND MORPHINE SENSITIVITY IN MICE FOLLOWING CHRONIC TREATMENT WITH ANABOLIC/ ANDROGENIC STEROIDS

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ABSTRACT

In male mice treated chronically with anabolic/androgenic teroids, morphine produced changes in exploratory behaviour similar to those normally associated with anxiolytic drugs. In addition, steroid treatment attenuated opioid-mediated stress-induced antinociception.

INTRODUCTION

It has been recognised for some time that opioid drugs disturb normal endocrine function (Meites 1980). In the case of male gonadal hormones, opioid agonists reduce circulating levels of testosterone by inhibiting the release of hypothalamic LHRH (Cicero 1980). Conversely, administration of opioid antagonists elevates plasma LH and testosterone. These findings, together with the detection of opioid-containing neurones in the hypothalamus, have led to the proposal that endogenous opioids are involved in the negative feedback control of LHRH release exerted by circulating testosterone (Cicero et al. 1982). It follows that administration of male sex hormones might influence endogenous opioid function in brain. In this paper, we report that chronic treatment of mice with anabolic/androgenic steroids alters some behavioural responses to opioids.

MATERIALS AND METHODS

Male mice (LACA strain ; 20 - 25 g) were used throughout. Steroids were given by weekly i.m. injections as slow-release preparations in ethyl oleate. Controls received appropriate volumes of ethyl oleate alone.

Antinociception

Testosterone, methyl-testosterone, or methenolone (each 0.4 mg/Kg) were given for 4 weeks. 3 - 4 days after each steroid injection morphine-induced antinociception (20 mg/Kg, i.p.) was assessed on a hot-plate (54°C).

Motor activity

Methyl-testosterone or methenolone (5, 10, and 20 mg/Kg) were given for 6 weeks. On the seventh week, motor activity was assessed 24 hr before and 30 min after 20 mg/Kg morphine i.p. Animals were injected with saline or morphine 30 min prior to being placed in an activity cage. After a 10 min settling in period X - Y motor activity was counted for 10 min.

Exploratory behaviour

Methyl-testosterone or methenolone (5 mg/Kg) were given for 6 weeks. On the seventh week, exploratory behaviour was assessed 24 hr before and 30 min after 20 mg/Kg morphine i.p. Animals were given saline

or morphine 30 min before being placed in a box maze (Davies and Wallace 1976). After a 1 min settling in period exploratory behaviour was counted for 3 min. Parameters measured were, dipping (pushing head down hole in middle of box section), rearing (placing both front paws on dividing walls), and crossing (moving from one box section to the next).

Stress-induced antinociception (SIA)

Methyl-testosterone or methenolone (1 and 5 mg/Kg) were given for 6 weeks. On the seventh week, SIA was assessed by recording reaction time on a hot-plate (54°C) before and 2 min after a 3 min swim at 20°C (Hart et al. 1983).

RESULTS

Antinociception

In naive mice, morphine increased hot-plate reaction time by 35 sec. In control mice, treated with ethyl oleate, there was a gradual decrease in morphine-induced antinociception such that, by the fourth week, there was no significant increase in reaction time; this was presumably due to tolerance induced by the weekly exposure to morphine for the antinociceptive test. A similar pattern was shown by mice treated with the steroids, there being no significant difference among the groups.

Motor activity

In control mice, morphine increased motor activity from 192 ± 10 to 252 ± 15 counts/10 min (mean ± s.e.). Methyl-testosterone treatment per se did not affect motor activity, but it abolished the stimulatory effect of morphine. A similar, but less pronounced, inhibition of morphine-induced motor activity was observed in methenolone treated mice.

Exploratory behaviour

The results from these experiments are shown in figure 1. In control mice, morphine reduced the number of dips, but did not affect rearing or crossing. Morphine also reduced the number of dips in mice treated with methyl-testosterone or methenolone. Further, in methyl-testosterone treated mice, morphine reduced the number of rears, while in methenolone treated mice morphine increased the number of crossings.

SIA

These results are given in table 1. In ethyl oleate treated mice the initial median reaction time on the hot-plate (T_1) was 12 sec; after a 3 min swim the reaction time (T_2) was increased by 9 sec. Treatment with methyl-testosterone or methenolone did not alter the initial reaction time; however, in methyl-testosterone treated mice (5 mg/Kg) the increased reaction time following a swim was attenuated. Methenolone (5 mg/Kg) also reduced the prolongation of reaction time after a swim, but in this case the reduction was just outside the levels of significance ($0.01>P>0.05$).

DISCUSSION

The results clearly indicate that chronic administration of

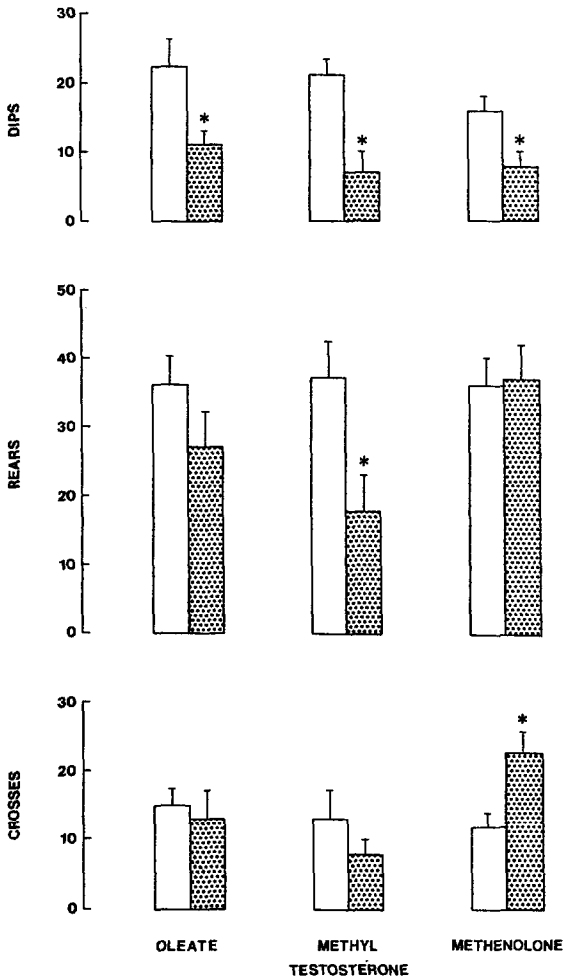

FIGURE 1. Histogram showing (mean ± s.e.) exploratory behaviour of mice on a box maze for 3 min. Open columns - mice given 6 weekly injections as indicated (steroids at 5 mg/Kg) ; hatched columns - same mice 30 min after 20 mg/Kg morphine. * P<0.05

anabolic/androgenic steroids alters the behavioural responses of mice to opioids. Two results are of particular interest. First, on the box maze morphine increased the number of crossings in mice treated with methenolone. Few drugs have been shown to have this effect, and the common property of those that do seems to be anxiolytic activity (J. K. Davies personal communication). The difference in morphine activity in methyl-testosterone and methenolone treated mice on the box maze may be a reflection of the different anabolic/androgenic ratios of the two steroids. Secondly, methyl-testosterone and to a lesser extent methenolone decreased SIA in a model which is known

		Median Reaction Times	
			$T_2 - T_1$
Saline	(12)	T_1	+10
Ethyl Oleate	(12)	12	+9
Methyl Testosterone 1mg/Kg	(6)	12	+9
5mg/Kg	(12)	11	+2.5*
Methenolone 1mg/Kg	(12)	9.5	+12
5mg/Kg	(12)	12.5	+6.5

TABLE 1. Median reaction times (sec) of mice on a hot-plate. T_1 was measured after 6 weekly injections of the treatment indicated. T_2 was measured 2 min after a 3 min swim. The increased reaction time ($T_2 - T_1$) represents SIA. Numbers in parentheses give number of mice.
* $P<0.05$

to involve the release of endogenous opioids (Hart et al. 1983). Thus, steroid administration not only influences responses to exogenous opioids but also behavioural activity resulting from endogenous opioid function. Both these observations may have relevance to one group of individuals known to take regular doses of anabolic steroids; that is athletes involved in strength-related events (Goldman 1984). In such athletes, the interaction between male sex hormones and opioids may be of importance during competition since both stress and exercise are potent stimuli for endogenous opioid release (Gibson et al. 1979 ; Gambert et al. 1981).

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OPIOIDS AND E-RECEPTORS INTERACTION: FURTHER STUDIES IN CULTURED CELLS

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ABSTRACT

In the present paper a further evidence of a functional link between opioids and ß-receptor system in cultured rat C6 cells is presented. We showed that C6 cell exposure to Desmethylimipramine (DMI) causes expression of opioid receptors and loss of ß-receptors. We now show that C6 cells exposure to DMI causes an increase of intracellular levels of opioid peptides and an increase of aminopeptidase activity. In addition, the presence of the aminopeptidase inhibitor bestatin during cell exposure inhibits DMI-induced R-receptor loss. These results indicate that cell exposure to DMI causes the full expression of the opioid peptide system. This effect could be related to changes of ß-receptor system.

INTRODUCTION

It has been recently found that C6 cell exposure to DMI causes expression of cell membrane opioid receptors (Tocque et al. 1984) and we have recently found that this effect was associated to the loss of R-receptors (Reggiani et al. in preparation). We also found that the simultaneous presence of opioids during DMI-exposure prevents ß-receptors down regulation and a similar modulation can be demonstrated on isoproterenol (ISO) induced desensitization in DMI-exposed cells. All these results suggest that, besides the actual mechanism of inducing ß-receptors loss opioids could operate to preserve ß-receptor function. In order to investigate whether DMI-induced opioid receptors appearance was associated to a wider influence on opioid peptide system experiments were carried out to study the effect of cell exposure to DMI on the intracellular synthesis of opioids and on their metabolic degradation. Furthermore, the effect of peptidase inhibitors on DMI induced loss of ß-receptors was tested in the attempt to verify whether peptidase inhibitors could mimic the exogenous opioid agonist action.

MATERIALS AND METHODS

Confluent monolayers of rat C6 glioma cells between passage 47th to 75th were used. Cells were grown in a humidified atmosphere 5% CO_2 - 95% air, using EMEM supplemented with 10% FBS and antibiotics as growth medium. ^{3}H-DHA and ^{3}H-DHM bindings were estimated as previously described by Fishman et al. (1981) and by Albouz et al. (1982) respectively. cAMP levels were measured on acid

extracts from cell suspension by using the RIA method of binding protein as recommended by Amersham. Met-Enkephalin (ME) content was measured on acid extracts from cell suspension by using rabbit antibody from Amersham (code RPM-1561) and 10 nM ^{3}H-ME as radioactive tracer. The hydrolysis of ^{3}H-Leu-Enkephalin (^{3}H-Le) by cultured C6 cells was estimated as follows: at the end of the proper treatment cells were challenged for their ability to hydrolyze peprides by exposing cultures for 30 min to 20 nM LE containing 10.000 cpm ^{3}H-LE. At the end, the medium was collected, boiled for 3 min and aliquots were applied for TLC separation by comparison with the proper standard mixtures.

RESULTS AND DISCUSSION

The influence of cell exposure to DMI on the intracellular content of opioid peptides was investigated by measuring ME content as probe of the opioid peptide concentration. As shown in table 1 naive cells have an intracellular level of ME comparable to the lowest estimate in whole brain and in other cultured cell lines (Gilbert et al. 1982, Glaser et al. 1982, Miller et al. 1978, Yang et al. 1977, Hughes et al. 1977). 24 hrs of cell exposure to different concentrations of DMI increased peptide content up to 5-6 times as much, suggesting that DMI exposure would trigger the expression of opioid peptides synthesis.

TABLE 1. Effect of C6 cell-exposure to different concentrations of DMI on the intracellular content of ME.

TREATMENT (24 hrsl	[M]	ME-CONTENT (fmol/mg protein)
Vehicle		277 ± 31
DMI	$5 \cdot 10^{-5}$	1784 ± 110 *
DMI	$5 \cdot 10^{-6}$	1055 ± 97 *
DMI	$5 \cdot 10^{-7}$	630 ± 78 *

* $P < 0.05$ vs vehicle treated cells
Kesults are the mean ± SE of 3 determinations in duplicate.

C6 exposure to DMI also influenced cell associated neuropeptidase activity as indicated by the results reported in table 2. According to these experiments, both in vehicle and in DMI treated cells most, if all, the neuropeptidase activity was due to an aminopeptidase since the major fragment formed was ^{3}H-Tyrosine (^{3}H-T). However, after cell exposure to DMI, the aminopeptidase activity was increased as indicated by the time dependent decrease in the

amount of intact ^{3}H-LE recovered after cell challenge associated to the time dependent increase of ^{3}H-T formed.

TABLE 2. Influence of cell exposure to DMI (5 . 10^{-5}M) on cell associated neuropeptidase activity versus exogenous ^{3}H-LE.

TREATMENT	TIME OF EXPOSURE (hrs)	RECOVERY AFTER CHALLENGE ^{3}H-LE (cpm)	^{3}H-T(cpm)
Vehicle	8	6549	2663
	16	6263	2606
	24	6423	2680
DMI	8	5345	3090
	16	4969	4270
	24	3770	5015

At the end of the treatment cells were challen ed for 30 min with 20 nM LE containing 10.000 cpm of intact ^{3}H-LE.

Among the neuropeptidase profile of the various peptidase inhibitors tested, only bestatin was found effective in interfering on DMI induced R-receptors loss of density and function. Table 3 summarizes the effects of bestatin on DMI induced decline of either O-receptors density or ISO stimulated cAMP accumulation in intact cells. In both cases the aminopeptidase inhibitor protected against DMI induced effects suggesting that the newly synthesized peptides mimic the action of exogenous peptides on ß-receptors system.

TABLE 3. Effect of bestatin on DMI induced decrease of R-receptors density and ISO-stimulated cAMP accumulation in intact C6 cells.

TREATMENT (24 hrs)	cAMP [a)] (pmol/mg protein)	^{3}H-DHA BOUND Bmax (fmol/mg protein)
Vehicle	2157 ± 169	255 ± 41
DMI	957 ± 181 *	123 ± 23 *
DMI+bestatin	1691 ± 171 ns	221 ± 35 ns
Bestatin	2258 ± 183 ns	241 ± 47 ns

* P < 0.05 vs vehicle ns: not significant vs vehicle
a) cAMP after 10 min of challenge with ISO 10^{-6}M
DMI concentration was 50 µM, bestatin was 10 µM
Results are the mean ± SE of 3 determinations in duplicate.

At the present time it is not known which is the actual biochemical mechanism involved in the reported effects of cell exposure to DMI. However, all these results could suggest that a common mechanism would activate opioid receptors' appearance, opioid peptide synthesis and aminopeptidase activity. Since the inhibition of endogenous opioid peptide degradation by bestatin prevents DMI induced B-receptor loss it can be speculated that the opioid system activation could be related to specific changes in B-receptor function.

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MU/DELTA OPIOID SITE SELECTIVITY OF SOME ANTIDEPRESSANTS

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ABSTRACT

Since pain and depression are influenced by opioids and antidepressants, it was of Interest to explore their interaction with common CNS receptors. A method for binding site selectivity was used to evaluate antidepressant specificities in inhibiting mu versus delta opioid binding. With the very specific opioid probes used, DAGO and DPDPE, a wide range of antidepressant selectivities was found. These may represent different modes of interaction between mu and delta opioid sites and antidepressant compounds.

INTRODUCTION

Chronic pain and depression are frequently associated, but the mechanisms underlying the efficacy of antidepressants in the management of pain syndromes are not clearly understood. The effectiveness of these drugs in pain relief may be secondary to their antidepressant activity, or may result from their own intrinsic analgesic activity. Since certain antidepressants inhibit opioid binding in vitro (Biegon and Samuel 1980; Somoza et al. 1981; Baraldi et al. 1983; Isenberg and Cicero 1984), it was of interest to examine the mu/delta opioid specificity of the interaction. The site selectivity method (Bárány 1973, Terenius and Wahlström 1976) was used to circumvent the potentially confounding effects of allosteric interactions between μ and δ opioid binding sites (Vaught et al. 1981, 1982; Rothman and Westfall 1982a and b).

MATERIALS AND METHODS

Male Sprague-Dawley rats (2-3 months of age) were decapitated and their brains removed. The forebrains were homogenized in 50 mM Tris-HCl (pH 7.4 at 25°C) in a teflon-glass homogenizer. The homogenate was centrifuged at 27,000 x g for 15 min and the pellet resuspended in Tris buffer (100 ml buffer/g brain tissue). One ml of this crude membrane fraction was used per assay tube; the final assay volume was 1.1 ml. Incubations (25°C, 1 hr) were terminated by filtration over Whatman GF/B filters with a Brandel cell harvester. Tubes and filters were rinsed 3 X 4 ml buffer. Radioactivity was counted in Protosol/Econofluor (New England Nuclear).

The experimental design was based on the site selectively method (Barany 1973; Terenius and Wahlström 1976). DAGO and DPDPE were used as indicator ligands for μ and δ opioid binding sites respectively. In each of two test solutions, both ligands were present at about their K_d values (previously determined to be:

DAGO, 0.4 nM; DPDPE 1.8 nM). In one solution DAGO was in the tritiated form, while DPDPE was radiolabeled in the other. A range of concentrations of the antidepressants examined was incubated in a series of tubes under each test condition. Controls for nonsaturable binding contained nonradlolabeled indicator ligand at a concentration of 0.1 μM. Binding of the indicator ligand in the presence of antidepressant was calculated as a percent of that observed when no test drug was present. The results were graphed according to Bárány (1973) and Terenius and Wahlström (1976).

Tritiated DAGO and DPDPE were from New England Nuclear, while the nonradiolabeled peptides were from Peninsula Labs. Imipramine and desipramine were from Sigma. Other antidepressants were kindly donated: fluoxetine by Eli Lilly, mianserin by Organon, amitriptyline by Merck, chlorimipramine by CIBA-GEIGY and nomifensine by Hoechst.

RESULTS

Previous work has demonstrated the excellent specificity of DAGO and DPDPE for μ and opioid binding sites, respectively (Mosberg et al. 1983; Paterson et al. 1984). In the present study, nonradiolabeled DAGO and DPDPE were also highly selective for the sites labeled by their tritiated analogs (fig. 1). DAGO inhibited only 5% of ^{3}H-DPDPE binding at a concentration which blocked 85% of ^{3}H-DAGO binding, while DPDPE prevented 98% of ^{3}H-DPDPE binding at a concentration which blocked only 5% of ^{3}H-DAGO binding.

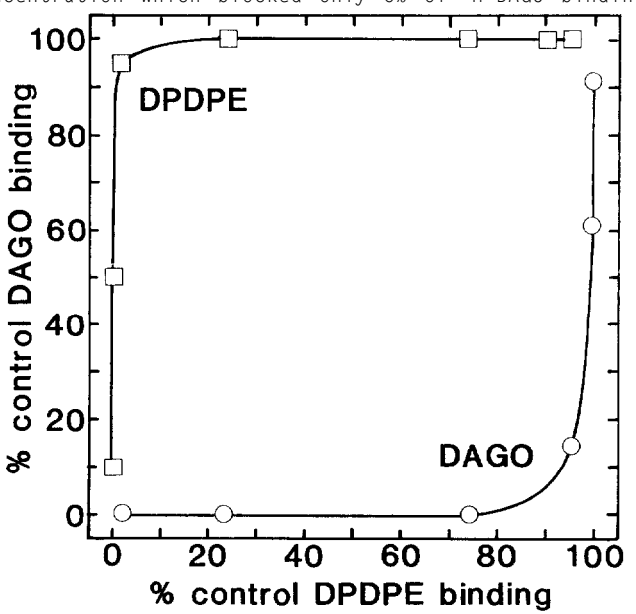

FIGURE 1. Relative inhibition of ^{3}H-DAGO and ^{3}H-DPDPE binding by nonradioableled DAGO and DPDPE (1 nM - 0.1 mM). Each point shown represents the mean of triplicate determinations of specific binding.

The mu/delta site selectivity of several antidepressants was evaluated in this test system and a range of selectivities was observed (fig. 2). Amitriptyline and mianserin competed equally well for DAGO and DPDPE binding sites; the graph of their inhibition data fell on the diagonal, indicating no preference for mu versus delta opioid binding sites. In contrast, fluoxetine had a 10-fold selectivity for sites labeled by the delta site ligand, DPDPE since its data points fell well above the diagonal. The other antidepressants tested Including imipramine, nomifensine, chlorimipramine and desipramine, had greater selectivities for mu binding sites since their competition data points were below the diagonal line (fig. 2). Thus, although all the antidepressants tested inhibited opioid binding in the range of concentrations tested, the drugs varied greatly in their preference for mu versus delta binding sites.

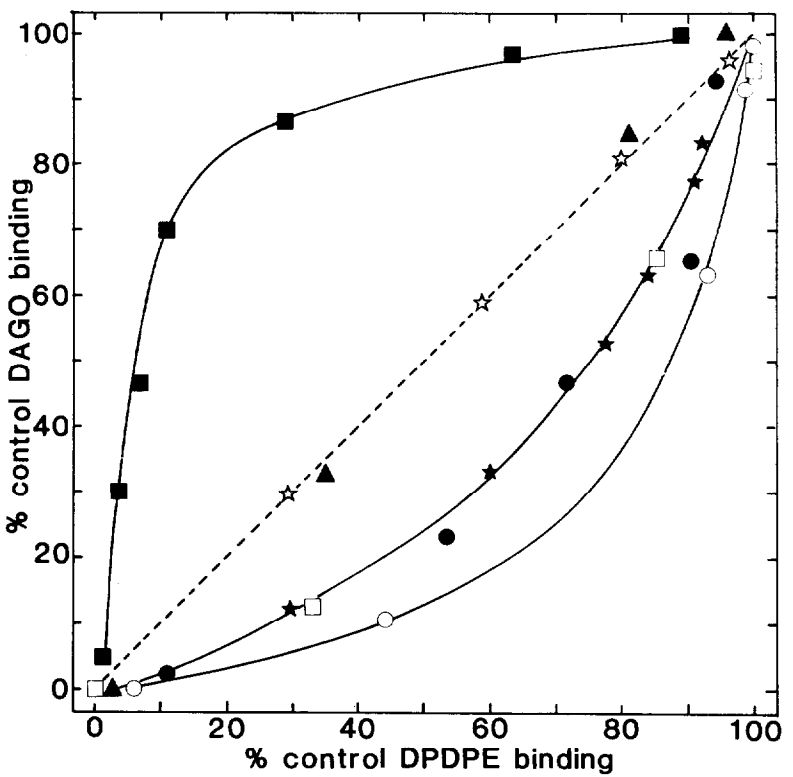

FIGURE 2. Relative inhibition of ^{3}H-DAGO and ^{3}H-DPDPE binding by some antidepressants. ■ - fluoxetine. 1- 300 PM, ▲ -- amitrptyline, 1 - 1000 µM; ☆ -- mianserin 6 - 100 PM; ★ -- nomifensine, 10 - 1000 µM; ● - chlorimipramine, 30 - 1000 µM; □ - imipramine, 0.1 - 1000 PM; 0 - desipramine. 0.1 - 1000 µM. The values shown are the means of triplicate determinations of specific binding.

DISCUSSION

Modulatory interactions between mu and delta ligands have been demonstrated in analgesic responses (Vaught and Takemori 1979; Lee et al. 1980; Vaught et al. 1982) and receptor binding (Vaught et al. 1981; Rothman and Westfall 1982a and b). Because of the proposed allosteric interactions between mu and delta opioid binding sites (Rothman and Westfall 1982a,b; Vaught et al. 1982). the site selectivity method was chosen to investigate antidepressant preference for opioid receptor subtypes. Because the molecular constituents of the test solutions used in this method are identical, their effects on the receptors will be identical (Terenius and Wahlström 1976). Under such conditions, DAGO and DPDPE exhibited a high specificity for their respective binding sites (fig. 1). An almost 100-fold concentration range exists for each ligand within which there is virtually no overlap in ligand binding between sites. Since the K_d of each ligand is well within that concentration range, the site selectively method is a powerful analytical tool when DAGO and DPDPE are used as the indicator ligands at their K_d concentrations.

A range of selectivities was found among the antidepressants tested. Patterns of selectivity included delta preferring (fluoxetine), mu preferring (desipramine, imipramine, chlorimipramine, nomifensine) and unselective (amitriptyline, mianserin). A similar range of preferences was observed by Isenberg and Cicero (1984) using dihydromorphine and DADL as mu and delta ligands. respectively. Presumably the use of more selective indicator ligands in the present study yielded greater discrimination of the selectivities of compounds tested.

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MOLECULAR MECHANISMS OF LIGAND BINDING TO OPIOID RECEPTORS: SELECTIVE INHIBITION BY ETHANOL ?

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ABSTRACT

Effects of varying concentrations of ethanol on the conformations of DAGO, a D-specific opioid ligand were monitored by FT-IR spectroscopy. The binding of ^{3}H-labeled DAGO (u-agonist) and DSLET (δ-agonist) to opioid receptors in the presence of varying concentrations of alcohol was also determined. The results indicate that the conformational features of the ligands are altered and conformational factors may play a role in ligand-receptor interaction.

INTRODUCTION

The concept of multiple receptors for the opioid ligands was originally proposed by Portoghese (1965). However, this concept was more widely accepted only after Martin et al. (1976) proposed that there are at least 3 types of opioid receptors, i.e., μ, κ, and σ from their pharmacological experiments. Later Lord et al. (1977) put forth evidence for a fourth type of receptor, δ. Presently, there are several methods that differentiate the various receptor types (Paterson et al. 1984). Hiller et al. 1981 proposed that alcohol selectively inhibits binding to δ-receptors, as &receptors are more strongly influenced by the alcohol-induced membrane fluidity changes than are the μ-receptors. They further stated that it is likely that alcohol affects the receptor rather than the peptides (ligands). As drug-receptor interaction involves rigid stereochemical and structural requirements, and in view of the importance of the conformational features for drug-receptor interaction, we have previously investigated the effects of ethanol on the secondary structural features of Met^{5}-enkephalinamide (Rapaka et al. 1986). We proposed that conformational features of the ligand may also play a role in drug-receptor interaction. We have presently extended these studies to both μ and δ-specific opioid peptide ligands.

MATERIALS AND METHODS

DAGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) and DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr-OH) were purchased from Bachem Inc., Switzerland.

^{3}H DAGO was purchased from Amersham, Boston, MA, and ^{3}H DSLET was purchased from New England Nuclear Corporation. FT-IR Spectroscopy was performed as reported by Rapaka et al. (1986). Rat brain membrane preparation was prepared as reported by Bhargava and Das (1986).

RESULTS AND DISCUSSION

FT-IR spectra of DAGO were obtained at room temperature with 0%. 2%, and 5% (v/v) ethanol in water. The background subtracted spectra are presented in figure 1. In water, DAGO manifests a doublet amide I band with an intense band at 1651 cm^{-1} and a less intense band at 1636 cm^{-1} with a shoulder around 1670-1680 cm^{-1}. From the conformational dependence of IR amide bands (Renugopalakrishnan et al. 1986; Byler et al. 1986), the intense band at 1651 cm^{-1} is assigned to either α-helical or "unordered" structure, the 1636 cm^{-1} band to ß-sheet structure, and the broad shoulder at 1670-1680 cm^{-1} to ß-turn structure. The occurrence of α-helical structure seems less likely, due to the short peptide backbone of DAGO. Hence, DAGO is expected, based on IR studies, to exist in a conformational equilibrium involving ß-turn, ß-sheet, and "unordered structures. In 2% aqueous ethanol, the pattern of IR bands remains unchanged but the intensities of the bands are slightly diminished. At higher concentrations of ethanol, i. e., 5% (v/v), the resolution-enhanced amide I region is simplified by the occurrence of two bands, an intense band at 1635 cm^{-1} and a less intense band at 1660 cm-1. Although the spectrum is simplified, further studies are required to clarify the details of assignment. In order to correlate the ethanol-induced changes in conformation with receptor binding parameters, binding of the tritiated ligands to receptors in the rat brain membrane preparation was studied. The effect of alcohol on binding of the tritiated ligands to the rat brain membrane preparation is presented in table 1.

TABLE 1

Effects of Varying Concentrations of Alcohol on the Binding of ^{3}H-Labeled Opioid Peptides to Opioid Receptors.

Alcohol Concentration % v/v	Specific Binding ^{3}H DAGO % Control	Specific Binding of ^{3}H DSLET % Control
Control	100	100
1	63.1	89.6
2	59.0	83.0
3	54.6	70.8
5	43.1	48.5

In our earlier studies (Rapaka et al. 1986), we investigated the conformational changes of Met^5-enkephalinamide upon the addition of different concentrations of ethyl alcohol to an aqueous solution

of Met^5-enkephalinamide. Met^5-enkephalinamide is an ideal molecule for these studies, as it exists in both ß-turn and ß-sheet conformations. FT-IR is ideally suited to follow the conformational transitions as changes in both ß-turn and ß-sheet conformations can be monitored simultaneously and the technique is simple and fast. Hence, in our continuing studies FT-IR was the method of choice.

Schiller and colleagues (Schiller and DiMaio 1980; DiMaio and Schiller 1980; DiMaio et al. 1982), from their studies on cyclic enkephalin analogs and the corresponding linear analogs, proposed that the opioid receptor subtypes differ in their conformational requirements of the ligand. Later several groups proposed that indeed the conformational features such as the ß-turns, ß-sheets, or other extended structures may play a role in receptor subtype recognition (for a review see Rapaka et al. 1985). Ethanol is

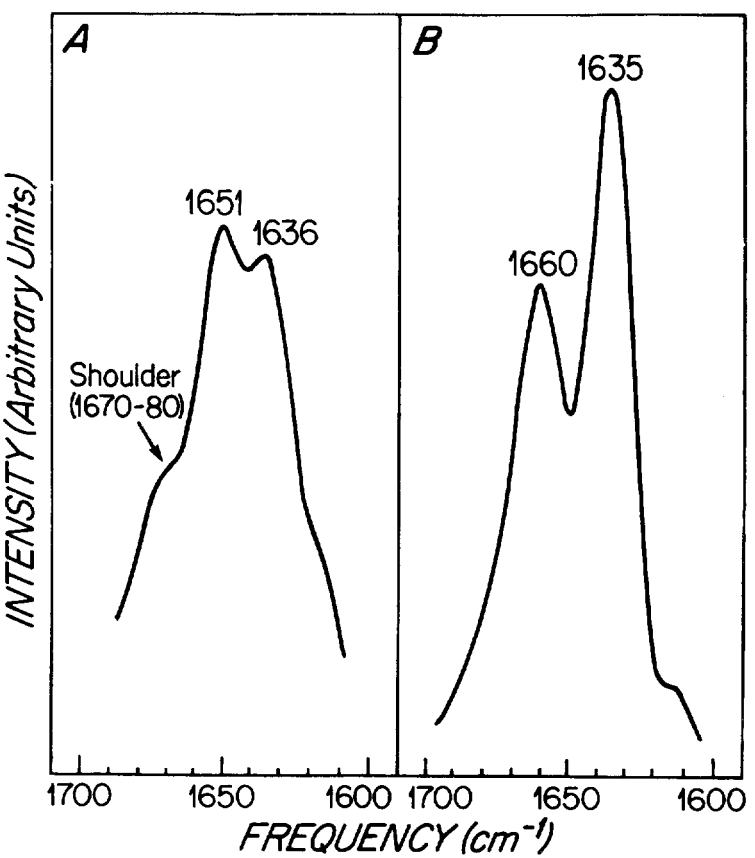

Figure. 1. Resolution-enhanced FT-IR spectra in the amide I region of DAGO in water (3 mM) at room temperature and in the presence of ethanol. (A) without ethanol; (B) with 5% ethanol.

known to induce conformational changes in polypeptides and proteins (Mishra and Ahluwalia 1983; Conio et al. 1970). Although Hiller et al. (1981) believe that alcohol may not affect the peptide, but rather the receptor, it appears likely that the effect may be primarily on the ligand. The ligands are usually small molecules and, especially, the enkephalins and their analogs are only pentapeptides and are known to exist in a conformational equilibrium. The FT-IR results indicate that both DAGO and Met^5-enkephalinamide exist in a conformational equilibrium involving ß-turns and ß-sheets. Addition of alcohol destabilizes these structures. Binding studies indicate that binding to both μ- and δ-ligands is inhibited in the presence of alcohol. We believe, the inhibition of binding by alcohol is not specific to δ-ligands only. Finally, it is also possible that alcohol may induce conformational changes in both the ligand and the receptor. Hence, the inhibition of binding may be due to loss of conformational features of the ligand or the receptor or both. Further studies are in progress.

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OPIOIDS AND INTAKE OF ALCOHOLIC BEVERAGES

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ABSTRACT

Rats were given a daily opportunity to consume a sweetened ethanol solution (ES) or water until they took about 2.0 g/kg of pure ethanol (E) during 1.5 hr/day. Then, rats were given one of a number of opioids before an opportunity to drink. Some opioids, e.g., morphine (MOR), methadone, and fentanyl, at small doses, increase ES-intake; some decrease intakes, e.g., naloxone (NX) and MR 2266. Diprenorphine, a mixed agonist-antagonist having no analgesic potential and blocking other opioids' analgesia, increases ES-intake across a wide range of doses beginning with 0.003 mg/kg.

INTRODUCTION

From older studies of a time when people could legally buy opium as well as alcohol (Seigel 1985), to studies of E's metabolism (Davis and Walsh 1970, Cohen and Collins 1970) and modern studies of receptor-dynamics (e.g., Hiller et al. 1981, Tabakoff 1981), a relationship between processes of opioids and E have been proposed. Not all E's effects are mediated by way of opioid receptors, since naloxone (NX) has limited or no effects on many E-induced events (Severs 1970). E has, however, many effects and endogenous opioids are multifaceted. It is unknown, however, whether or not all effects of E are unrelated to opioid-mechanisms. Since, there are no known E-receptors, E may achieve its ability to sustain its own use by way of opioid-mechanisms. If this is the case, at least some opioids should increase and NX should decrease E-intake. Large doses of morphine (MOR), however, decreased E-intake (Sinclair 1974) and NX reduces intake of many ingesta including ESs (Reid 1985). So, these early studies of opioids' effects on E-intake did not support the idea that opioids had specific effects on taking ES.

A problem with testing drugs' effects on E-intake is that rats do not readily take meaningful amounts of ESs across the brief time that relevant doses of drugs might be effective. To overcome this, a sweetened ES was used and it was found that rats eventually take, over days, substantial amounts of E during brief daily opportunities to do so. After rats were taking considerable ES, small doses of MOR (e.g., 2 mg/kg) were tested and they produced marked increments in E-intake. Studies of the small-dose-MOR-effect indicate that (a) the increment in intake is not related to flavoring of ES, (b) it does not

tolerate, and (c) it is not dependent on variables such as housing conditions, age, or sex of the subjects (Hubbell et al. 1985, Reid et al. 1986). ES-intake was also shown to be more sensitive to NX than water-intake. So, the basic theoretical requirement of an agonist increasing and an antagonist decreasing E-intake was met. Also, benzodiazepines and a barbiturate did not produce similar effects and a dopamine receptor antagonist did not modify the intake of an ES (Mudar et al. 1986). These initial results led us to test other opioids and some of those tests are reported here.

MATERIALS AND METHOD

Male, Sprague-Dawley rats were placed on a daily regimen for more than 21 days before testing. The daily regimen involved water-deprivation until the time of presentation of ES and water for 1.5 hr/day (food was always available). ES was 5 g sucrose, 12 g pure E and water added to yield 100 g of solution. Across days, rats gained weight as those given unlimited water. So, these healthy rats can take or not take ES. At first, rats take very little, but, across days they take more and more until they take about 2.0 g/kg of E during the 1.5 hr-period (an amount often producing visible drunkenness).

After stable intakes are achieved, rats were given placebo, and then the next day, an opioid. Subsequent to a test of one dose, a number of days intervened and then another placebo and dose were tested. Rats were given a limited number of doses before being retired to avoid carryover-effects; although in direct tests for carryover, we have yet to observe any as long as 2 days intervened between tests. Each dose was tested with 10 or more rats. Injections were given subcutaneously, 15 min before a test. Doses range from very small to those large enough to interfere with drinking of any kind. Consequently, the largest doses of all opioids are expected to reduce intakes.

A number of indices of rats' intake of E are possible, e.g., (a) preference ratio, i.e., the amount of ES taken/total fluid-intake, or (b) g of pure E taken/kg of body weight (g/kg). Since these two measures were highly correlated, only g/kg are reported in detail here, but preference ratios are mentioned when they give a different perspective. The mean difference between intake under placebo and a dose is determined and dependent t-tests calculated.

RESULTS AND DISCUSSION

Small doses of MOR as well as small doses of methadone and fentanyl increase intake of ES. With large doses of MOR, preference ratios are often increased since large

doses reduce intake of water more than ES. These agents can be analgesics and are readily self-administered. Not all opioid analgesics, however, increase E-intake, e.g., ethylketocyclazocine (EKC) did not, except in a narrow range of doses. Also, levorphanol and dextrorphan both increase E-intake at small doses. NX reduces E-intake. When MOR and NX are given concurrently, the resultant E-intake is as if NX alone was given. MR2266, in doses similar to NX. decreases intake.

Diprenorphine is not an analgesic and blocks other opioids' analgesia. Diprenorphine, however, increments pressing for rewarding brain stimulation and establishes a conditioned place preference, both of which are indices of an opioids' ability to induce positive affect (Beaman et al. 1984, Pollerberg et al. 1983). Diprenorphine across a wide range of doses increases E-intake and, if anything, reduces water-intake. When diprenorphine and NX are given concurrently, the effect is that of NX.

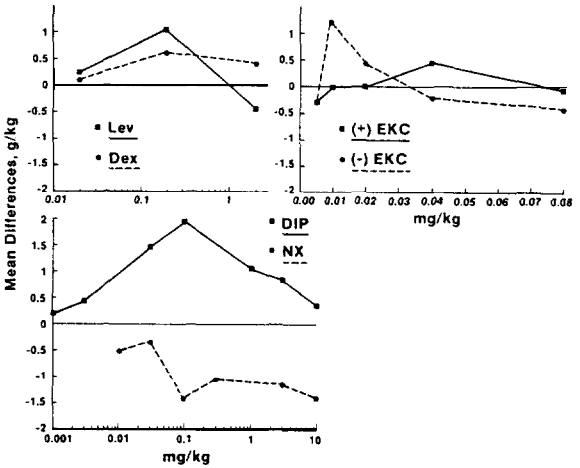

FIGURE 1. Mean difference scores are depicted (drug-placebo), so a positive score indicates the dose produced that increment in g/kg of E-intake. Only the value for 0.2 mg/kg for levorphanol tartrate (LEV) and 0.2 and 2.0 mg/kg dextrorphan tartrate (DEX) represent reliable ($p < 0.05$) differences. Only the value for 0.1 for (-)Ethylketocyclazocine (EKC) is reliably different than placebo-scores of all the values plotted in the top-right panel. Doses greater than 0.3 mg/kg of NX HCl represents reliable decreases in E-intake. All values representing diprenorphine's effects, except the smallest (0.001 mg/kg) and the largest dose (10 mg/kg), are statistically significant. Doses are represented as the salt, except for diprenorphine and EKC.

When diprenorphine and MOR are given concurrently, the increase in E-intake is greater than when either is given alone. Diprenorphine increases intakes of unflavored ES (Reid et al. 1986). It is clear, small doses of some opioids produce marked increments in E-intake. The pattern of different opioids' effects indicate that the ability to increment E-intake is not related to opioids' analgesic potential: some analgesics produce decreases in intake and others little or no effect. Diprenorphine, an antagonist with respect to analgesia, produces increases. A pattern that seems to relate to opioids' enhancement of drinking ES is the pattern of opioids' effects on pressing for rewarding brain stimulation. In general, the doses of opioids that increase pressing also increase E-intake.

These data lead to the conclusion that an opioid-mechanism could be involved in E's ability to sustain its own intake and involved in promoting "excessive" E-intake. Perhaps, it is the same mechanism that is involved with increasing pressing for rewarding brain stimulation. The receptor for which diprenorphine is an agonist, which is also sensitive to NX's antagonism, seems particularly germane.

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(+)-NALOXONE: ANTAGONISM OF PENTOBARBITAL-INDUCED NARCOSIS

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ABSTRACT

Intracerebroventricular injection of (+)-naloxone shortened pentobarbital-induced sleeping times in rats. It does not appear that opiate receptors regulate the duration of narcosis.

INTRODUCTION

The role of opiate receptors and the endogenous opioid peptides in the production of general anesthesia is unclear. Intracerebroventricular administration of racemic (+)-naloxone dose-relatedly shortened the duration of sleeping time induced by pentobarbital, halothane and ketamine in rats. The fact that equivalent molar doses of naltrexone, a more potent opiate antagonist, had no antianesthetic effect suggested that naloxone's activity was unrelated to an opiate receptor mechanism (Kraynack and Gintautas 1982). Naloxone exhibits biopharmacological effects unrelated to opiate receptor activity. Parallel experiments with the inactive enantiomer, (+)-naloxone, may provide additional evidence concerning the opioid agonism theory of anesthesia.

This study was designed to investigate the modification of narcosis in laboratory animals by the inactive enantiomer of naloxone, (+)-naloxone. The effects of centrally administered (+)-naloxone on the duration of sleeping time (ST) has been determined in rats anesthetized with pentobarbital.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Indiana) weighing 125-150 g, were given an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Immediately after the loss of righting reflex (LRR), groups of rats received an intracerebroventricular (ICV) injection of 120 or 240 ug of (+)-naloxone according to a method described previously (Kraynack and Gintautas 1982). Control ST were determined in rats receiving 0.9 percent saline ICV. A total volume

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of 12 or 24 microliters of the test drug or 0.9 percent saline was injected into the right lateral ventricle of the brain. The (+)-naloxone was dissolved in 0.9 percent saline for injection. There were 10 rats per treatment group. Awakening was defined as regaining the righting reflex (RRR). Sleeping time (ST), defined as the period between LRR and RRR, was recorded for all experimental groups.

Data were expressed as the mean ± SEM. Bonferroni (DUNN) t- test was used to evaluate pairwise differences of naloxone induced changes. Critical differences was set at alpha = 0.05. Significant differences between values was assumed when $p<0.05$.

RESULTS

There was no statistically significant difference between mean sleeping times of saline treated rats receiving 12 or 24 ul injections. The data were pooled for comparisons. The mean sleeping time induced with pentobarbital (ICV saline treatment) in this study demonstrated no statistically significant differences from that previously reported by us (pairwise differences $p<0.05$; t-test).

Intracerebroventricular administration of (+)-naloxone shortened the ST duration of rats anesthetized with pentobarbital. A dose-related shortening of ST was not observed. Sleeping time was shortened by 17 and 21 percent in rats receiving 120 or 240 ug ICV of (+)-naloxone (table 1).

Treatment group	Mean Sleeping Time (ST) (min ± SEM)
Saline	140.9 ± 3.7
(+)-naloxone	
120 ug	116.3 ± 6.9*
240 ug	111.0 ± 12*

*Significantly different from control at 0.05 level

Thirty and forty percent of the rats in the groups treated with 120 or 240 ug, respectively, of (+)-naloxone died. These animals exhibited gasping respiratory movements accompanied by cyanosis, defecation and micturition.

DISCUSSION

The present study clearly demonstrates that centrally administered (+)-naloxone shortens ST induced by pentobarbital. Enantiomeric specificity was not observed. As shown by Ijima et al. (1978), (+)-naloxone has no more than 1/1.000 - 1/10,000th the activity of (-)-naloxone as an opiate receptor antagonist in severe1 in vitro systems. Our studies revealed that siqnificantly higher doses of (+)-naloxone, and (+)-naloxone are needed to shorten ST, than would be expected if the drugs acted strictly as opiate receptor antagonists. In addition, naltrexone, a more potent narcotic antagonist, has no antianesthetic effect in rats anesthetized with pentobarbital, halothane or ketamine. In accordance with our data, is the fact that barbiturates do not modify the stereo-specific opioid receptor binding of ^{3}H-naloxone (Pert and Snyder 1973).

The mechanism of naloxone's antianesthetic action remains obscure, but we may conclude that narcosis (as measured by the righting reflex), the predominant effect of general anesthetics, is not related to pharmacologic competition for opiate receptors. Clearly, pentobarbital does not mediate its effect on the righting reflex (i.e., narcosis) through pharmacologic antagonism of a receptor sharing comparable affinities for naloxone, naltrexone and morphine. Furthermore, Way et al. (1984) found no evidence that anesthetics act by increasing endogenous opioid peptide activity and concluded that analgesia associated with general anesthesia is not mediated by beta-endorphin; nor, as suggested by Shingu et al. (1981) do general anesthetics alter sensitivity or facilitate opioid peptide actions on nociceptive neurons. These findings concur with those of Deady et al. (1981) who concluded that the righting reflex is depressed through a mechanism separate from that responsible for analgesia.

Consequently, these in vivo findings enhance our understanding of the many inconsistant and often contradictory results reported concerning naloxone antagonism when different indices (narcosis, analgesia, cardiorespiratory effect) are used. The inference that these effects of general anesthesia share a common mechanism mediated by opiate receptors appears unwarranted.

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RAT STRIATAL DOPAMINE RELEASE MECHANISMS OF COCAINE

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ABSTRACT

Centrally problematic in the study of drugs of abuse is the addictive process itself. Addictive drugs affect dopaminergic nigrostriatal and mesolimbic neuronal circuitry during brain reward. These findings show that cocaine produces a decrease in dopamine release from rat striatum and prevents the surge of dopamine release which occurs post-mortem. The results suggest that dopaminergic control over striatal neuronal membrane may be important in neurochemical mechanisms underlying brain reward.

INTRODUCTION

Cocaine's commonality with opiates is "abuse potential." A neuronal reward circuitry in brain exists (Olds and Milner 1954) and activation of this circuitry is necessary for reinforcement (Wise 1982). Psychomotor stimulants and opiates both appear to have their effect in the nigrostriatal and mesolimbic areas of the descending reward fibers of the medial forebrain bundle (Corbett and Wise 1980;Wise 1981). Whereas opiates affect the level of cell bodies, stimulants affect terminals of the dopaminergic nerve fibers (Wise 1984).

The critical response for the rewarding property of cocaine is an action at the dopaminergic synapse. Cocaine actually prolongs the activity of dopamine by presynaptic reuptake inhibition, an action shared by amphetamine (Fuxe et al. 1967). Although amphetamine has been shown to augment dopamine release (Broderick et al. 1983), there have been no previous in vivo studies on the dopaminergic release mechanisms of cocaine. The present study serves to further elucidate the relationships between cocaine, striatal dopaminergic presynaptic mechanisms and brain reward.

METHODS

The effect of cocaine (20 mg/kg sc) on dopamine release from striata of male, Sprague-Dawley rats was studied by semiderivative electroanalyses, an in vivo electrochemical methodology. Chloral hydrate, anesthetized rats (body temperature maintained at 37° C) underwent stereotaxic surgery for positioning of a teflon-coated working graphite electrode (150-175μ) (stearate modification) (Blaha and Lane 1983) in anterior striatum. A Ag/AgCl reference electrode and stainless steel auxiliary electrode was placed in contact with the rat cortex. Semiderivative voltammograms were recorded every ten minutes.

Potentials were applied between -200 and +500 mv, at a scan rate of 10 mv sec^{-1}. In calibration experiments, the electrochemical response increased linearly with elevated concentrations of dopamine without interference from serotonin, other amines, associated metabolites and ascorbic acid.

RESULTS AND DISCUSSION

The results show that cocaine, at a dose sufficient to produce euphoria, caused a decreased dopamine release from rat striatum. The dopamine signal returned to normal within the hour period studied. These results are consistent with the known half-life of cocaine (Nayak et al. 1976).

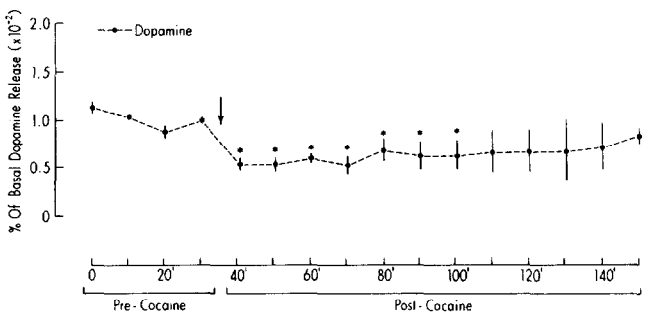

FIGURE 1. A line graph showing the time course characteristics of the effect of cocaine (20 mg/kg sc) on dopamine release from rat striatum. The x axis represents time (in minutes) before and after the administration of cocaine. The y axis represents dopamine release as percent of control. The percent of controls were calculated by averaging the first four scans and dividing all values by that average. This was done for each animal. The results were considered statistically significant ($p<0.05$) if the data points were outside the 95% confidence limits of the control.

Other addicting drugs, such as morphine and enkephalinamides, also decrease dopamine release from rat striatum (Broderick et al. 1983; Broderick et al. 1984;Broderick 1985). Post-mortem studies of rat striatal dopamine re-

lease after cocaine treatment show that cocaine caused an inhibition of the significantly increased surge of dopamine release which occurs at death (Phebus et al. 1986; Broderick 1986).

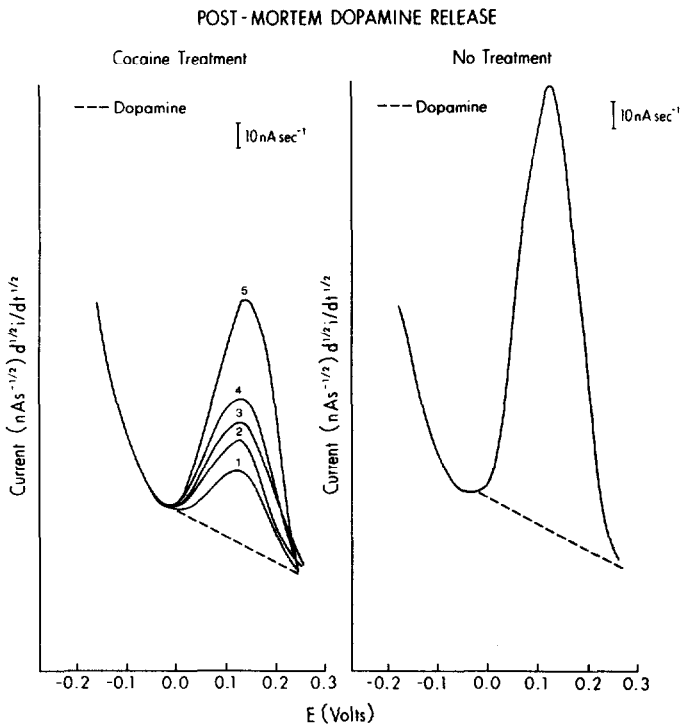

FIGURE 2. Semiderivative voltammograms showing post-mortem differences between dopamine release in cocaine treated (left voltammogram) and untreated animals (right voltammogram). The x axes represent increasing oxidation potentials in volts; the y axes represent current in nA sec^{-1}. The electrochemical signal for dopamine (1) represents dopamine release pre-mortem. The electrochemical signals for dopamine (2,3,4,5) represent dopamine release post-mortem, (ten minute intervals), in a cocaine treated animal. In contrast, the right voltammogram represents dopamine release ten minutes post-mortem in an untreated animal. The signal is increased approximately six-fold over that dopamine release indicated by voltammogram (1) pre-mortem.

Taken together with previous studies which show that cocaine inhibits reuptake (Fuxe et al. 1967), these findings lend explanation to a report showing unchanged striatal dopamine concentrations after cocaine (Lasley et al. 1985). In aqreement with others (Rice et al. 1985), these studies show that in vivo electrochemistry primarily estimates release. Finally, the results suqqest that control of dopaminergic function, perhaps across membrane (Sershen et al. 1984), may be as important in brain reward mechanisms than is augmentation of dopaminergic function.

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CENTRAL CLUCACON ANTAGONIZES MORPHINE- AND STRESS-INDUCED ANTINOCICEPTION IN THE MOUSE

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ABSTRACT

Previously, we have demonstrated that glucagon antagonizes morphine-induced antinociception in the rat (Malcolm et al. 1985). In the present studies we have shown that central injection of glucagon antagonizes morphine- and stress-induced antinociception in the mouse. Since stress-induced antinociception is mediated by the activation of endogenous opioid systems, these findings provide the first evidence for glucagon's ability to antagonize endogenous opioid actions.

INTRODUCTION

Clucagon and its receptor have been identified within the mammalian central nervous system (CNS)(Tager et al. 1980, Hoosein and Curd 1984). However, with the exception of reports indicating that intracerebroventricular (icv) injections of glucagon elevate circulating levels of somatostatin (Abe et al. 1978) and glucose (Marubashi et al. 1985), very little is known about the physiological and pharmacological actions of glucagon in the CNS.

We have previously reported that central injection of glucagon antagonizes morphine-induced bradycardia and antinociception in the rat (Malcolm et al. 1986) suggesting that glucagon may functionally interact with endogenous opioid systems. This interpretation is supported by the observations that (1) glucagon and opioid receptors share many similarities in neuroanatomical distribution (Akil and Watson 1983, Hoosein and Curd 1984) and (2) other brain-gut peptides (e.g. ACTH, somatostatin, cholecystokinin) have been shown to possess some anti-opioid properties (Terenius 1975, 1976, Faris et al. 1983, ltoh et al. 1982). In the present studies we sought to determine whether central injection of glucagon antagonizes opioid actions in another species, the mouse, and furthermore to determine whether central glucagon can modulate the action of endogenous opioid systems. Since endogenous opioid systems are thought to mediate immobilization stress-induced antinociception, we chose to test glucagon's ability to antagonize stress-induced antinociception as a means to assess glucagon's effects on endogenous opioid actions.

MATERIALS AND METHODS

All studies were carried out using male mice (ICR, CD-1 strain) weighing approximately 30 g and having free access to food and water. Central (icv) injections of drugs were performed in conscious mice according to the method of Haley and McCormick (1957). Immobilization stress was induced by restraining animals for 30 minutes in a restraining apparatus for mice (Amir

et al. 1981). Animals were injected icv with either saline (control) or glucagon (1 or 10 µg, n=5-10; Eli Lilly and Co., Indianapolis, IN) just prior to subcutaneous (SC) injection of morphine sulfate (5 mg/kg, n=8; Merck and Co., Inc., West Point, PA) or immobilization. Thirty minutes after morphine or 30 min after the onset of immobilization stress, animals were placed on a hot plate (52°C) and jump/escape latencies were recorded. Maxiumum time allowed for an animal to remain on the hot plate was 90 seconds.

Data were analyzed using analysis of variance followed by Duncan's Multiple Range test for comparison. The level of statistical significance was chosen at $p < 0.05$.

RESULTS AND DISCUSSION

Compared to control nociceptive response latencies (29.3±11 sec), morphine administration elevated jump/escape latencies 2-fold (65.1±11 sec) by 5 minutes post-injection (fig. 1). Pretreatment with 10 but not 1 µg glucagon icv significantly attenuated morphine-induced antinociception at 15 and 30 minutes by 66% and 68%, respectively. Mice not treated with morphine (fig. 1, saline/saline group) exhibited a progressive decrease in nociceptive response latencies when tested over time, and this decrement most likely reflects the normal learning curve for these animals. These findings in the mouse confirm our previous observations in the rat and support the hypothesis that central glucagon antagonizes morphine actions and furthermore indicate that glucagon's anti-opioid effects occur across species.

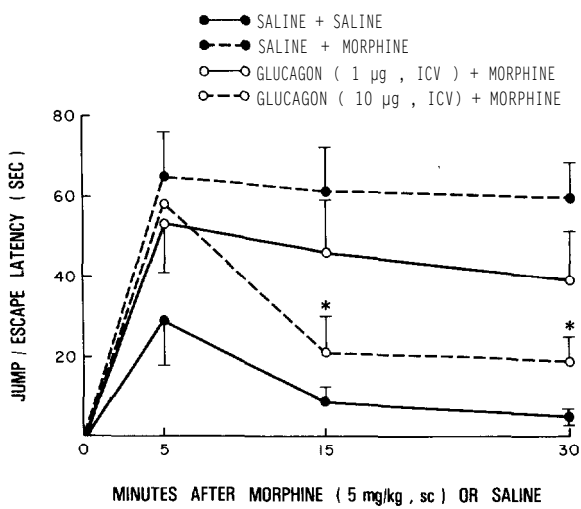

Figure 1. Effects of icv injection of glucagon on morphine-induced antinociception in the mouse. Clucagon at 10 µg but not 1 µg icv, significantly ($p<0.05$) attenuated morphine-induced antinociception at 15 and 30 min as compared to the saline plus morphine-treated group (n=5-10).

Thirty minutes of immobilization elevated nociceptive response latencies 2.3-fold from 32.6±6 to 76.6±8 sec. (fig. 2). Pretreatment with 1 but not 10 µg glucagon icv completely prevented the stress-induced antinociception (fig. 2). In both studies, glucagon treatment (1 and 10 µg, icv) alone did not alter baseline nociceptive response latencies at the times tested. This suggests that glucagon's antagonism of these opioid effects is not due to a direct lowering of the threshold to pain but rather may represent a functional antagonism between glucagon and the opioids. Since glucagon receptors have been localized in areas of the brainstem (Hoosein and Curd 1984) known to be involved in the regulation of pain transmission, it is possible that glucagon's antagonism of opioid-induced antinociception may be mediated through glucagon receptors at these brainstem sites.

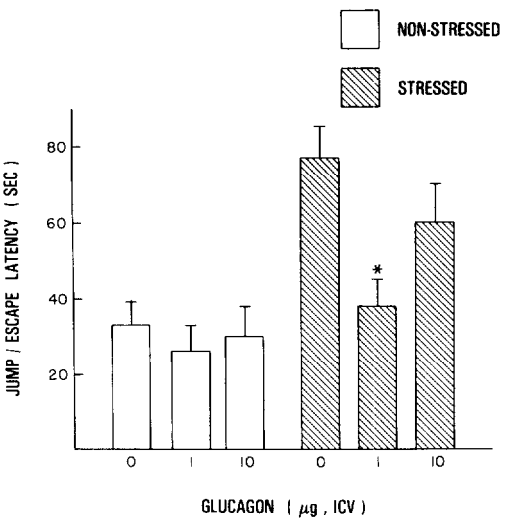

Figure 2. Effects of icv injection of glucagon on stress-induced antinociception in the mouse. Clucagon at 1 µg but not 10 µg, icv, completely prevented the stress-induced antinociception at 30 minutes post-injection. Clucagon treatment alone (1 and 10 µg, icv) did not alter baseline nociceptive response latencies at the times tested.

In summary, these data confirm our previous findings in another species and more importantly demonstrate that central glucagon blocks stress-induced ant inociception. Since stress-induced antinociception is mediated by the activation of endogenous opioid systems, these findings provide the first evidence for glucagon's ability to antagonize endogenous opioid actions.

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ENDORPHINS CONTRIBUTE TO THE LOSS OF GLUCOSE HOMESTASIS IN ANAPHYLACTIC SHOCK

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ABSTRACT

Central injection of naloxone attenuated the development of hyperglycemia following induction of non-fatal anaphylaxis in mice, but it failed to reverse the hyperglycemia in this model. In contrast, central TRH both blocked as well as reversed the hyperglycemic response. These results suggest a possible role for central endorphin mechanisms in the hyperglycemic response to anaphylactic shock in mice. Further, they demonstrate a novel action of TRH to block shock-induced hyperglycemia. This antihyperglycemic action of TRH is independent of opiate mechanisms.

INTRODUCTION

Endorphins have been implicated in the cardiovascular depression of circulatory shock because treatment with the opiate antagonist naloxone reversed the hypotension and improved survival in several experimental shock states, including endotoxic, hemorrhagic, spinal, and anaphylactic shock (Holaday 1983). More recent studies have shown that endorphins may also contribute to the glucose dishomeostasis which characterizes shock since naloxone was found to block the hyperglycemic response to endotoxin administration (Amir and Harel 1983) or hemorrhage (Bereiter et al. 1983). The involvement of endorphins in the hyperglycemic response to shock was further investigated in the present study by evaluating the effect of central or systemic naloxone on the plasma glucose responses to non-fatal anaphylaxis in mice. In addition, the effect of the physiologic opiate antagonist thyrotropin-releasing hormone (TRH) on the plasma glucose responses to anaphylaxis was investigated. TRH was found in earlier studies to block fatal anaphylaxis in mice (Amir et al. 1984) and to antagonize opiate-induced hyperglycemia in this species (Amir 1985).

MATERIALS AND METHODS

Subjects were normally fed male ICR mice, 28-30 g. Anaphylaxis was induced by subcutaneous (SC) injection of the histamine releaser compound 48/80 (C 48/80 [Sigma] 4 mg/kg in 0.2 ml saline). Naloxone (Endo) and TRH (Sigma) were dissolved in sdline and injected intracerebroventricularly (ICV) or intravenously (IV) one min before or 15 min after C 48/80. The ICV injections were made according to the method of Haley and McCormick (1957) using a Hamilton microsyringe bearnig a 27 gauge needle 2.5 mm long. Animals were sacrificed by decapitation at different times after C 48/80 administration. The trunk blood was collected and centrifuged to obtain plasma. Plasma

glucose concentrations were detenmined by the glucose oxidase method using a Bekckman glucose analyzer.

RESULTS AND DISCUSSION

Induction of anaphylaxis by SC challenge with 4 mg/kg C 48/80 resulted in a rapid and sustained increase in the levels of circulating glucose (fig. 1). This hyperglycemia was attenuated by 10 ug naloxone given ICV one min before C 48/80. In contrast, naloxone increased the plasma glucose of shocked mice when given IV (100 ug) one min before C 48/80 (fig. 2).

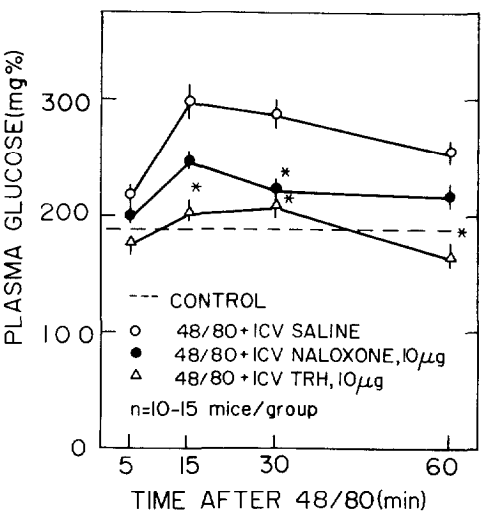

FIGURE 1. Effect of ICV naloxone or TRH pretreatment on plasma glucose of C 48/80-treated mice. The symbols and vertical lines represent means ± SEM of plasma glucose concentrations. Asterisks indicate significant difference from C 48/80 alone ($p<0.05$ t tests).

As with naloxone, ICV pretreatment with 10 ug TRH strongly blocked C 48/80-induced hyparglycemia (fig. 1). However, TRH also blocked the hyperglycemic response when given IV (100 ug, fig. 2).
Finally TRH (1 ug ICV), but not naloxone (1 ug ICV), reversed the hyperglycemic response to C 48/80 when given 5 min after induction of shock (fig. 3).

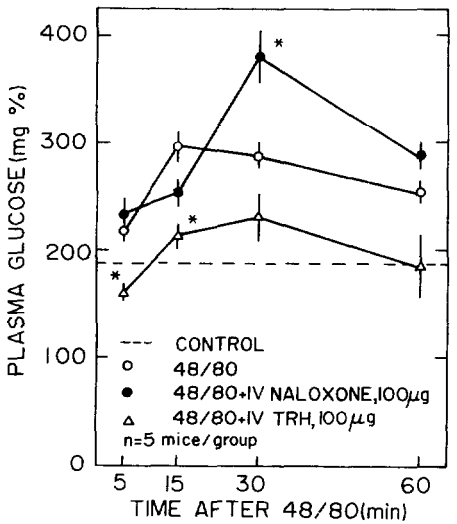

FIGURE 2. Effect of IV naloxone or TRH prtreatment on plasna glucose of C 48/80-treated mice. See legend to fig. 1 for details.

The results with naloxone suggest that activation of edorphin mechanisnms in the CNS may contribute to the loss of glucose homeostasis and development of hyperglycemia following induction of anaphylactic shock in mice. Morever, failure of systemic naloxone to mimic the central action of the drug on hyperglycemia further suggests that endorphins may affect central and peripheral glucoregulatory mechanisms differentially. In contrast to naloxone TRH not only blocked C 48/80-induced hyperglycemia but also rapidly reversed it. This suggests that the antihyperglycemic action of TRH is independent of endorphin mechanisms. Thus, while naloxone probably affects the development of hyperglycemia following C 48/80 by antagonizing a central opiate receptor-mediated action of endorphins to stimulate glucose production (Van Loon and Appel 1981), TRH may act in the CNS to physiologically oppose the hyperglycemia by stimulating insulin secretion and enhancing glucose uptake. This possibility is consistent with recent finding that TRH lowers the plasma glucose of normoglycemic mice by a parasympathetic-mediated insulin-dependent mechanism (Amir et al. 1985).

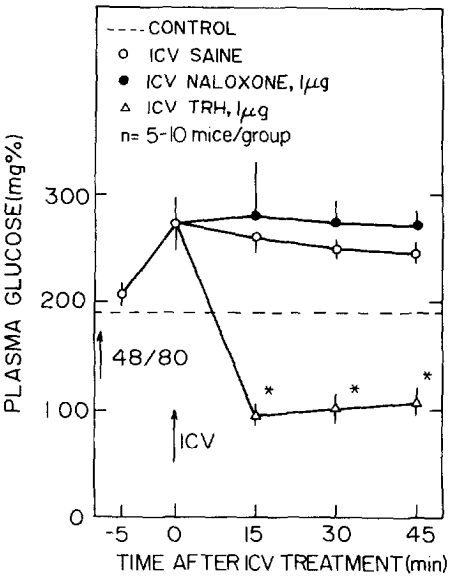

FIGURE 3. Action of ICV naloxone or TRH to reverse C 48/80-induced hyperglycemia. See legend to fig. 1 for details.

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EFFECTS OF OPIOID ANALGESICS ON LOCAL CEREBRAL GLUCOSE UTILIZATION

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ABSTRACT

The autoradiographic 2-deoxy-D-[1-[^{4}C]glucose ([14-C]DG) method was used to map the effects of morphine, oxymorphone and nalbuphine on local cerebral glucose utilization (LCGU), an index of local brain function. The mu agonists (morphine, oxymorphone) decreased LCGU in thalamic nuclei, including some which have been implicated in somatosensory processing. Nalbuphine did not produce these effects, but stimulated LCGU in nuclei of the spinal trigeminal tract. The findings suggest that different supraspinal mechanisms mediate the actions of mu vs. kappa opioids, and that the [14-C]DG procedure is helpful in elucidating the anatomical areas involved.

INTRODUCTION

Opioid analgesics vary in their spectrum of side effects and abuse potential. The differences may reflect varying activities at opioid receptor subtypes. Oxymorphone is a relatively pure mu agonist, morphine has mu and kappa agonist properties, and nalbuphine is a partial agonist at ka a receptors with minimal sigma agonist and mu antagonist properties (Jaffe and Martin 1985). The purpose of this study was to compare the distributions of the in vivo effects of these drugs on rates of LCGU, an index of local cerebral function. Studies of LCGU have been used to elucidate the in vivo metabolic responses to drugs of various classes, including dopaminergic agents (McCulloch 19821, cholinomimetics (Dam et al. 1982), and GABA-mimetics (Palacios et al. 1982).

MATERIALS AND METHODS

Male Fischer-344 rats, 3 mo of age (Charles River Breeding Laboratories) were acclimated to a vivarium (25°C, 12h:12h light:dark cycle) at the Addiction Research Center for about 4 weeks before the drug studies. [14-C]DG, specific activity 59 mCi/mmol, Amersham Corp.) was rechromatographed to ascertain purity. Oxymorphone HCl and nalbuphine HCl were supplied by DuPont Pharmaceuticals; morphine sulfate was obtained from Mallincbrodt. Inc.

The rats were fasted overnight before being prepared with indwelling, femoral venous and arterial catheters under light halothane anesthesia. After surgery, they were placed in a sound-insulated box where they were allowed to recover from surgery for about 3 h before drug treatments.

Opioids were administered s.c. 15 min before the radiotracer. Control animals received s.c. injections of 0.9 % NaCl. Effects on arterial blood gases and pH were assessed at several times.

LCGU was determined after injection of [14-C]DG (125 μCi/kg body weight, i.v.) as previously described (Sokoloff et al. 1977). arterial blood samples were collected for assesment of plasma glucose and [14-C]DG. Animals were killed by a pentobarbital overdose 45 min after the [14-C]DG injection, and brains were rapidly removed and frozen in 2-methylbutane at -50°C.

Frozen 20 μM cryostatic sections were cut from the brains at -18 to -20°C thaw-mounted onto glass coverslips, and exposed to x-ray film (Kodak SB5) for one week in a cassette containing [14-C]methyl-methacrylate standards. Brain radioactivity was determined by quantitative autoradiography using a Leitz microscope and the MPV-DADS scanning microdensitometry system (E. Leitz, Inc.). Brain structures were identified using standard rat brain atlases. LCGU was calculated from brain and plasma radioactivities and plasma glucose concentrations using an operational equation (Sokoloff et al. 1977).

RESULTS

Morphine and oxymorphone, but not nalbuphine decreased the pH of arterial blood (mean pH ± SEM: control, 7.403 ± .006; morphine, 7.336 ± .013; oxymorphone, 7.336 ± .009; nalbuphine, 7.391 ± .008). Oxymorphone significantly increased arterial blood $PaCO_2$ and greased $PaCO_2$ 51 min after the drug administration ($PaCO_2$, PaO_2: control, 32.8 ± 0.9, 94.0 ± 2.0; oxymorphone 38.0 ± 1.4, 80.0 ± 4.0). At this time, morphine produced similar changes in blood-gases (35.9 ± 2.8, 82.0 ± 3.0), but to a smaller degree which did not reach statictical significance.

Drug effects on LCGU were examined in 62 brain regions, and significant differences from control were obtained in twelve of the regions (Table I). Morphine and oxymorphone decreased LCGU in several thalamic nuclei and in the dorsal tegmental nucleus and the median eminence. Nalbuphine stimulated LCGU in the spinal nuclei of the trigeminal nerve, and in the globus pallidus.

DISCUSSION

The results support the view that opioid systems are involved in several distinct analgesic mechanisms that are mediated by different subpopulations of opioid receptors. The LCGU decrements in thalamic nuclei induced by the mu agonists are consistent with mu receptor localizations and with the hypothesis that these opioids can produce analgesia not only by activating descending inhibitory pathways but also by inhibiting ascending somatosensory pathways at a supraspinal level. There are especially high concentrations of mu receptors in midline and ventral thalamic nuclei (Goodman et al. 1980). In contrast, the kappa system has been localized to other brain areas, including the striatum, hypothalamus, and trigeminal sensory nuclei (Goodman and Snyder 1982; Watson et al. 1982). Nalbuphine's effect on LCGU in sensory nuclei of the spinal trigeminal tract is consistent with an active inhibition of pain pathways originating at a spinal level (Dubner and Bennett 1983).

The findings indicate that the [14-C]DG method can be used to delineate CNS areas mediating the effects of opioid analgesics, distinguishing between effects of ligands for various opioid receptor subtypes.

TABLE 1. EFFECTS OF OPIOID AGONISTS ON GLUCOSE UTILIZATION

	Saline Control	Morphine 8 mg/kg	Nalbuphine 16 mg/kg
Paratenial Nucleus	95 ± 8.9	69 ± 3.4*	94 ± 6.6
Central Medial Nucleus	104 ± 7.5	79 ± 2.3*	107 ± 6.3
Ventrolateral Nucleus	79 ± 8.3	62 ± 1.3*	83 ± 5.3
Ventroposterior Nucleus			
Medial Part	86 ± 7.4	63 ± 1.3*	90 ± 5.7
Lateral Part	74 ± 6.1	58 ± 1.8*	81 ± 3.9
Interanteromedial Nucleus	100 ± 8.8	79 ± 2.8*	114 ± 7.7
Median Eminence	46 ± 3.1	37 ± 1.7*	51 ± 3.2
Dorsal Tegmental Nucleus	90 ± 5.6	70 ± 3.2*	84 ± 7.8
Spinal Trigeminal Nerve			
Oral Part	51 ± 2.9	52 ± 3.3	63 ± 4.6*
Interpositus Part	49 ± 3.3	43 ± 2.3	61 ± 6.6*
Caudal Part	52 ± 2.7	46 ± 3.4	64 ± 4.6*
Globus Pallidus	41 ± 3.9	43 ± 1.9	53 ± 3.2*

Each value represents the mean ± SEM for 6 rats (μmol/100 g/min). Values obtained in rats treated with 0.4 mg/kg oxymorphone were similar to those from morphine-treated rats.

*Significant from control by one-way analysis of variance and Duncan's multiple range test, $p < 0.05$.

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EFFECTS OF MORPHINE AND CALCIUM ANTAGONISTS ON PLASMA GLUCOSE IN MALE RATS

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ABSTRACT

Subcutaneous administration of morphine sulfate (4-16 mg/kg) did not significantly alter the plasma glucose concentration in fasted adult male Fischer-344 rats. All rats treated with calcium channel antagonists, verapamil or diltiazem (10 mg/kg, with or without morphine), showed marked and significant hyperglycemia, persisting at high levels throughout the 2 h experiment.

INTRODUCTION

Calcium channel antagonists reduce calcium influx through voltage-dependent calcium channels (for reviews see Chaffman and Brogden 1985; Schramm and Towart 1985). Opioids also inhibit Ca^{2+} influx in a naloxone-reversible manner (for reviews see Ross and Cardenas 1979; Chapmand and Way 1980), suggesting that opioids might interact with calcium channel antagonists in affecting various physiological parameters. In this regard, calcium antagonists potentiate morphine induced antinociception and hypothermia in rats (Benedek and Szikszay 1984), but attenuate the respiratory depressant effect of morphine (Szikszay et al. 1986). The major objective of the present work was to determine if calcium antagonists and morphine interact to alter plasma glucose levels in rats.

MATERIALS AND METHODS

Seventy seven male 3-4 month old Fischer-344 rats were fasted for approximately 15 h prior to surgery, and then were prepared with a femoral arterial catheter under halothane anesthesia. They were partially immobilized as described previously (London et al. 1981), so that results would be pertinent to future studies of cerebral glucose utilization. Three hours later, arterial blood samples were taken from awake rats 5 min before and at 0.25, 0.5, 1 and 2 h after drug treatments to assess plasma glucose concentrations by a Beckman Glucose Analyzer 2.

Morphine sulfate, verapamil HCl and diltiazem HCl were dissolved in 0.9% NaCl. Morphine sulfate was administered at 4, 6, 8 and 16 mg/kg, and calcium antagonists were given at 10 mg/kg, expressed as the salts. All doses of morphine *were* tested with verapamil, but only 4 mg/kg was tested with diltiazem. Drugs were injected s.c. either alone or in combination (5-6 rats/group). Effects of the treatments were assessed by a two-way (diltiazem) or three-way (verapamil) analysis of variance with repeated measures *over* time. Post-hoc comparisons were performed using Tukey's w-procedure.

RESULTS AND DISCUSSION

At all doses tested, morphine did not significantly alter the plasma glucose concentration as compared to values in saline-treated rats [main effect of morphine (4,43); F = 0.61. Although a hyperglycemic effect of morphine has been recognized for many years, most of the previous experiments were performed in cats and dogs (Borison et al. 1962; Feldberg and Shaligram 1972). In rodents, some studies have shown altered glucoregulation in response to morphine, depending upon the strain, gender and dose. For example, Simon et al. (1981) did not show significant effects of 4 or 8 mg/kg morphine on blood glucose in female mice. In immature (120-140 g) female Sprague-Dawley rats, 30 mg/kg morphine increased the glucose level by about 40% (Muller et al. 19671, and in male non-fasted, anesthetized Wistar rats, 20 mg/kg morphine increased plasma glucose by about 50% (Nakaki 1981). The dosages of morphine used in our experiments were much smaller and apparently were not sufficient to cause hyperglycemia in awake male Fischer-344 rats.

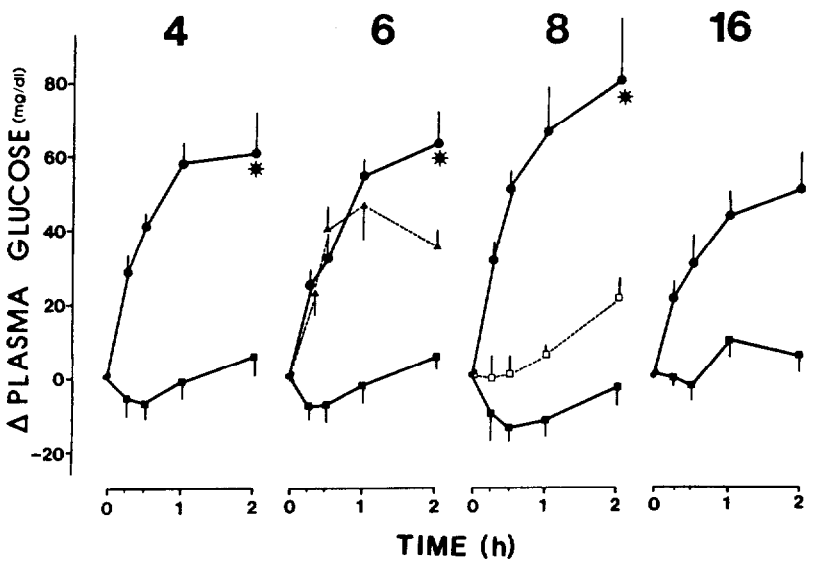

FIGURE 1. Effects of different morphine doses and verapamil on plasma glucose levels. Measurements were taken before (0) and at four times after the s.c. injection of 0.9% NaCl (□), 10 mg/kg verapamil (▲), morphine alone (■) and in combination with verapamil (●). Abscissae: time in hours. Ordinate: mean deviation from the baseline values (114.3 ± 4.1 mg/dl). *Significant $p<0.05$ (verapamil vs. morphine + verapamill.

In contrast, rats treated with verapamil, alone or in combination with morphine, showed marked and significant elevations of arterial plasma glucose levels [main effect of verapamil (1,43); F = 2141. Moreover, there were significant interactions of verapamil not only with time but also with dose of morphine (Fig 1). The highest plasma glucose levels were observed in verapamil-treated rats after the lower doses of morphine; whereas rats treated with 16 mg/kg of

morphine + verapamil had approximately the same plasma glucose level as rats treated with verapamil alone [morphine by verapamil interaction: (4,43); F = 5.81. Plasma glucose levels rose more sharply during the first hour and tended to level off or increase at a lower rate during the second hour after treatment [verapamil by time interaction: (3,129); F = 20.21. The significant three-way interaction of morphine by verapamil by time (12,129; F =3.5) reflected the difference between verapamil alone as compared to verapamil in combination with the three lower doses of morphine at 2 h only. Diltiazem alone or in combination with 4 mg/kg morphine produced a level of hyperglycemia similar to that obtained with verapamil (52-60 mg/dl over baseline, data not shown).

Although verapamil inhibits both calcium entry and uptake in the pancreatic beta cells and glucose-induced insulin release in vitro (Malaisse et. 19771, effects of calcium channel antagonists on blood glucose concentrations in vivo have been inconsistent. For example, nifedipine produced hyperglycemia and increased blood glucagon levels in human subjects (Charles et al. 1981), but verapamil (0.5 mg/kg, i.p.) did not alter blood glucose in rats (Luyckx and Lefebvre 1976). Our results indicate that a much higher dose of verapamil or diltiazem can produce hyperglycemia in rats, suggesting an overall hyperglycemic effect of calcium antagonists.

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DYNAMIC CHANGES IN THE LEVELS OF STRIATAL PROENKEPHALIN mRNA AND LARGE MOLECULAR WEIGHT ENKEPHALIN CONTAINING PEPTIDES FOLLOWING ELECTROACUPUNCTURE

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ABSTRACT

Electroacupuncture has been shown to increase met- and leu-enkephalin contents in rat striatum and hypothalamus. Its mechanism was further studied by measuring the levels of the large molecular weight enkephalin containing peptides (LECP), processed intermediates, free leu-enkephalin as well as proenkephalin mRNA in the striatum of rats decapitated at different times after the termination of 30 min electroacupuncture. A prominent increase of leu-enkephalin was seen in rats decapitated immediately after electroacupuncture. This increase returned to control level at 0.5 h. The levels of LECP and intermediates began to elevate at 1 h after electroacupuncture, reached a plateau at 8 h and lasted for 96 h. A second phase of smaller but longer lasting increase of leu-enkephalin appeared at 8-96 h. The proenkephalin mRNA amount measured by dot-blot procedure increased 3-5 folds starting at 1 h and lasting for at least 48 h.

INTRODUCTION

It has been shown that acupuncture increases the contents of met- and leu-enkephalin immunoreactivities in the striatum and the hypothalamus (Tsou et al. 1980). This effect was attenuated by icv administration of the protein synthesis inhibitor cycloheximide suggesting that acupuncture accelerates enkephalin synthesis (Wu et al. 1980). In order to gain some insight into this mechanism, we followed the levels of the enkephalin precursors, the processed intermediates and leu-enkephalin in the rat striatum simultaneously after electroacupuncture for 96 h. To answer if it also involves gene transcription, the proenkephalin mRNA was quantitated by dot-blot procedure at various times after electroacupuncture.

MATERIALS AND METHODS

Electroacupuncture

Rats weighing 200-250 g were restrained in lucite containers. One stainless steel electrode was implanted in the sciatic nerve region of each hind limb. The electrodes were connected to a therapeutic acupuncture stimulator, which produces intermittent high and low frequency biphasic waves. The rats were stimulated for 30 min with pulses just above the threshold of slight vocalization.

Chromatographic fractionation and enzymatic digestion

The striata of 10 rats from the same group were pooled and homogeni-

zed in a 10 times volume of 1 M acetic acid. The homogenate was centrifuged for 20 min at 10,000 rpm, 4°C. The supernatant was fractionated on a Sephadex G 75 column. From each collecting tube 1 ml of eluates was lyophilized. The lyophilized material was resuspended and incubated first with TPCK treated trypsin solution and then with carboxypeptidase B. The enzymes were subsequently inactivated in a 90°C water bath for 20 min. Radioimmunoassay was carried out as described (Tsou et al. 1930).

Proenkephalin mRNA quantitation

RNA was extracted and purified according to Feramisco et al (1982). A 918 base pair cDNA complementary to the proenkephalin mRNA sequence of human pheochromocytoma was a gift of Professor E. Herbert. This cDNA was cloned into the Pst 1 site of pBR 322 (Comb et al. 1982 and propagated in E. coli RRI. The cDNA probe was labelled with (α-^{32}P) MT? by nick-translation to a final specific activity of 1-5x10^8 cpm/µg. Equivalent amount (20 µg) of RNA from each sample was used for dot-blot assay on a nitrocellulose filter paper, which was then washed and exposed on to a X-ray film at -7O°C. The autoradiograms were scanned with a Shimadzu CG-930 scanning densitometer.

RESULTS AND DISCUSSION

When the rat striatal eluates from the Sephadex G 75 column were monitored at 254 µM, two peaks of absorption were observed. Radioimmunoassay of leu-enkephalin content in each collecting tube revealed immunoreactivities only in those tubes containing small molecular size peptides. This peak of immunoreactivites coincided with the second peak of UV absorption. In the tubes containing higher molecular weight peptides and proteins, leu-enkephalin immunoreactivities only appeared after trypsin and carboxypeptidase B digestion. Therefore the eluates from tube No. 3-24 (corresponding to the first peak of UV absorption) were pooled as the total amount of the enkephalin precursors, from tube No. 25-48 as the intermediates and from tube No. 49-68 (corresponding to the second peak of UV absorption) as the free leu-enkephalin. A composite figure (figure 1) could be drawn with changes of precursors, intermediates and free leu-enkephalin levels in the rat striatum after the termination of 30 min electroacupuncture. A prominent increase of leu-enkephalin content appeared immediately (0 h) after electroacupuncture. This rapid increase declined quickly to the control level at 0.5 h. During this initial period no change in the levels of precursors and intermediates was found. At 1 h after the termination of electroacupuncture, there were elevations of both the precursor and intermediates levels, which rose steadily until reaching a plateau at about 8 h and lasted for at least 96 h. A second phase of smaller hut longer lasting increase of leu-enkephalin appeared at 8 h and lasted for more than 96 h. These results suggest that a huge amount of enkephalin is released during acupuncture. The high level of leu-enkephalin represents the released and the newly formed but still unreleasable forms. The quick decline of leu-enkephalin was due to rapid degradation of the released form. Although during this initial period no change was found with the precursor and the intermediate , the rate of biosynthesis and processing might have already increased

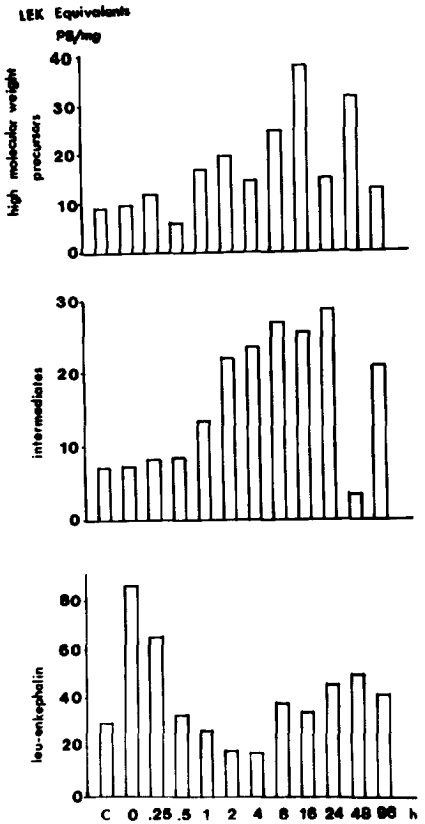

FIGURE 1. Changes in the levels of enkephalin precursors, processed intermediates and leu-enkephalin after electroacupuncture. Each column represents the content in the extract of pooled striata of 10 rats.

so that their levels could remain unchanged. After the cessation of electroacupuncture for about 1 h, the rate of enkephalin utilization might slow down leading to an increase in the levels of enkephalin precursors and processed intermediates. Their elevation would eventually lead to a second phase of smaller but longer lasting increase of leu-enkephalin.

Proenkephalin mRNA was quantitatad by hybridization with a human proenkephalin cDNA probe using dot-blot procedure. A 3-5 fold increase of proenkephalin mRNA level in the striatum could be detect-cd as early as 1 h after the termination of 30 min electroacupuncture. This proenkephalin mRNA induction continued for at least 48 h (fig 2).

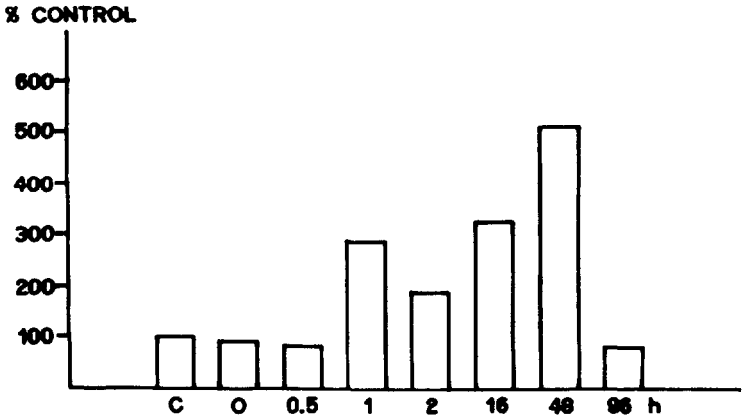

FIGURE 2. Temporal effect of electroacupuncture on rat striatal proenkephalin mRNA level. C: conrol; 0.5, 1,2, 16, 48 and 96 are the time intervals (h) between the termination of electroacupuncture and the sacrifice of the animals.

Since no change in the proenkephalin mRNA level was observed in the first hour after electroacupuncture, the initial rapid enkephalin increase in the striatun is most likely due to accelerated translation of the mRNA and post-translational processing of the precursor. It is also postulated that the large release of enkephalin during electroacupuncture may lead to a depletion of the releasable form. 'This depletion in turn brings about an enhanced transcription of the proenkephalin gene. Such a mechanism may play an important part to compensate the depletion by electroacupuncture.

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DOPAMINERGIC REGULATION OF PROENKEPHALIN-A GENE EXPRESSION IN THE BASAL GANGLIA

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ABSTRACT

Dopaminergic denervation by 6-hydroxydopamine (6-OHDA) increased proenkephalin-A gene expression in the striatum as evidenced by increases in the levels of Met[5]-enkephalin (ME), precursor and preproenkephalin mRNA (PE-mRNA). This and other lines of evidence support the hypothesis that nigrostriatal dopamine input is inhibitory to striatal enkephalin neurons and that the removal of this inhibitory influence leads to an acceleration of ME biosynthesis. These findings suggest that the enkephalin system may be associated with the progression of adaptive changes subsequent to dopaminergic dysfunction in basal ganglia.

INTRODUCTION

Enkephalins are found in high concentrations in the same forebrain structures which contain high levels of dopamine (Atweh and Kuhar, 1977; Hong et al. 1977; Johnson et al. 1980). Treatments that alter dopaminergic transmission also alter the enkephalin system, for example, chronic dopamine receptor blockade with haloperidol leads to an increase in ME biosynthesis (Hong et al. 1978; Tang et al. 1983; Sabol et al. 1983; Hong et al. 1985; Mocchetti et al. 1985; Sivam et al. 1986). In addition, unilateral substantia nigra lesions also increase the ME level in the ipsilateral striatum (Thal et al. 1983). Parkinson's disease and Lesch-Nyhan syndrome are two clinical disorders in which dopamine deficiency has been found to be the major component. Therefore, elucidation of the interrelationship between enkephalin and dopamine in basal ganglia function is important and the present work addresses this issue.

METHODS

Adult Sprague-Dawley rats received 6-OHDA (200 µg, free base) intracisternally in a volume of 25 µl. Behavioral evaluation to assess dopaminergic supersensitivity following 6-OHDA lesions was made by measuring locomotor activity. Animals were decapitated, brains were removed, the striata and hippocampi were dissected out and used for biochemical determinations.

Dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined utilizing the reverse phase high-performance liquid chromatography (HPLC) procedure. The content of ME (native ME) was determined by radioimmunoassay (RIA) as described earlier (Hong et al. 1978). Precursor content as reflected by the cryptic ME level was determined following sequential digestion of the tissue proteins with trypsin and carboxypeptidase-B (Sivam et al. 1986). Dynorphin-A (1-8)-like immunoreactivity was determined by RIA.

Total RNA was isolated from striatal tissues following the method described by Chirgwin et al. (1979). Northern blot and dot-blot analyses were carried out using a nick-translated ^{32}P cDNA probe from rat brain (Yoshikawa et al. 1984). The blots were subjected to autoradiography followed by densitometry. The data were evaluated for significance by analysis of variance. Post hoc comparisons between group means were made with Fisher's Least Significant Test.

RESULTS AND DISCUSSION

Rats lesioned with 6-OHDA exhibited an enhanced locomotor response (table 1) to LY-171555, a D_2-dopamine receptor agonist. These data

Table 1. Locomotor responses to dopamine agonist in rats lesioned with 6-OHDA.

Treatment	Drug	Activity Counts (counts/150 min)
Unlesioned control	Saline	884 ± 92
Unlesioned control	LY-171555	2954 ± 757*
6-OHDA lesion	Saline	876 ± 176
6-DHDA lesion	LY-171555	31403 ± 2000*

LY-171555 (0.3 mg/kg), a D_2 agonist. There are at least 8 rats in each group. $p<0.05$ compared to the respective saline group.

document a functional supersensitivity of dopamine receptors and confirm previous observations (Breese et al. 1985a,b). The 6-OHDA-induced lesion reduced dopamine (>90%) in striatum; the dopamine metabolite DOPAC and HVA were also markedly reduced. These data clearly indicate the effectiveness of 6-OHDA treatment to destroy dopaminergic fibers.

Table 2. Effect of 6-OHDA-induced lesions on various biochemical parameters in the striatum.

Parameters	Treatment	
	control	6-OHDA lesion
Dopamine system[a]		
Dopamine	41.8 ± 1.8 (8)	3.2 ± 0.6 (8)
DDPAC	5.8 ± 0.4 (8)	0.7 ± 0.1* (8)
HVA	2.2 ± 0.2 (8)	0.5 ± 0.1* (8)
Proenkephalin-A system[b]		
Native ME	1.34 ± 0.07 (12)	1.99 ± 0.17* (13)
Cryptic ME	0.19 ± 0.02 (12)	0.31 ± 0.02* (12)
PE-mRNA	100 ± 0 (4)	157 ± 5* (4)
Dynorphin system[c]		
Dynorphin-A (1-8)	20.6 ± 1.43 (12)	21.3 ± 1.53 (12)

* $p<0.01$ compared to respective control group. Values are mean ± SE of the indicated number of samples given in parentheses. a) Dopamine and its metabolites were estimated by HPLC; b) ME was estimated by radioimmunoassay and PE-mRNA was quantitated by RNA-cDNA hybridization; c) dynorphin-A (1-8) was measured by radioimmunoassay.

The 6-OHDA lesioned group had elevated levels of ME in the striatum as compared to levels observed in unlesioned controls (table 2). Dynorphin-A (1-8) content was also evaluated, but no significant change was observed between the groups. The hippocampal content of ME or dynorphin-A (1-8) was not altered by the 6-DHDA treatments. These results indicate regional specificity and selectivity with regard to the increase in ME as a result of 6-DHDA-induced lesions. In order to investigate the mechanism of this increase, the proenkephalin content as reflected by the cryptic ME content, as well as the PE-mRNA abundance, was assessed in the striatum. It was found that both precursor content and PE-mRNA abundance were increased after 6-DHDA-induced lesions (table 2).

These data provide evidence that dopaminergic denervation by 6-OHDA administration increases major biosynthetic indices of ME in the basal ganglia, suggesting increased transcription and/or translation processes for this peptide. These results are consistent with other lines of evidence (see Introduction) that dopamine can modulate the enkephalinergic neurons in the striatum. These studies support the hypothesis that dopaminergic neurons are inhibitory to ME-containing neurons in the striatum and that the removal of this control results in disinhibition of enkephalinergic neurons (Tang et al. 1983; Thal et al. 1985; Hong et al. 1985) which, in turn, leads to enhanced synthesis of ME. The adaptive changes in the enkephalin system resulting from the dopamine deficiency may have relevance to clinical syndromes with dopamine deficiency.

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INCREASED ENKEPHALIN GENE EXPRESSION IN THE HIPPOCAMPUS FOLLOWING SEIZURES

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ABSTRACT

Bilateral, recurrent seizures were induced in adult male rats by a unilateral, electrolytic lesion of the dentate gyrus hilus. This treatment led to a bilateral depletion of enkephalin-like immunoreactivity in the hippocampal mossy fibers by 12 hours (h) post-lesion, a rebound above normal levels by 24 h, a further rise by 4 days then a fall to control values by 10 days post-lesion. The mRNA for preproenkephalin was elevated in the granule cells by 3h post-lesion, reached a maximal 24-fold rise by 30 h then fell again to control values by 4-10 days post-lesion.

INTRODUCTION

The mossy fiber axons of the rat hippocampus have been shown to contain the opioid neuropeptides enkephalin and dynorphin (Gall et al. 1981, McGinty et al. 1983). This topographic and exclusively ipsilateral projection from dentate gyrus granule cells to CA3 pyramidal cells represents a principal relay in the circuit of excitatory connections within the hippocampal formation. In contrast to enkephalin's typically observed inhibitory actions, in hippocampus enkephalin has been demonstrated to lead to a net excitation of pyramidal cells either through inhibition of inhibitory neurons (Zieglgansberger et al. 1979) or by changing somatic dendritic coupling (Lynch et al. 1981). Several previous studies have demonsytrated that enkephalin-like immunoreactivity or preproenkephalin mRNA change in the hippocampus in response to a variety of seizure-producing stimuli (Gall et al. 1986; Hong et al. 1980; Hong et al. 1985 a,b). Similarly, we have demonstrated that enkephalin peptide biosynthesis is stimulated 14-fold following a unilateral electrolytic lesion of the dentate gyrus hilus and that this rise in peptide biosynthesis is preceeded by a rise in the content of preproenkephalin mRNA (White et al. 1986). In the present study we wished to monitor the time course over which enkephalin-like immunoreactivity and preproenkephalin mRNA change in the granule cells following the seizure-producing hilus lesion.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (200-250 gm) were used for these studies. Reagents for nucleotic acid techniques were generally from Sigma and Fisher. [α] ^{32}P-CTP (300 - 600 Ci/mmol) was from Amersham or ICN. GeneScreen Plus membrane was from New England Nuclear.

Surgical procedures

Experimental animals were anesthetized and a lesion was placed in the right dentate gyrus hilus using an insulated steel wire and an anodal current of 0.8mA for 8 sec. as described (White et al. 1986). Control (sham lesion) animals received identical surgical procedures without current. Behavioral seizures were rated using the scale of Racine (1972) and only those animals displaying scale 4-5 seizures were used for the study.

Immunohistochemistry

Coronal sections through the hippocampus were processed for enkephalin-like immunoreactivity by the peroxidase antiperoxidase technique as described elsewhere (Gall et al. 1981).

Tissue Harvest and Nucleic acid techniques

At designated intervals following the lesion, the rats were sacrificed by decapitation, the hippocampus contralateral to the lesion was dissected free and divided longitudinally into region CA3 and CA1/dentate gyrus. The latter samples were used for RNA measurement.

Total nucleic acid was prepared by extraction in urea/SDS followed by phenol/chloroform extraction and ethanol precipitation. Total RNA was obtained by treating these samples with RNase-free DNase, phenol/chloroform extraction, and ethanol precipitation. Identical concentrations of RNA was electrophoresed on denaturng agarose gels, transferred to nylon membrane and hybridized with ^{32}P-labeled cRNA probe to rat preproenkephalin mRNA. Following washing, the blot was apposed to X-ray film. The resulting autoradiographs were scanned densitometrically for quantitation. Details are given in White et al. (1986).

RESULTS AND DISCUSSION

In previous studies, we have demonstrated that a unilateral lesion of the hilus leads to the generation of bilateral, electrophysiologically identified episodes of epileptiform activity which are initiated approximately 1-2 hours post-lesion and that recur for 12 hours thereafter (Gall et al. 1986). Additionally, we have demonstrated that this seizure-producing paradigm leads to a dramatic increase in enkephalin biosynthesis in the granule cells. Thus, 24 hours post-lesion, there is a 14-fold increase in enkephalin peptide biosynthesis, as measured by the technique of *in vivo* radiolabeling, and a similar rise in preproenkephalin mRNA (White et al. 1986). Preliminary studies suggested that enkephalin levels gradually returned to normal levels and the present studies were initiated to verify and extend these initial observations.

Figure 1 illustrates the changes in the intensity of immunohistochemical staining for enkephalin-like immunoreactivity in intact, contralateral mossy fibers at various times post-lesion. As

the figure indicates, enkephalin levels are low in control animals (a) but fall rapidly to non-detectable levels by 12-hours post-lesion (b). By 24 hours post-lesion the staining has attained supranormal levels and enkephalin-like immunoreactivity increases further by 2-4 days post-lesion (c). However, by 10 days post-lesion the staining has returned to control values.

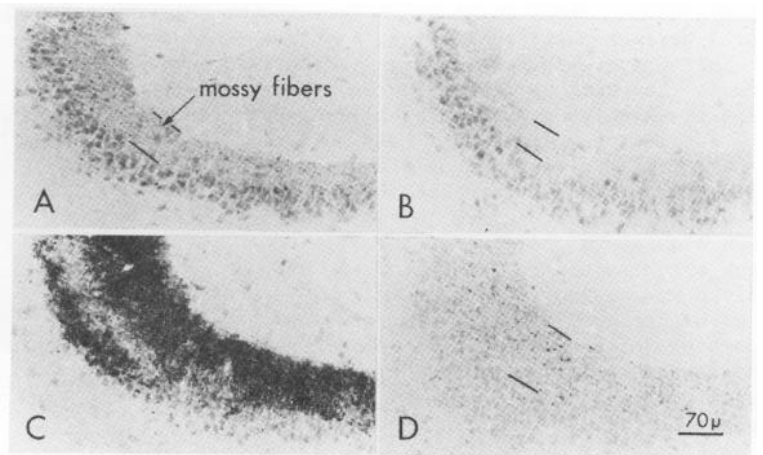

FIGURE 1: Enkephalin-like immunoreactivity in the mossy fibers of region CA3 of a control animal (a) and, rats sacrificed at 12-hours (b), 2 days (c) and 1 month (d) post-lesion. Side contralateral to lesion is shown.

Preproenkephalin mRNA levels

The above described changes in enkephalin-like immunoreactivity were accompanied by a similar change in the content of preproenkephalin mRNA in the dentate gyrus granule cells following the hilus lesion. As shown in figure 2, preproenkephalin mRNA was elevated in the granule cells as early as 3 hours post-lesion (2-fold above control), reaching a peak by approximately 30 hours post-lesion (24-fold) then declining to near baseline levels by 10 days post-lesion.

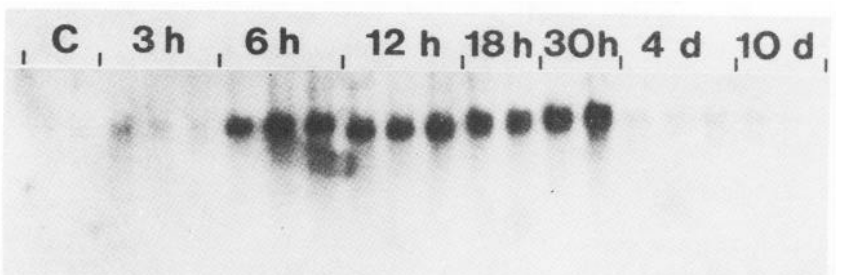

FIGURE 2: Preproenkephalin mRNA in the granule cells: The autoradiogram of the Northern blot analysis of preproenkephalin mRNA content in animals sacrificed at the indicated post-lesion intervals is shown above. C=control, h=hours, d=days.

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The present experiments have demonstrated that enkephalin gene expression is rapidly influenced by the consequences of the hilus lesion. These changes appear to be relatively transient in that enkephalin peptide and preproenkephalin mRNA levels return to control values by 10 days following the cessation of seizures. It is worth noting that the hilus lesion-induced changes in enkephalin gene expression are not accompanied by changes in the relative amounts of the several Met-enkephalin containing peptides present in the mossy fibers. That is, despite the large increase in enkephalin peptide biosynthesis induced by the seizures, the ratio of Met-enkephalin, Met-enkephalin-ArgGlyLeu, Met-enkephalin-ArgPhe, and BAM 18P does not change, suggesting a tight physiological control of the post-translational processing of proenkephalin (White et al. 1986). The rapid and dramatic increase in enkephalin biosynthesis within identified hippocampal neurons in response to seizure activity makes the hilus lesion a promising paradigm for the elucidation of mechanisms which regulate enkephalin gene expression in brain. Given the well-known anatomy, physiology and putative neurotransmitter makeup of hippocampal circuitry, it will now be possible to evaluate the separate roles played by peptide release and depletion of cellular storage pools, neuronal excitation per se and the activation of specific post-synaptic receptors in the regulation of enkephalin gene expression.

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REGULATION OF PROOPIOMELANOCORTIN (POMC) mRNA LEVELS IN PRIMARY PITUITARY CULTURES

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ABSTRACT

The effect of a variety of neurotransmitters and of corticotropin releasing factor (CRF) upon levels of mRNA coding for proopiomelanocortin (POMC) was studied in primary cultures of rat pituitary. Total nucleic acid was extracted from cells, fixed on nitrocellulose and hybridized to a single-stranded (α-^{32}P-labeled DNA complementary to 150 bases of mouse POMC mRNA. After two days incubation with CRF (10 M), the levels of POMC mRNA in both the anterior (AL) and the intermediate lobe (IL) cells increased by about 100%. A similar increase in the IL cells was seen after incubation with the ß-adrenergic agonist isoprenalin (10^{-6}M). In contrast, dopamine (10^{-6} M) and GABA (10^{-6} M) decreased POMC mRNA levels in the IL cells to about 50% of control levels without affecting POMC mRNA levels in the AL cells. In both pituitary lobes, POMC mRNA was decreased by the Ca^{2+}-channel blocker D_{600} (10^{-5}M) and increased by forskolin (10^{-6}M). This indicates that both cAMP and Ca^{2+} ions play an important role as intracellular messengers for the control of POMC gene expression in normal melanotrope and corticotrope cells.

INTRODUCTION

The proopiomelanocortin (POMC) gene is expressed in both the melanotrope cells of the intermediate (IL) and the corticotrope cells of the anterior (AL) lobe of the pituitary. Processing of the peptide precursor, however, is different in the two structures. In addition, there exist major differences in the regulation of the release of POMC-derived peptides from the two pituitary lobes. Thus, the secretion of POMC peptides from corticotrope cells is stimulated by CRF and inhibited by glucocorticoids. The secretion from the melanotrope cells of IL is under inhibitory control of dopamine (DA) and γ-aminobutyric acid (GABA) (Demeneix et al. 1986). In contrast to CRF, which stimulates both melanotrope and corticotrope cells (Vale et al. 1983, Proulx-Ferland et al. 1982), DA and GABA only interact with IL cells. There is accumulating evidence that these factors not only modulate peptide secretion but also induce a readjustment of peptide biosynthesis. Recently, we and others have shown that POMC gene expression at the mRNA level can be modulated by pro-

longed treatment with glucacorticoids, CRF and dopaminergic drugs in vivo (Birnberg et al. 1983, Bruhn et al. 1984, Höllt and Haarmann 1984, Höllt et al. 1982, Chen et al. 1983) (for a review see Civelli et al. 1984). However, from these in vivo results it is difficult to interpret whether these drugs act directly at the level of pituitary cells to change POMC mRNA levels or indirectly by altering factors released by the hypothalamus. Therefore, we measured the effect of various drugs on primary cultures of IL and AL of rat pituitary.

MATERIALS AND METHODS

Cells were cultured as previously described (Loeffler et al. 1985). Briefly, intermediate (IL) and anterior pituitary lobes (AL) from 30 male Sprague-Dawley rats (200 g) were dispersed and washed with phosphate-buffered saline (PBS) supplemented with kanamycin (50 µg/ml), 0.1% collagenase, 0.2% dispase II, 0.1% hyaluronidase, 0.05% DNase I and 1% bovine serum albumin (BSA). Dispersion was completed after shaking for 1 h at 37°C. IL and AL cells were plated on 24 and 48 dishes (Costar), respectively, in BM 86 WISSLER medium (Boehringer, Mannheim, F.R.G.) supplemented with 10% foetal calf serum. After two days of culture, cells were kept in serum-free medium to which drugs were added during two additional days. The cultured cells were lysed in 300 µl of 20 mM HEPES, 1 mM EDTA and 1% SDS and the total nucleic acids (TNAs) were extracted with phenol-chloroform. The TNAs were bound to nitrocellulose filters according to the dot-blot technique of White and Bancroft (1982). For hybridization, a single-stranded cDNA complementary to 150 nucleotides of mouse POMC mRNA was used. The mouse POMC DNA originally cloned in pBR 322 (a generous gift from Dr. J. Roberts, New York) (Roberts et al. 1979) was subcloned in M13 mp8 vector. A single-stranded M13 recombinant containing the message-sense insert was used as a template for the synthesis of a cDNA with high specific activity (10^9 cmp/µg DNA). Relative POMC mRNA levels are expressed as percentage of control.

RESULTS

Treatment of cells for two days with dopamine (10^{-6} M) and GABA (10^{-6} M) resulted in a marked decrease in POMC mRNA levels in the IL, but not in the AL cells of the pituitary (Fig. 1). Isoprenaline (10^{-6} M), a B-receptor agonist, increased the POMC mRNA levels in the IL cells by about 100% (its effect on the AL cells has not been measured). CRF (10^{-8} M) doubled POMC mRNA levels in both IL and AL (Fig. 1).
The Ca^{++} channel blocker D_{600} (10^{-5} M) reduced the POMC messenger in both pituitary lobe cells (Fig. 2) by about 50%, whereas forskolin (10^{-5} M), an agent which stimulates the adenylate cyclase, increased the POMC mRNA levels in AL and IL by more than 100% (Fig. 2).

Fig. 1: After two initial days of culture, IL and AL cells were treated for 48 h in serum-free medium with various drugs in the presence of 1% ascorbic acid. Dopamine (10^{-6} M) (DA), GABA (10^{-6} M), CRF (10^{-8} M) and isoprenalin (10^{-6} M) (Isop.) were used. Data are given as percentage of control. Significance of differences are assessed with Student's t test (*when $p < 0.05$).

Fig. 1

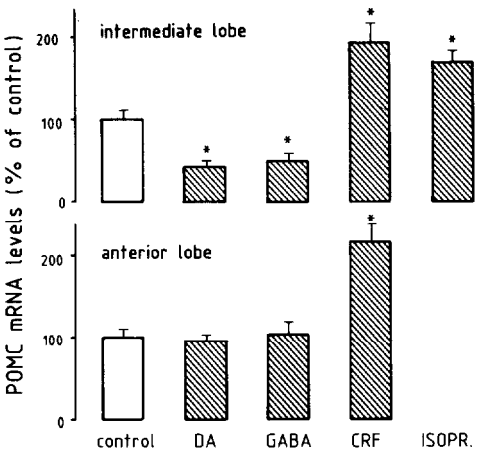

Fig. 2

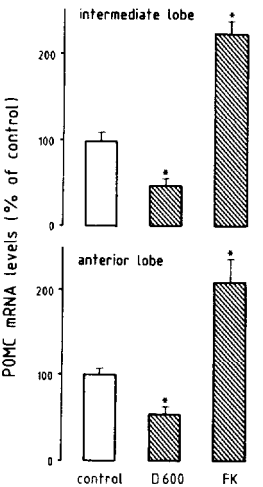

Fig. 2: IL and AL cells were treated for 48 h in serum-free medium with Ca^{++} channel antagonist D_{600} (10^{-5} M) or with the adenylate cyclase activation forskolin (10^{-6} M) (FK). Data are given as percentage of control. Significance of differences are assessed with Student's t test (*when $p < 0.05$).

DISCUSSION

The results of this study showed that in primary cultures of rat pituitary, various neurotransmitters directly modulate the gene expression of POMC at the mRNA level. This regulation can be different in the distinct pituitary lobes; thus, dopamine and GABA decrease POMC mRNA in the IL but not in AL cells. However, for CRF a similar increase in the POMC mRNA levels in both AL and IL has been found (Fig. 1). In earlier experiments, we found a marked decrease in the levels of POMC-mRNA in the IL of rats which had been chronically infused with low doses of CRF (Höllt and Haarmann 1984). Thus, it appears that CRF induces changes in the activity of unknown transmitters in vivo, which counteract its own up-regulating effect on POMC mRNA in the IL. In both lobes, cAMP appears to play a major role as second messenger for the changes in POMC gene expression at the mRNA level. Indeed, forskolin,

which elevates intracellular cAMP by activating the adenylate cyclase, increases POMC mRNA levels. Moreover, isoprenalin, a ß-receptor agonist, increases CAMP levels and enhances POMC mRNA in the IL. Similarly, CRF, which increases cAMP levels in both lobes, increases POMC mRNA levels. The direct involvement of cAMP in the POMC mRNA enhancing effect of CRF was recently demonstrated in AtT_{20} cells (Reisine et al. 1985). Besides cAMP, Ca^{2+} appears to have an important function in the control of POMC gene expression in both lobes, since the Ca^{2+}-antagonist D_{600} inhibited POMC mRNA levels. It may be that GABA exerts its effect on IL cells by decreasing their electrical activity and thus causing a decrease in Ca entry (Demeneix et al. 1986, Taraskewich and Douglas 1982).

In conclusion, we have shown that a variety of substances (dopamine, GABA, CRF, ß-adrenergic agonist) directly interact with melanotrope or corticotrope cells to modulate POMC gene expression. These effects appear to be mediated by cAMP and/or Ca^{2+} ions.

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REGULATION OF HYPOTHALAMIC ß-ENDORPHIN AND DYNORPHIN RELEASE BY CORTICOTROPIN-RELEASING FACTOR (CRF)

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ABSTRACT

Synthetic rat-human corticotropin releasing-factor (CRF), in doses between 10^{-12}M and 10^{-8}M, significantly stimulates the release of immunoreactive ß-endorphin and dynorphin-A from rat hypothalamic slices superfused in vitro: With prolonged exposure to CRF, there is a reduction in the release of both opioids which can be reversed by the addition of naloxone. The latter result indicates the involvement of autoregulatory mechanisms in the control of opioid neuronal function.

INTRODUCTION

The opioid peptide ß-endorphin is found in both the pituitary and hypothalamus (Rossier et al. 1977). While several studies have shown that corticotropin releasing factor (CRF) is one important modulator of pituitary ß-endorphin (Bruhn et al. 1984; Höllt and Haarmann 1984; Vale et al, 1980), the role of CRF in regulating hypothalamic ß-endorphin has been only speculated upon (Nikolarakis et al. 1986; Sirinathisinghji 1985). The studies described here were done to test the effects of CRF upon hypothalamic ß-endorphin release. In addition, CRF effects upon the release of dynorphin-A (dynorphin) were also investigated. Dynorphin is found in pituitary and brain (Höllt et al. 1980), but there is no information available as to whether its secretion is modulated by CRF.

MATERIALS AND METHODS

Hypothalami were obtained from adult male Wistar rats, sliced (250 µM) and superfused, in pairs, with Medium 199 (100 ul.min^{-1}) in chambers maintained at 37°C. Superfusates were collected every 10 min., snap-frozen and later assayed for ß-endorphin and dynorphin immunoreactivities. CRF was added to the superfusion medium at concentrations ranging from 10^{-18} M to 10^{-8}M. In separate experiments, naloxone (10^{-6}M) was added to the slices together with CRF (10^{-8}M).

RESULTS AND DISCUSSION

CRF was found to significantly stimulate the release of ß-endorphin and dynorphin from rat hypothalamic slices in-vitro, when presented at doses of 10^{-12}M to 10^{-8}M. The data therefore support earlier speculations about the stimulatory effects of CRF upon hypothalamic ß-endorphin (Nikolarakis et al. 1986; Sirinathisinghiji 1985) and provide evidence that CRF also stimulates the release of dynorphin, an opioid derived from a precursor different to the one from which ß-endorphin is derived.

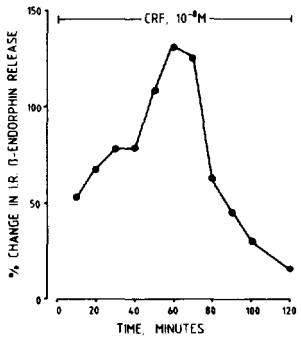

Fig. 1. Time course of ß-endorphin release after superfusion with CRF 10^{-8}M. Basal release rates were in the range of 3-5 fmol•100µl^{-1}.10 min^{-1}.

Figure 1 shows the ß-endorphin response to CRF; the pattern of response for dynorphin was similar, except that stimulation of release occured some 30-40 min. later. Of particular interest in fig. 1 is the decline in the amount of opioid released upon continued exposure to CRF. This feature suggested to us that CRF stimulates the release of opioids which may then act presynaptically to turn off their own release (reminiscent of the way neurotransmitters limit their own release). Indeed, when the opiate receptor antagonist naloxone was superfused together with CRF the attenuation of opioid peptide release was not observed. Furthermore, there was a tendency for opioid release to increase when naloxone was presented on its own: and these effects persist in the presence of tetrodotoxin (TTX, 10^{-6}M), suggesting them to be presynaptic events. Current studies are focussed on finding the type of ligand(s) involved in this inhibition of opioid peptide release.

We conclude that: (1) CRF can stimulate the release of ß-endorhin and dynorphin from the rat hypothalamus; (2) prolonged exposure to CRF leads to an attenuation of opioid peptide release; and (3) naloxone prevents the loss of response to CRF, demonstrating the involvement of autoregulatory mechanisms in the control of opioid peptide neurones.

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INHIBITION OF LH RELEASE BY CRF MAY BE PARTIALLY MEDIATED THROUGH HYPOTHALAMIC ß-ENDORPHIN RELEASE

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ABSTRACT

Corticotropin releasing factor (CRF) given i.c.v. at doses of 0.02-2 nmol, significantly inhibited luteinising hormone (LH) secretion in castrate male rats. The luteinising hormone releasing hormone (LHRH) release from hypothalamic slices Superfused in vitro with CRF (10^{-12}M - 10^{-8}Ml was markedly reduced. In rats that had been infused with naloxone 2 days prior to testing with 0.2 nmol CRF, the effect of the CRF was significantly reduced. Together, these findings indicate that CRF inhibits LH secretion by inhibiting LHRH release from the hypothalamus, and that the latter action involves endogenous opioid peptides.

INTRODUCTION

Injections of corticotropin-releasing factor (CRF) into the mesencephalic central gray area of the brain inhibits sexual receptivity in the female rat (Sirinathsinghji 1985), and its injection into the third ventricle reduces serum luteinising hormone (LH) concentrations (Rivier and Vale 1984). Since opioids inhibit LH secretion (see Millan and Herz 1985) and CRF modulates ß-endorphin levels in the pituitary (Bruhn et al. 1984; Höllt and Haarmann 1984) and hypothalamus (Almeida et al. this volume), an involvement of opioids in the CRF inhibition of LH has been postulated (Sirinathsinghji 1985; Nikolarakis et al. 1986). In view of our recent data, showing CRF stimulating the release of ß-endorphin and dynorphin from the hypothalamus (Almeida et al. this volume), the possibility that hypothalamic, rather than pituitary, opioids might mediate the CRF action arises.

MATERIAL AND METHODS

1. In vivo experiment:
   Adult male Wistar rats were implanted with i.c.v. stainless steel guide cannulae. 5 days later they were bilaterally castrated and had a cannula placed into a jugular vein. Some rats were also implanted with osmotic minipumps containing naloxone (released at a rate of 9.6 mg/kg/day), starting 48h prior to, and during, treatment with CRF. CRF was applied i.c.v. 5 days after castration in doses ranging from 0.02 to 2 nmols. Blood was withdrawn at various time intervals via the jugular cannula and the serum was assayed for LH concentrations.
2. In vitro experiment:
   Hypothalami were dissected from normal adult male Wistar rats following decapitation, and superfused as described elsewere (Nikolarakis et al. 1986). CRF was

added in the superfusion medium at concentrations ranging from 10^{-12} to 10^{-8}M. Ten minute pools of superfusate were collected and assayed for LHRH.

RESULTS AND DISCUSSION

CRF at all doses tested (0.02, 0.2 and 2 nmoles) produced significant reductions in serum LH levels within 30 min of i.c.v. administration. LH levels remained significantly lower in rats receiving 0.2 or 2 nmoles for up to 6 hours, whereas LH secretion was not significantly different 2 hours after treatment with the lowest dose. In the presence of the opioid antauonist naloxone the CRF (0.2 nmol) inhibition of LH was significantly reduced (fig. 1).

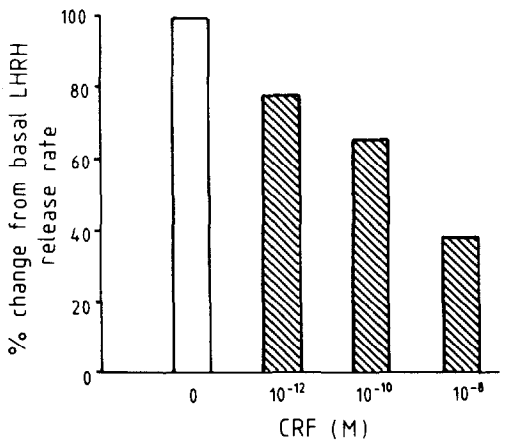

Fig. 1. Serum LH levels after CRF in castrates pretreated with either water or naloxone via osmotic mini-pumps. LH levels in untreated castrates shown for comparison.

Furthermore, rats treated with naloxone showed a faster recovery of LH levels than did the animals that received CRF alone. In our in vitro experiments basal LHRH release was suppressed in a dose dependent way after CRF application (fig. 2).

Our findings are in accordance with a previous report (Rivier and Vale 1984) showing that centrally administered CRF is a potent inhibitor of LH secretion in recently castrated male rats. Although naloxone could block the CRF inhibition of lordosis behaviour in the rat (Sirinathsinghji 1985), suggesting an opioid involvement, the application of a different antagonist, naltrexone, did not affect the CRF inhibition of LH (Rivier and Vale 1984), leading the latter authors to question an involvement of opioids. Our results, showing that chronic opiate blockade leads to a reduced inhibition at LH by CRF, indicates an opioid involvement in this CRF action. CRF probably inhibits LH by reducing the release of hypothalamic LHRH. The inhibitory actions of CRF within the hypothalamic neuronal pathways affecting LHRH

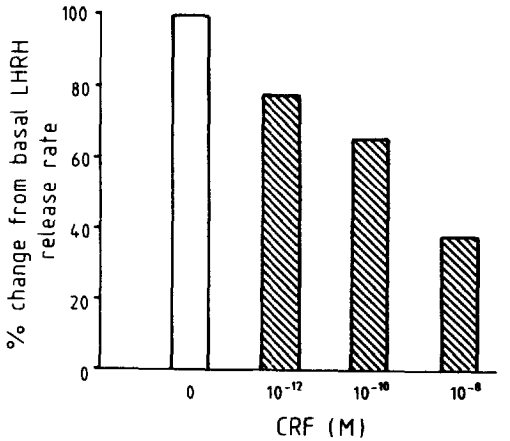

Fig. 2. Dose-response inhibition of LHRH release from hypothalamic slices in vitro.

release could include the release of opioids. In fact, we have found that CRF increases both ß-endorphin and dynorphin release from hypothalamic slices (Almeida et al. this volume.

In conclusion, we suggest that CRF inhibits LHRH and LH secretion by stimulating the release of opioids such as ß-endorphin and dynorphin from hypothalamic sites.

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INCREASED SPINAL CORD DYNORPHIN mRNA DURING PERIPHERAL INFLAMMATION

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ABSTRACT

Injection of a complete Freunds adjuvant-saline emulsion into the hindpaw of the rat induces a peripheral inflammatory process accompanied by pronounced increase (3 fold) in spinal cord dynorphin content. A marked increase (-9 fold) in the mRNA coding for the preprodynorphin precursor accompanies the increase in peptide. The parallel elevations of the mRNA and peptide product suggest that an increased activity of dynorphin neurons occurs in spinal cord in response to altered afferent input due to the peripheral inflammatory process.

INTRODUCTION

Chronic inflammatory states, as exemplified by the adjuvant-induced arthritis model, have been associated with an increase in spinal cord met^5-enkephalin content (Cesselin et al. 1980) and more recently dynorphin (Millan et al. 1985, 1986). Using a peripheral, more acute inflammation model we have found that spinal dynorphin is subject to a rapid elevation in its content (Iadarola et al. 1985). The increase is specific to the spinal segments innervating the inflamed limb and peaks between 5-8 days. A basic problem in interpreting these data revolves around whether an elevated peptide content reflects an increase or decrease in peptide utilization. We have measured the content of preprodynorphin mRNA in spinal cord tissue during the peripheral inflammation. Our results indicate that the dynorphin peptide increase is accompanied by a pronounced increase in preprodynorphin mRNA accumulation.

MATERIALS AND METHODS

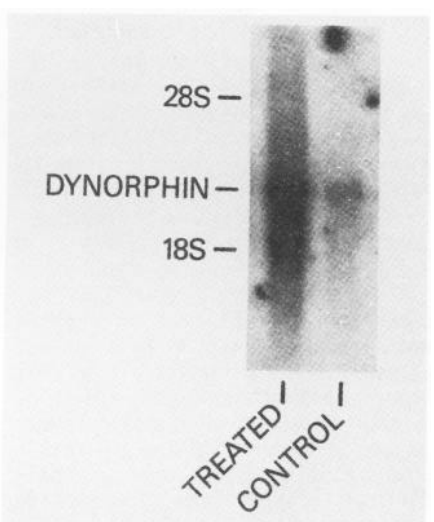
Inflammation of one hind paw was induced by intraplantar injection (100 ug) of a saline-Freunds adjuvant (Calbiochem) emulsion in 350 g male Sprague Dawley rats. Treated rats were killed by decapitation after 5 days of inflammation; a group of non-treated rats was killed at the same time. The whole spinal cord was removed from the spinal canal by injection of saline at the sacral vertebral level (De Sousa & Horrocks 1979). The dorsal lumbar spinal cord was dissected and frozen on dry ice until use. RNA was prepared as described by Chirgwin et al. (1979) using homogenzation in guanidine thiocyanate

and centrifugation of RNA through cesium chloride. Dynorphin peptides were assayed in the guanidine thiocyanate supernatants from the above RNA preparation as described by Naranjo et al. (1986); the specificity of the antibody is described in Iadarola et al. (1986).

Northern blots were prepared with poly A^+ enriched RNA and were hybridized with plasmids pBgBa and pBaBa labeled with ^{32}P-dCTP and -dATP to a specific activity of 1-3 x 10^5 cpm/ng. The two plasmids each contain 800-900 base pairs of genomic DNA from the major exon and represent, respectively, the protein coding and non-coding regions of rat preprodynorphin mRNA (Civelli et al. 1985). After autoradiography the blots were washed (Byrd et al. 1986; Milner and Sutcliffe 1983) and probed again with a cDNA to the rat ß-actin message (Nudel et al. 1983) in order to standardize each lane for slight variation in the amount of poly A^+ RNA applied. Both autoradiograms were scanned with a laser densitometer and the data are expressed as a ratio of the dynorphin densitometric signal to the ß-actin signal.

RESULTS

A single hybridization band was observed after agarose gel electrophoresis conducted under denaturing conditions (Tang et al. 1983). This indicates that no heterogeneity in message size (Civelli et al. 1985) occurs in dorsal spinal cord preprodynorphin mRNA in the inflamed condition compared to control (Fiagure 1). At 5 davs of inflammation a marked elevation of preprodynorphin mRNA is evident.



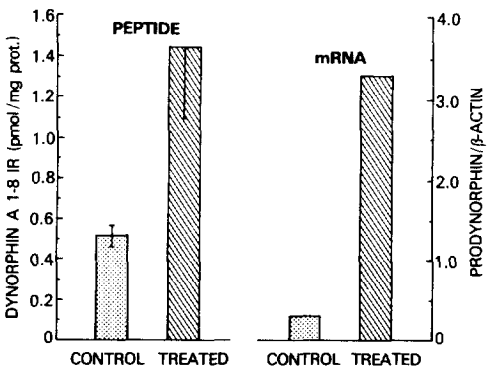
The RNA blot was hybridized with labeled dynorphin DNA probes. Dorsal spinal cord RNA was extracted at day 5 of inflammation (treated) and processed in parallel with untreated rats (control). The increased density of the band from the inflamed condition is apparent. There appears to be some nonspecific binding or tailing into the nearby band of 18 S rRNA; this was not included in the densitometric analysis.

Figure 1
Increase in preprodynorphin mRNA after inflammation

This same blot was rehybridized with a rat ß-actin cDNA (not shown). Normalization of densitometric scan from the dynorphin blot with that

from the ß-actin blot showed that about a 9 fold increase in dynorphin mRNA had occurred in the inflamed condition (Figure 2).

Measurement of dynorphin A 1-8 content from the guanidine thiocyanate supernatent from this experiment and several other similar RNA preparations showed that the mRNA increase was accompanied by approximately a 3 fold increase in this dynorphin peptide (Figure 2).



The peptide content of several guanidine thiocyanate RNA preparations was analyzed (Naranjo et al. 1986) for dynorphin A 1-8 content (left side). The histograms at the right show the ratio of dynorphin mRNA to ß-actin mRNA based on the areas under the respective peaks after densitometric scanning. The ß-actin cDNA probe was used to standardize the lanes for the amount of poly A^+ RNA applied.

FIGURE 2
Comparison of increase in dynorphin A 1-8 peptide and dynorphin mRNA after inflammation

DISCUSSION

The data presented here show that a peripheral inflammation induces an increase in dynorphin mRNA content and dynorphin A 1-8 immunoreactivity. These observations are consistent with the idea that the increase in peptide occurs as a result of an activation of dynorphin gene transcription in neurons located in dorsal lumbar spinal cord. Since mRNA transcription and translation generally occur in the neuronal cell body, our data also minimize the possibility that the dynorphin peptide increase arises from alternative neuronal sources such as the dorsal root ganglia (Botticelli et al. 1981, Basbaum et al. 1986) or a supraspinal nucleus.

The data suggest that spinal cord dynorphin-containing neurons are in an altered state of activity produced by the afferent input arising from the inflamed limb. The concurrent elevation of both mRNA and peptide product support the idea of an increase in peptide synthesis and utilization. However, the precise mechanism whereby the increase

in mRNA occurs (for review see Schwartz and Costa 1986) and whether peptide release is increased requires further examination.

In conclusion, the data presented suggest an enhanced activity of spinal cord dynorphin neurons during a peripheral inflammation and indicates the participation of these neurons in the sensory processing or modulation of pain at the spinal level.

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DIFFERENTIAL EFFECTS OF SELECTIVE BRAIN LESIONS ON HYPOTHALAMIC AND PITUITARY IR-DYNORPHIN

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ABSTRACT

Selective radiofrequency and neurotoxic lesions of the serotonergic pathways caused in the rat a significant reduction of ir-dynorphin A and Bin the hypothalamus but not at pituitary level. These data confirm that dynorphin-related peptides are regulated independently in these areas.

INTRODUCTION

Several studies support the view that differential neural and endocrine factors participate in the regulation of immunoreactive dynorphin (ir-DYN) at hypothalamic and pituitary level (Höllt et al.1981, Spampinato et al. 1986a. Spampinato et al. 1986b). Thus, it seems reasonable to hypothesize that hypothalamic and pituitary ir-DYN pools may be influenced by aminergic neurons, since biogenic amines act as neurotransmitters in these areas. Millan et al. (1984) reported that noradrenergic neurons regulate ir-DYN in the neurointermediate lobe of the pituitary. However, little is known about possible interactions between the dynorphinergic and serotonergic systems. To ascertain this possibility we examined the changes of hypothalamic and pituitary ir-DYN A and B following neurotoxic and radiofrequency lesions of serotonergic neurons.

MATERIALS AND METHODS

Neurotoxic lesions

Male Sprague-Dawley rats (220-250 g) bearing chronic intracerebroventricular (icv) cannulae received 5,7 dihydroxytryptamine creatinine sulphate (5,7 DHT, 200 µg/rat, icv) combined with desipramine (25 mg/kg, i.p., 60 min prior to 5,7 DHT).

Lesions of the medial and dorsal raphe nuclei

Male Sprague-Dawley rats (220-250 g) were anaesthetized and placed in a stereotaxic apparatus. Radiofrequency lesions were performed by nickel-chrome electrodes, according to the Pellegrino et al. (1979) stereotaxic atlas and pilot studies. The coordinates adopted for the medial raphe nucleus were: antero-posterior (AP) -6.3 mm, lateral (L) 1.3 mm, depth (D) -8.6 mm at an angle of 8°; for the dorsal raphe nucleus were: AP -6.2 mm, L 0.8 mm, D -6.3 mm, at an angle of 7°.

Sham lesions were performed in an identical manner with unactivated electrodes. Histological examinations were made in all lesioned animals to ascertain the correct position and size of lesions.

Preparation and processing of tissue extracts

Two weeks after lesions rats were killed by decapitation. Hypothalamic and pituitary tissues were processed and ir-DYN A and B measured by radioimmunoassay as extensively reported (Spampinato et al.1986a, Cone and Goldstein 1982).

RESULTS AND DISCUSSION

As shown in table 1, ir-DYN A and B were significantly reduced in the hypothalamus after the 5,7 DHT treatment, while there was no change in the pituitary gland. This neurotoxin causes a prolonged reduction of serotonin in whole brain, including the hypothalamic area, as a consequence of the destruction of serotonergic neural terminals (Baumgarten et al. 1977). It is likely therefore, that the reduction of serotonin levels in the hypothalamus might affect in some way the dynorphinergic system.

Table 1

Effect of 5,7 DHT treatment on hypothalamic and pituitary content of ir-DYN A and ir-DYN B.

Area examined	Treatment	ir-DYN A (fmol/µg protein)	ir-DYN B (fmol/µg protein)
Hypothalamus	Control	0.44±0.09	1.81±0.11
	Treated	0.27±0.04**	1.25±0.13**
Anterior pituitary lobe	Control	1.70±0.11	7.51±0.76
	Treated	2.44±0.29	9.05±1.42
Neurointermediate pituitary lobe	Control	9.30±0.96	47.04±4.09
	Treated	11.56±1.81	51.14±5.76

Each group consisted of 8-10 rats.
** $p < 0.05$ vs control rats of the same group (Student's two-tailed t-test).

After destruction of dorsal and medial raphe nuclei, from which originates the major serotonergic input to the hypothalamus (Van de Kar and Lorens 1979). a decrease of ir-DYN A and ir-DYN B occurred in this area (table 2). On the contrary, the levels of both peptides remained unmodified in the pituitary gland (table 2). Thus, these results are

in agreement with those reported above and, taken together, give evidence of an influence of the serotonergic system on hypothalamic dynorphins.
This hypothesis is further supported by the well known interactions between other opioid peptides and the serotonergic neurons at hypothalamic level (Spampinato et al. 1979).

Table 2

Effect of medial and dorsal raphe nuclei lesions on hypothalamic and pituitary content of ir-DYN A and ir-DYN B.

Area examined	Treatment	ir-DYN A (fmol/µg protein)	ir-DYN B (fmol/µg protein)
Hypothalamus	Control	0.40±0.08	1.98±0.17
	Lesioned	0.26±0.05**	1.47±0.27**
Anterior pituitary lobe	Control	1.34±0.05	8.79±0.39
	Lesioned	1.28±0.08	9.53±0.84
Neurointermediate pituitary lobe	Control	10.01±1.37	64.08±1.60
	Lesioned	8.64±1.54	63.22±3.25

Each group consisted of 8-10 rats.
** $p < 0.05$ vs control rats of the same group (Student's two-tailed t-test).

The existence of a differential regulation of dynorphin-related peptides by the serotonergic system is consistent with a recent paper of Majeed et al. (1985), who found a decrease of duodenal, but not hypothalamic, ir-DYN after an acute treatment with drugs known to activate the serotonergic system. However, the discrepancy with our findings as regards hypothalamic ir-DYN, may be related to the fact that we achieved a chronic and marked depletion of brain serotonin.

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MODULATION OF ß-ENDORPHIN SECRETION FROM MOUSE PITUITARY TUMOR CELLS BY CALMODULIN INHIBITOR W7

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ABSTRACT

The involvement of calmodulin in the secretion of ß-endorphin from the mouse anterior pituitary tumor cell line, AtT-20, was investigated. The calmodulin inhibitor W7 potentiated secretion produced by 8-BrcAMP, and induced a secretory response to arginine vasopressin, which did not elevate ß-endorphin levels when added alone. Release of hormone in response to CRF was not affected. Calmodulin phosphodiesterase inhibitor 8-MeOMeMIX produced a dose-dependent increase in 8-BrcAMP stimulation, suggesting that inhibition of cAMP degradation is the mechanism of enhancement of 8-BrcAMP-induced secretion in the presence of W7.

INTRODUCTION

The mouse anterior pituitary tumor cell line AtT-20 secretes ACTH and R-endorphin in response to a variety of receptor-mediated events and to cAMP analogues (Reisine et al. 1982; Axelrod and Reisine 1984). The participation of the adenylate cyclase/protein kinase A pathway AtT-20 cells share with corticotrophs and other endocrine cells in the anterior pituitary (Bilezikjian and Vale 1983; Brazeau et al. 1982; Labrie et al. 1982; Aguilera et al. 1983). It has recently been demonstrated that Ca^{2+}-channel antagonists are capable of elevating cAMP levels and CAMP-dependent responses in the presence of growth hormone releasing factor and forskolin in somatotrophs and GH4 cells respectively (Barinaga et al. 1985; Waterman et al. 1985). This Ca^{2+}-dependent modulation of cAMP processes suggests a Ca^{2+} -sensitive component in either the biosynthesis or degradation of the cyclic nucleotide. As there is evidence that the calmodulin (CaM)-dependent phosphodiesterase can contribute to decreasing cAMP levels (Miot et al. 1984; Erneux et al. 1985), and Murakami et al. (1985) have demonstrated the involvement of CaM in ACTH secretion in anterior pituitary (Murakami et al. 1985), experiments were conducted with a CaM inhibitor and an inhibitor of the CaM-dependent phosphodiesterase to investigate any contribution this system might have in AtT-20 cell secretion.

MATERIALS AND METHODS

Release Experiments

AtT-20 cells were plated in 24 well Primaria plates (Falcon) at an initial density of 1×10^5 cells/ml/well in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10% fetal calf serum (HiClone), and grown at 37°C in 5% CO_2. Incubations with regulators were conducted three or four days after plating in 0.1% bovine serum albumin (BSA, Sigma) in DMEM.

CaM inhibitor W7 (6-aminohexyl-5-chloronapthalene-1-sulfonamide, Sigma) and CaM-dependent phosphodiesterase inhibitor 8-MeOMeMIX (1-methyl-3-isobutyl-8-methoxy methylxanthine (a gift from Dr. Jack Wells, Vanderbilt University, Nashville) were dissolved in ethanol and DMSO respectively, diluted in DMEM and added 15 minutes before the secretagogues. Corticotropin releasing factor (CRF, Penninsula) arginine vasopressin (AVP, Sigma) and 8-BrcAMP (8 bromo adenosine 3':5'-cyclic monophosphate, Sigma) were introduced to the wells and the incubation continued for a further three hours at 37°C. The medium was then removed and stored at -20°C or assayed immediately.

RIA

The ß-endorphin immunoreactivity (B-End I.R.) was assayed using Sugar antisera (a gift of Dr. Richard Allen). The antibody recognizes the mid portion of ß-endorphin and has a titre of 1:2,000.

Aliquots of medium were incubated with antibody and ^{125}I-labelled ß-endorphin overnight in 200 ul PBS, 0.1% BSA, 0.1% Triton X-LOO at 4°C and antibody-bound label was precipitated the next day with 500 ul 2% goat antirabbit gamma globulin, 0.2% normal rabbit serum (both from Breit Laboratories) in 1.2 M ammonium sulphate in 10 mM sodium phosphate buffer, pH 7.2, at 4°C. Tubes were centrifuged in a Beckman J6B centrifuge and pellets were counted on a Micromedic Apex 10/600 gamma counter.

RESULTS

CaM inhibitor W7 at 10 uM did not affect basal secretion, and no effect on CRF-stimulated secretion was observed (fig. la), however, the response to 8-BrcAMP was considerably enhanced, and the addition of W7 appeared to increase R-End I.R. in the presence of AVP.

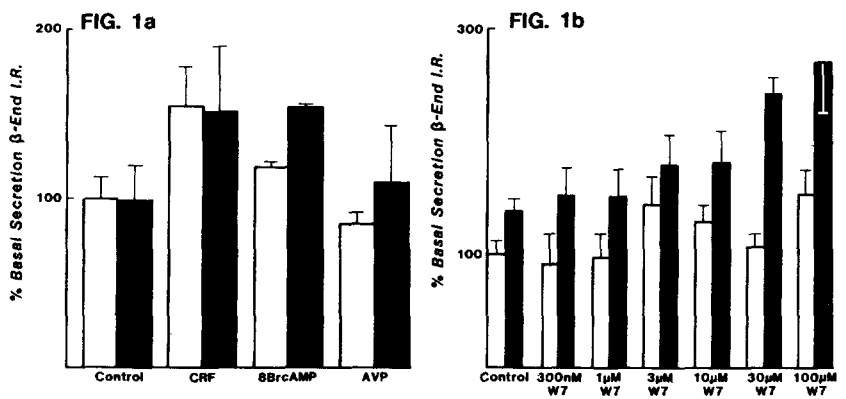

FIGURE 1a. Incubation of AtT-20 cells with regulators of secretion in the presence (closed bars) and absence (open bars) of 10 uM W7 and conducted as described in materials and methods. Concentrations of compounds were as follows: CRF, 30 nM; 8-BrcAMP, 500 uM; AVP, 100 nM. Bars show the mean and standard deviation of at least two experiments conducted in triplicate.

FIGURE 1b. Dose-response curve of W7 in the presence (closed bars) and absence (open bars) of 500 uM 8-Br CAMP. Each point represents the mean and standard deviatio of a representative experiment conducted in triplicate.

Figure 1b shows the dose-related increase in ß-end I.R. in response to W7 in the presence of 500 uM 8-BrcAMP. The increase over control is particularly marked at 30 uM W7, which represents the ID_{50} for this inhibitor when tested in intact cells (Hidaka and Tanaka 1985).

The capacity of 8-MeOMeMIX to potentiate 8-BrcAMP secretion is shown in figure 2. No increase is apparent at 2 uM, the reported ID_{50} for this compound (Miot et al. 1984); only at 30 uM and 100 uM 8-MeOMeMIX is an increase over 8-BrcAMP alone apparent.

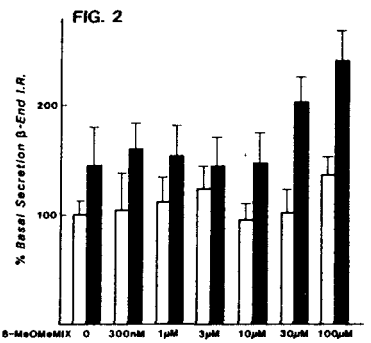

FIGURE 2. Dose-response curve of 8-MeOMeMIX in the presence (closed bars) and absence (open bars) of 500 uM 8-BrcAMP. The experiments were conducted twice with similar results. The values shown are the means and standard deviations from a representative experiment conducted in triplicate.

DISCUSSION

The data presented here suggests the involvement of an inhibitory calmodulin function, perhaps at the level of cAMP degradation, in AtT-20 cells.

Murakami et al. (1985) found that in pituitary halves, W7 inhibited CRF-stimulated secretion at 100 uM, a concentration which potentiated 8-BrcAMP-stimulated secretion in AtT-20 cells in the experiments described in figure 2. This difference could be due to an altered signal transduction mechanism in AtT-20's or to the difference in secretagogues used. In the above paper a small increase in both ACTH release and cAMP levels in response to CRF was observed at 10 uM W7.

In the absence of direct measurement of cAMP levels, the role of a calmodulin dependent phosphodiesterase can only be inferred by the data presented here. In thyroid and astrocytoma cells this type of phosphodiesterase activity is associated with capacity of muscarinic agonists to attenuate increases in cAMP (Miot et al. 1983; Miot et al. 1984; Meeker and Harden 1982), and occurs

concomitantly with phosphatidylinositol turnover (Masters et al. 1984). Although the contribution of this system in AtT-20 cells has not been described, the basal activity of the CaM phosphodiesterase may indicate that this activity could be increased in response to a receptor mediated event.

It is clear from the data presented here and elsewhere that further work is needed to clarify the effects observed when cAMP and CAMP-mediated functions are elevated by calcium-channel blockers, calmodulin inhibitors and calmodulin-sensitive phosphodiesterase inhibitors.

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RELEASE OF I-R METENKEPHALIN FROM RAT AMYGDALA SLICES IN VITRO

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ABSTRACT

Release of immuno-reactive metenkephalin has been demonstrated from slices of rat amygdala in vitro. This release was calcium dependent and the amount released was related to the concentration of potassium ions in the releasing stimulus. The effect of drugs such as clonidine, an alpha 2 adrenoceptor agonist, and idazoxan, an antagonist, on this release was measured. Neither drug had a significant effect on the amount of ir-metenkephalin released. Thus although release of noradrenaline can be affected by opioids it seems that release of endogenous opioids is not affected by drugs which interact with noradrenaline.

INTRODUCTION

Several groups of workers have studied the stimulated release of ir-metenkephalin (ir-ME) from slices of rat brain taken from various regions e.g. Henderson et al. (1978) corpus striatum, Iversen et al. (1978) qlobus pallidus, Bayon et al (1978) qlobus pallidus, Richter et al. (1980) corpus striatum, Lindberg and Dahl (1981) corpus striatum and Del Rio et al, (1983) corpus striatum and periaqueductal grey. The majority of the groups have confined their studies to areas which are relatively rich in their content of enkephalins. In this study we have investigated an area of the brain which has a lower content of enkephalins (Hong et al.1977) but which shows a high level of alpha-2 adrenoceptor agonist binding (Unnerstall et al, (1984), and we have examined the effects of drugs active at alpha-2 adrenoceptor sites on the potassium stimulated release of ir-metenkephalin in order to examine if these drugs can have any effects on enkephalin release. There have been reports of interaction between alpha-2 agonist druqs and opioid induced changes in neuronal function (Aghajanian 1978). and also in behavioural studies (Tsenq et al. 1975), but the exact nature of the sites of interaction of these two groups of drugs is still not clear.

MATERIALS AND METHODS

Adult male Wistar rats (200-300 g body wt.) were decapitated and the brains removed onto ice. The amygdalae were dissected out, and tissue slices (1.0x0.4x0.4mm) were prepared on a McIlwain tissue chopper. The slices were placed in superfusion chambers and super-fused with an oxygenated physiological saline solution (PSS) pH7.2 containing (mM) NaCl 118, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.0, $CaCl_2$ 0.75, $NaHCO_3$ 25, glucose 11, ascorbic acid 1.0. In addition 1g/l bovine serum albumen and 50mg/l bacitracin were included to reduce peptide breakdown. The slices were superfused for ten minutes to

equilibrate at a rate of 0.5ml/min. Four eight minute fractions were then collected with the first four minutes of the third fraction beinq exposed to PSS containing an elevated potassium ion concentration (balanced bv a reduction in sodium ion concentration). Normally this elevated K^+-concentration was 35mM. At the end of each experiment the tissue was removed from the chambers and processed for radioimmunoassay as described by Morris & Livingston (1984). The fractions were extracted on Sep-Pak disposable ODS columns and also processed for radioimmunoassay. The radioimmuno-assay used an antibody to the sulphoxide form of metenkephalin which was generously provided by the group working at St. Bartholomew's Hospital, London. This antibody was highly specific and showed negligible cross-reactivity, and a sensitivity down to 1 femtomole per assay tube (Clement-Jones et al.1980). The amounts of ir-ME released were calculated as a fractional release of the total present in the tissue at the start of the release period for each fraction.

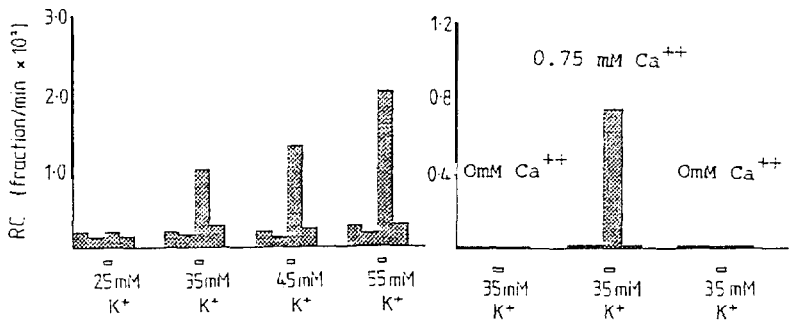
RESULTS

Preliminary experiments showed that the ir-metenkephalin released produced parallel displacement curves in the radioimmunoassay with authentic metenkephalin and that the PSS and raised K^+ had no intrinsic effects on the radioimmunoassay. The recovery of ir-ME, calculated by adding a known amount of ME to the PSS, was 35%. Basal release was 5-20 pq/min representing about 0.1-0.2% of tissue stores. The amount of ir-ME released was dependent on the K^+ concentration used, 25mM K^+ produced only a slight rise in release, 35mK^+ raised release to about 0.8%, 45mM K^+ raised release to about 1.2% and 55mM K^+ raised it to about 2%. The release of ir-ME was also dependent on the presence of Ca^{++}ions and omission of Ca^{++} ions completely abolished 35mM K^+ stimulated release (Fig. 1). 35mM K^+ was chosen as the release stimulus for the subsequent experiments. In the presence of 1 µm clonidine, the alpha-2 adrenoceptor agonist, from the start of perfusion there was no significant change in 35mM K^+ stimulated release of ir-ME. similarly in the presence of 1 µm idazoxan (RX781094), the alpha-2 adrenoceptor antagonist, there was no significant change (Fig. 2).

DISCUSSION

The recovery rates of ir-ME found in these experiments are similar to those obtained by Iversen et al.(1978) and Bayon et al.(1978), however some workers (Richter et al.1979) have found higher recoveries using faster perfusion rates. It would appear that recovery rates do vary with the experimental conditions used. The values for basal release of 0.1-0.2% agree well with most of the other reports (Iversen et al.1978, Sawynok et al.1980, Lindberg and Dahl 1981). Similarly, the degree of increase in release in response to K^+ stimulation was comparable with the other reports although comparison is difficult because of the variety of values for K^+ utilised in the different studies. The concentrations of clonidine and idazoxan used were sufficient to produce significant effects in studies using stimulated [^{3}H] noradrenaline release from rat amygdala under similar experimental conditions, but at these levels had no effect on ir-ME release. These findings are in accord

FIGURE 1.



Effect of K^+ concentration and presence of Ca^{++} ions on release of ir-ME from rat amygdala. Each example represents a single experiment.

with our earlier report (Morris & Livingston 1986) that administration of clonidine to rats did not affect ir-ME levels in this region. Thus we would conclude that although opioids may affect noradrenaline release and there are reports of alpha-2 adrenerqic modulation of the effects of chronic opioid administration, there is no evidence that alpha-2 adrenergic active drugs can effecf the release of endogenous metenkephalin from the rat amygdala in vitro.

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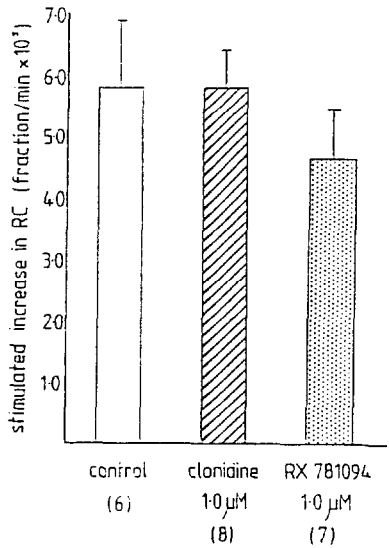
FIGURE 2.



Effect of clonidine and idazoxan on the release of ir-ME from rat amygdala slices in vitro. Mean ± SEM from 6-8 determinations of the fractional rate of release stimulated by 35mM K^+.

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RELEASE OF OPIOID PEPTIDES FROM THE SPINAL CORD OF RATS SUBJECTED TO CHRONIC PAIN

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ABSTRACT

Chronic localized pain increased the level of the opioid peptides, dynorphin (DYN), α-neoendorphin (ANEO), Met-enkephalin (MET) and Met^5-enkephalin-Arg^6-Gly^7-Leu^8 (MEAGL), in the lumbar enlargement of the rat spinal cord. It was accompanied with a reduction of the spontaneuos and K^+-stimulated release of ANEO and MEAGL from spinal cord slices in vitro and a decreased release of ANEO from the spinal cord in vivo. The results indicate that the reduction in the activity of endogenous opioid peptide systems might occur in the spinal cord of rats subjected to chronic pain.

INTRODUCTION

Chronic localized pain, as well as chronic polyarthritis increase the level of opioid peptides derived from prodynorphin (Przewlocki et al. 1984, Millan et al. 1985) and proenkephalin (Cesselin et al. 1980, Faccini et al. 1984, Przewlocki et al. 1984) in rats. these changes occur mostly in the lumbar enlargement of the spinal cord. They are accompanied with an increase in the analgesic potency of various opioid agonists and opioid peptides when applied directly into the spinal cord (Przewlocki et al. 1984). The obtained results indicate that supersensitivity of opioid receptors develops in rats subjected to chronic pain. Furthermore, the above observation posed a question whether chronic pain was associated with the enhancement or decrease of the activity of spinal opioid peptide systems. Therefore we studied an in vitro and in vivo release of opioid peptides from the spinal cord of rats subjected to prolonged localized pain. In addition, we evaluated the activity of enkephalin convertase, an enzyme thought to be responsible for the enkephalin synthesis in the nervous system (Strittmatter et al. 1984).

MATERIALS AND METHODS

Male Wistar rats (250-300 g) were intradermally injected with 0.1 ml of Freund's adjuvant into the hind limb sole in order to induce a chronic local inflammation. Three weeks after inoculation the rats were killed by decapitation and their spinal cord was immediately dissected and divided, and the tissue was processed to estimate immunoreactive DYN, ANEO, MET and MEAGL by specific RIAs as described previously (Przewlocki et al. 1983). For the in vitro release the tissue from dorsal halves of the lumbar enlargement was sliced (300 µm) with Mc Ilwain tissue chopper. Slices weighing about 100 mg were transferred to 1.5 ml superfusion chambers filled with a Ca^{2+}-free medium. After 20 min of washing the slices were continously super-

fused (flow rate 0.25 ml/min, temp 30°C with Krebs-bicarbonate solution modified according to Chavkin et al. (1983). Fractions (1 ml) were collected and ANEO and MEAGL were measured by RIAs. The in vivo release was performed according to the techniques described by Yaksh and Tyce (1980). To determine the stimulated release 30 and 57 mM K^+ and 20 µM capsaicin were used in respective solutions. Aliquots (usually 250 µl) of the collected frations were used directly for RIAs. The enkephalin convertase activity, as well as the ^3H-guanidinoethylmercaptosuccinic acid (GEMSA - an inhibitor of enkephalin convertase) binding to the enzyme were assessed according to Strittmatter et al. (1984).

RESULTS

As is shown in tab. 1, a chronic localized pain induced an increase in the level of DYN, ANEO, MET and MEAGL locally in the lumbar (but not cervical or thoracic) part of the spinal cord.

TABLE 1. The influence of chronic localized pain (CP) on the levels (pmol/g) of opioid peptides in lumbar spinal cord of rats

	DYN	ANEO	MEAGL	NET
control	26.1±2.3	12.8±1.3	93.6±3.6	401.2±34.0
C P	55.8±5.5*	26.5±1.8*	139.0±13.0*	551.5±62.6

p* <0.05

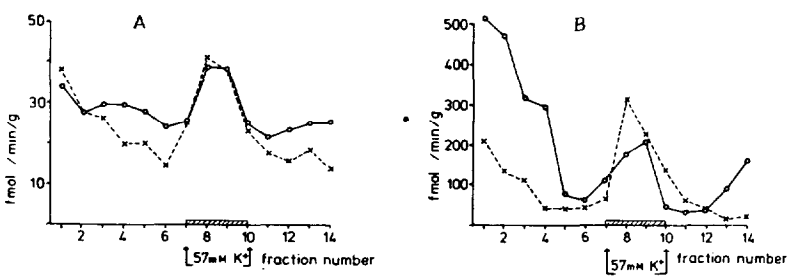

FIGURE 1. In vitro release of ANEO (A) and MEAGL (B) from the spinal cord of rats subjected to chronic pain. Control x----x, chronic pain o——o.

No changes in the peptide level were observed when tested in different brain structures (hypothalamus, thalamus, cortex). An increase in the β-endorphin content was noticeable in the anterior,

but not intermediate part of the pituitary (not shown). Increased levels of the peptides were accompanied with a slight increase in the basal in vitro release of ANEO and MEAGL from the dorsal part of the lumbar spinal cord. The stimulated release (57 mM K^+) was not significantly different when both groups of rats were compared (fig. 1). When the release was calculated as per cent of the tissue content, it showed that the basal and K^+-stimulated release was significantly lower in rats subjected to chronic pain (0.16 ± 0.01 vs 0.1 ± 0.01, and 0.34 ± 0.04 vs 0.15 ± 0.02 in controls and rats with chronic pain, respectively). The release of ANEO was further examined using an in vivo superfusion technique. The results are shown in fig. 2. As can be seen, in viva basal and stimulated release (30 mM K^+ or 20 µM capsaicin) of ANEO was significantly lower in rats subjected to chronic pain.

The activity of enkephalin convertase, an enkephalin synthetizing enzyme was not significantly changed though a slight decrease in the binding affinity of the enzyme inhibitor ^{3}H-GEMSA occurred in the lumbar spinal cord of rats subjected to chronic pain (K_D = 4.46 ± 0.1 vs K_D = 6.17 ± 0.2, respectively).

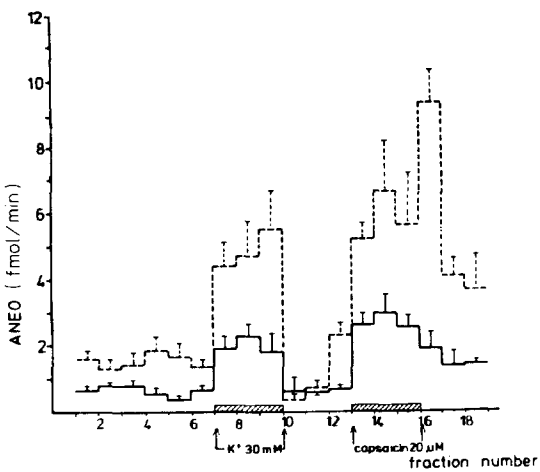

FIGURE 2. In vivo release of ANEO from the spinal cord of rats subjected to chronic pain. Control ----, chronic pain ——.

DISCUSSION

The present results demonstrate that chronic localized pain induces changes in the level and release of opioid peptides from the spinal cord. An elevated level of the peptides in the lumbar part of the spinal cord was accompanied with a decrease in the release of peptides derived from prodynorphin and proenkephalin in both in vitro and in vivo studies. Similar results were currently obtained by Cesselin et al. (1984), who examined release of Met-enkephalin in polyarthritic rats. It appears that prolonged painful stimuli most probably induced a decrease in the opioid peptide release, which

leads to their accumulation in neuronal terminals and, in consequence, decreases the availability of the peptides at a synaptic cleft. This might further induce supersensitivity of opioid receptors; actually, this phenomenon was demonstrated in our laboratory in rats subjected to chronic localized pain (Przewlocki et al. 1984), as well as in polyarthritic rats (Kayser and Guilbad 1983). Similar phenomena might also occur in man, since some authors reported reduction in the opioid peptide content in the cerebra-spinal fluid of patients suffering from chronic pain (Almay et al. 1978).

In conclusion, our results indicate that both prodynorphin and proenkephalin systems in the rat spinal cord adapt themselves to chronic painful stimuli by reducing the system activity, which in consequence, may result in their insufficiency in physiological mechanisms of pain control.

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RELATIONS BETWEEN GABAERGIC AND ENKEPHALINERGIC SYSTEMS:

EFFECTS OF SODIUM VALPROATE ON RAT BRAINENKEPHALINS.

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ABSTRACT

The acute effects of a Gaba-mimetic drug, sodium Valproate, are studied on brain enkephalin contents in rat brain. Valproate administered at both 300 and 600 mg/kg doses induces an increase of enkephalin levels in striatum, hypothalamus, brain stem and cortex. No effect is seen in the hypophysis.

INTRODUCTION

When relations between monoaminergic and enkephalinergic systems are extensively studied, the interactions between opioidergic and Gabaergic systems present an interesting development of opiate physiology. Both anatomical evidences (Aronin et coll 1984) and pharmacological arguments (De Feudis 1984) underly the concept of a Gaba/enkephalin link. Some studies were realized about the effects of Gaba-mimetic drugs on opiates. Roughly, administration of a Gaba A agonist is generally followed by a reduction of opioid 'levels (Duka and coll 1980) or release (Bourgoin and coll 1982). In opposition, the Gaba-B mimetic drugs as Baclofen enhance enkephalin release from brain rat slices (Sawynok and Labella 1981).
Sodium Valproate (VPA) is an extensively used anti-epileptic drug, enhancing Gaba levels by blocking the degradation enzymes of this neuro-transmitter (Johnston and Slater 1982). We studied the acute effect of this drug on Mat-Enkephalin and Leu-Enkephalin levels in five areas of rat brain. VPA was administered at doses eliciting an anti-epileptic effect on kindled seizures in rat (Tulloch and coll 1982)

MATERIEL AND METHODS

Three groups of 7 albinos male rats (300g +/- 20) from Iffa Credo were used for the experiments. Animals of each group were administered I.P. respectively by two doses of 300 and 600 q/kg of VPA (Depakine, Labaz laboratories, France), and 0.5 ml of distillated water (drug vehicle). The rats were killed by decapitation, without anaesthesia, between 10:00 and 12:00 am, to avoid a possible effect of circadian ryhtms.
Both tissue samples preparation and RIA procedure was carried out as previously described (Cupo and Jarry 1985). ME antibodies exhibit following properties: IC50 = 9.1 pM and cross-reactive factor (f) with analog peptides always greater than 175. LE antibodies were obtained by an original method (Cupo and coll to be published) and present the following characteristics: IC50 = 23 pM and f > 80 in

all cases.

RESULTS

Injection of VRA at anti-epileptic doses is followed by an increase of both LE (table 1) or ME (table 2). This effect is significant at a 600 mg/kg dose for LE levels and significant for the 2 doses for ME levels. Note the lack of effect in the hypophysis.

TABLE 1. Acute effect of sodium valproate on Leucine-enkephalin levels in five areas of rat brain. Two doses were injected intra-peritonealy: 300 and 600 mg/kg. Animals were sacrified 30 minutes after injection. Results are given in picomole of wet tissue (+ or - sem). St= striatum, Ht=Hypothalamus, Hph=Hypophysis, Br.St.=Brain stem, and Cx=cortex.

Groups	St.	Ht.	Hph.	Br St.	Cx
Controls	0.033 (0.004)	0.033 (0.003)	0.26 (0.012)	0.013 (0.001)	0.0023 (0.001)
VPA 300 mg/kg	0.038 (0.004)	0.039 (0.006)	0.25 (0.016)	0.012 (0.012)	0.005 (0.002)
VPA 600 mg/kg	0.065 (0.010) ***	0.068 (0.021) ***	0.23 (0.016)	0.029 (0.005) ***	0.005 (0.004) **

Statistical analysis: variance analysis. ***, p<0.001; **,p<0.01; *, p<0.05.

When administration of two doses of VPA induces an increase of enkephalinergic system activity in four brain areas, the lack of this effect in hypophysis suggests that, in 30 minutes, VPA presents store of a neuro-neuronal action than a neuro-endocrine one. In this point of view, if the effect of VPA per se on enkephalinergic neurons must not be discarded, there are many evidences that there is a close correlation between plasmatic concentrations of VPA and synaptic Gaba levels (Iadarola and Gale 1981). The nature of the effect of VPA on ehkephalinergic systems seems to mimic the effects of Baclofen and suggests a preferential implication of Gaba B receptors (Sawynok and Labela 1981).
The small delay between the injection of VPA and the sacrifice of the animals is a good argument for an increase of enkephalin levels in relation to an increase in pro-enkephalin consumption more than to an increase of pro-enkephalin processing. Preliminary results on octapeptide (an other peptide derived from pro-enkephalih) show that the same doses of VRA induce a reduction of octapeptide levels suggesting a precursor consumption and/or a differential maturation of the pro-ehkephalin related peptides (Vion-Dury and coll to be published)

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This work raises an important question: are the therapeutic or adverse effects of VPA partially related to enkephalinergic pathway activation ? When action of opioid peptides or agonists on epilepsy is very complex and very discussed (Frenk 1983), it is probable that the anti-epileptic action of VPA is mainly related to an increase of Gaba-ergic transmission (Johnston and Slaters 1982).

TABLE 2. Acute effect of sodium valproate on Methionine-Enkephalin levels in the same areas of rat brain. For legend, see table 1.

Groups	St.	Ht.	Hph.	Br.St.	Cx
Controls	0.58 (0.06)	0.52 (0.016)	2.49 (0.42)	0.19 (0.016)	0.033 (0.003)
VPA 300 mg	0.82 (0.11) **	0.77 (0.08) **	2.51 (0.34)	0.24 (0.03)	0.041 (0.003) *
VPA 600 mg	0.80 (0.13) ***	0.76 (0.11) **	2.37 (0.48)	0.33 (0.04)	0.051 (0.004)

Yet VPA (as others Gaba-mimetic drugs) exhibits also important analgesic properties at lower doses than those used for the anti-epileptic treatment (Mesdjian and coll 1983) Some preliminary dosages show that VPA a 100mg/kg (analgesic doses), induces an increase of opioid peptides in striatum(Vion-Dury and coll to be published). In this point of view, it must be noted that Mesdjian and coll (1983) suggested an non implication of opioidergic transmission because the effect of VPA was not reversed by naloxone. They conclude that the effects of VPA was related to an increase of Gabaergic transmission. But, if it is assumed that 1) weak doses of VPA induce an increase of enkephalinergic transmission, and 2) the synaptic organization of Gaba/enkephalin link is underlyed by a parallel circuitry (Andree and coll 1983), the lack of the effect of naloxone can be easily explained: the naloxone antagonism of enkephalin effect would be masked by Gaba-mimetic action of VPA on pain pathways.

Increase of enkephalinergic transmission after VPA treatment might also explain the apparition of a tolerance and dependence to analgesic action of both morphine and Gaba agonists in mice receiving injections of THIP(Andree and coll 1983). If the action of VPA on enkephalinergic pathways is underlyed by a preferential activation of Gaba-B receptors, our results might also explain the syndrome of a Baclofen overdose, which mimics the syndrome of opiate overdose (De Feudis 1984).

So, from these data, it is possible to try to complete the schema proposed by Andrée and coll (fig 1) and to suggest the hypothesis of a parallel organization of Gaba-ergic and enképhalinergic pathways with a fine modulation of enkephalinergic activity by Gaba-ergic

system involving several Gaba-ergic receptors as suggested by Bourgoin and coll (1981).

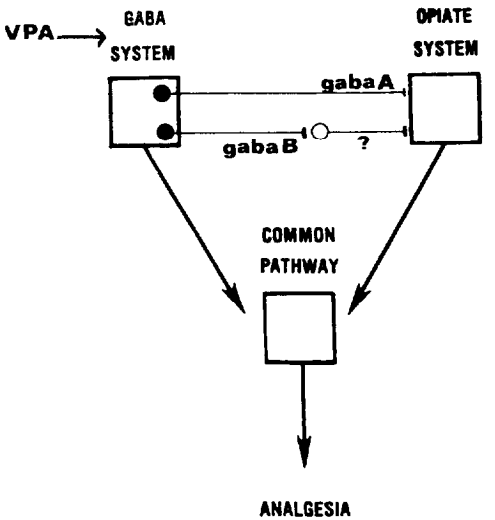

FIGURE 1: Possible organization of the relations between Gabaergic and enkephalinergic systems (from Andree and coll 1983): Commentary in the text

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[MET]ENKEPHALIN-ARG^6-PHE^7 IN HUMAN CSF - INCREASED LEVELS IN LATE PREGNANCY

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ABSTRACT

The opioid heptapeptide, [Met]enkephalin-Arg^6-Phe^7 was measured in CSF of women at term pregnancy and during the luteal phase by RIA. Levels turned out to be significantly increased in pregnant women. These results are in agreement with an earlier paper in which we reported increased levels of receptorassayed opioid peptides deriving from proenkephaln A in CSF of women in term pregnancy compared to levels in nonpregnant, nonpuerperal women.

INTRODUCTION

C-terminal extensions of the pentapeptides [Met]enkephalin and [Leu]-enkephalin (so called ECPs) were in fact isolated from brain and peripheral tissues a few years prior to the sequencing of the opioid peptide precursors proenkephalin A and proenkephalin B. One of these C-terminally extended peptides, [Met]enkephalin-Arg^6-Phe^7, was described by Stern et al. 1980. On a molar basis, Rossier et al. (1980) found this heptapeptide eight times more analgesic than [Met]enkephalin in the tail flick test. In a previous study we reported elevated levels of so-called FII endorphins both in term pregnancy and in early puerperium compared to levels in nonpregnant, nonpuerperal women. We suggested these elevated FII endorphins to derive from the [Met]enkephalin precursor (Lyrenäs et al. 1986). In the present study we measured CSF levels of [Met]enkephalin-Arg^6-Phe^7 by RIA in individual pregnant women and in nonpregnant women during the luteal phase of the menstrual cycle.

MATERIAL AND METHODS

The study included CSF from two groups of women:

1. This group comprised of 12 pregnant women who underwent elective cesarean section at term due to pelvic disproportion. The operations were performed during spinal anesthesia and five ml samples of CSF were removed prior to administration of the anesthetic drug. CSF levels of [Met]enkephalin-Arg^6-Phe^7 were measured by RIA.
2. A control group composed of eight nonpregnant nonpuerperal healthy female volunteers; CSF samples were collected during the luteal phase of the menstrual cycle. Lumbar punctures were performed at the level of L:3-L:4 with the same technique as used in group 1. CSF levels of [Met]enkephalin-Arg^6-Phe^7 were estimated by RIA.

CSF samples were taken in ice cold polyethylene tubes prepared with EDTA, immediately frozen and kept at -70°C until analyzed.

Materials: Peptide standards used in this study were obtained from

sources previously noted (Nyberg et al. 1983). Reversed phase silica gel cartridges (SepPak C-18) were purchased from Waters Associates (Milford, Mass., U.S.A.). All other chemicals and solvents were of analytical reagent grade from commercial sources.

Sample work up: Prior to RIA, the thawed CSF sample (two ml) was subjected to SepPak separation. Before sample application, the cartridge was washed consecutively with 10 ml methanol containing 0.04 % trifluoroacetic acid (TFA), 10 ml methanol and 10 ml water. The CSF sample was applied in a volume of two ml and the cartridge washed with 2.5 ml of water. Elution was carried out with aqueous mixtures of TFA (0.04 %) and increasing concentrations of methanol. The heptapeptide-containing fraction was eluted with 60 % methanol and evaporated. The dried fractions were redissolved in 100 µl 1M acetic acid and incubated for 30 min. at 37°C with 1 % H_2O_2. All samples were evaporated a second time before RIA analysis.

The radioimmunoassay for [Met]enkephalin-Arg^6-Phe^7 was based on the charcoal adsorption technique and conducted in conformity with those described for other peptides (Christenson-Nylander et al: 1985). The antibodies were raised against the heptapeptide sulfoxide ([Met]-O-enkephalin-Arg^6-Phe^7) and the iodinated oxidized peptide was used as tracer. The detection limit of the RIA was around 20 fmol/tube. Crossreaction with [Met]enkephalin and other proenkephalin A or B derived peptides was less than 0.1 %. Runs with the synthetic heptapeptide added to artificial CSF at a concentration of 50 fmol/ml indicated a recovery of approximately 70 % of the extraction procedure.

High performance liquid chromatography (HPLC): A HPLC system (LKB, Bromma, Sweden) was used for identification of the measured peptide. Pooled aliquots of the assayed fractions were analyzed in a single run. The column (Spherisorb C-18, 5 µ, 4.5 x 250 mm) was eluted with a 40 min. linear gradient of acetonitrile (10-45 %) containing TFA (0.04 %). Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min. and evaporated prior to RIA analysis.

RESULTS

Figure 1 shows the levels of radioimmunoassayable CSF [Met]-O-enkephalin-Arg^6-Phe^7 in groups 1 and 2. The mean level (SD) in term pregnancy was 32.8 (12.0) fmol/ml and in the luteal phase 22.4 (7.1) fmol/ml. The difference is statistically significant ($p < 0.05$; Student's t-test). HPLC identification showed that the major part of RIA activity eluted like the standard (figure 2). Minor activity eluting with higher retention time was also observed.

DISCUSSION

The complexity of the proenkephalin systems is considerable, and a whole family of enkephalin peptides can be identified in human CSF (Nyberg et al. 1986). We have earlier used a receptorassay to identify so-called Fraction II opioid peptides, which mainly derive from proenkephalin A. In CSF from women at term pregnancy and in the early puerperium, fraction II levels were increased (Lyrenäs et al. 1986). One major component in Fraction II is [Met]enkephalin-Arg^6-Phe^7 (Nyberg et al. 1983). The present study using a specific RIA for this peptide, confirmed the elevation of enkephalin peptides at term pregnancy. Assuming this elevation to reflect increased activity,

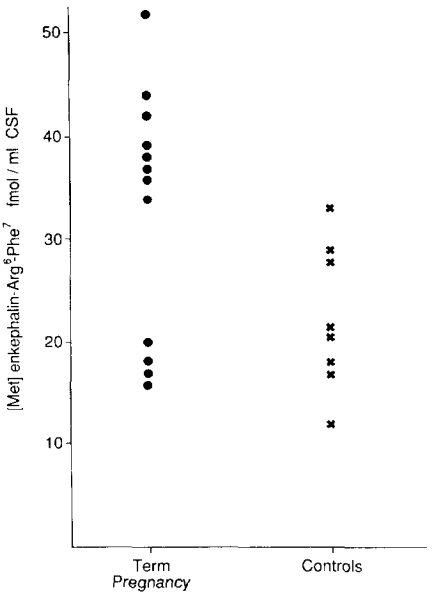

FIGURE 1. CSF levels of [Met]enkephalin-Arg^6-Phe^7 in term pregnancy and during the luteal phase measured by RIA. Antibodies used in the RIA were raised using the heptapeptide sulfoxide.

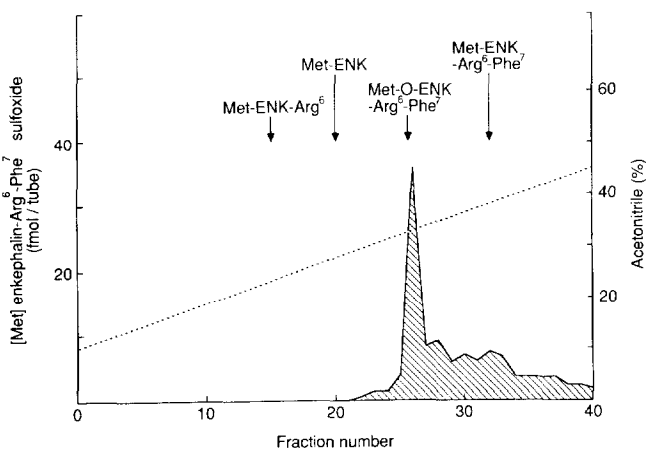

FIGURE 2. High performance liquid chromatography of [Met]enkephalin-Arg^6-Phe^7 sulfoxide immunoreactivity. Calibration runs were performed with synthetic standards as indicated by arrows (ENK = enkephalin). Further details are given in the text.

enkephalin pathways appear to be under higher tonic activity in late pregnancy and the early puerperium than in non-pregnant, non-puerperal women. Similar information is available with regard to plasma levels, i.e. the hormonal secretion of β-endorphin and [Met]enkephalin in late pregnancy and early puerperium (Pancheri et al. 1985).

The general elevation of endorphin activity in late pregnancy and early puerperium may be considered a primary reaction subserving a protective role or be a secondary reaction to the stress involved. Further studies will be needed to distinguish between these possibilities and to elucidate the basic mechanisms.

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EXPRESSION AND REGULATION OF KAPPA OPIATE RECEPTORS IN RAT SPINAL CORD-DORSAL ROOT GANGLION COCULTURES

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ABSTRACT

We have been using rat spinal cord-dorsal root ganglion primary cocultures (SC-DG) as a model system for exploring K receptor regulation. During the first 10 days in culture, the total number of opiate receptors increased markedly, reaching a Bmax of 180 fmoles/mg protein for K sites and a Bmax of 50 fmoles/mg protein for μ sites. Following this period of development, the K and μ receptor number did not change significantly. No detectable δ sites were observed at any time of culture. The binding of [^{3}H]diprenorphine to K sites was found to be saturable, of high affinity and stereospecific. After chronic agonist treatment of cultured cells, K receptors did not down-regulate, whereas more than 50% of the μ receptor sites did. Following chronic antagonist treatment, μ receptors were markedly up-regulated (260% of control), while K sites exhibited a weaker up-regulation response (160% of control). These data demonstrate that K opiate receptors are expressed at high concentrations in SC-DC cultures andthatcontraryto μ receptors in spinal cord and δ receptors in NG10815 cells, K binding sites are less susceptible to modulation following chronic agonist or antagonist treatment. This suggests that K receptors may be regulated by different control mechanisms.

INTRODUCTION

Exposure of cells to agonists or antagonists often results in a change in the concentration of their surface receptors. Up-regulation of μ and δ opiate receptors in rat brain has been induced by chronic administration of antagonists (Tempel et al. 1985). Ample documentation of μ and δ receptor down-regulation has been provided by agonist treatment of aggregated fetal brain cells (Lenoir et al. 1984) and of cultured. NG10815 cells (Law et al. 1983). On the other hand, very little is known about the regulation of K opiate receptors in nervous tissue. We have been using SC-DG cultures as a model system for exploring K receptor regulation since spinal cord has been proved to be a rich source of K sites (Attali et al. 1982).

METHODS

SC-DG cultures were prepared according to Ransom et al. (1977). After 2 weeks in culture, the cells were treated for the time indicated. Cells were washed 3 times with PBS and incubated 15 min at O°C in 100 mM NaCl and 50mH Tris-HCl (pH=7.4). Cells were homogenized and centrifuged for 30 min at 27,500xg, pellets resuspended in 100mM NaCl, 50mM Tris-HCl (pH=7.4) and incubated for 15 min at 37°C to allow bound drug to dissociate from the receptor. The membranes were further washed twice in 50mM Tris-HCl (pH=7.4) and resuspended in the

same buffer for binding assay. In control untreated cells, equal concentrations of the same agonists or antagonists were added to culture dishes immediately before cell harvesting. K binding sites were laballed by [^{3}H] diprenorphine (30 min, 37°C) in the presence of DAGO (0.1µM) and DADL (0.1µM) to suppress the contribution of µ and δ sites. [^{3}H] DAGO and [^{3}H]DSTLE were used for labeling (30 min, 37°C) µ and δ receptor sites, respectively. Non-specific binding was determined in the presence of 10 µM diprenorphine.

RESULTS

No detectable µ, δ or K sites were observed on day of plating. During the first 10 days in vitro, the total number of opiate receptors increased markedly, reaching a Bmax of 180±44 fmoles/mg protein (n=11) for K sites and a Bmax of 50±6 fmoles/mg protein (n=3) for µ sites. Following this period of development, the K and µ receptor number did not change significantly. Interestingly, no detectable δ sites were observed at any time of culture (up to 4 weeks in vitro). The binding of [^{3}H] diprenorphine to K sites and that of [3H]DAGO to µ sites were found to be saturable and of high affinity (Kd=O.85±0.15nM, n=11 and Kd=2.4±0.5nM, n=3, respectively).

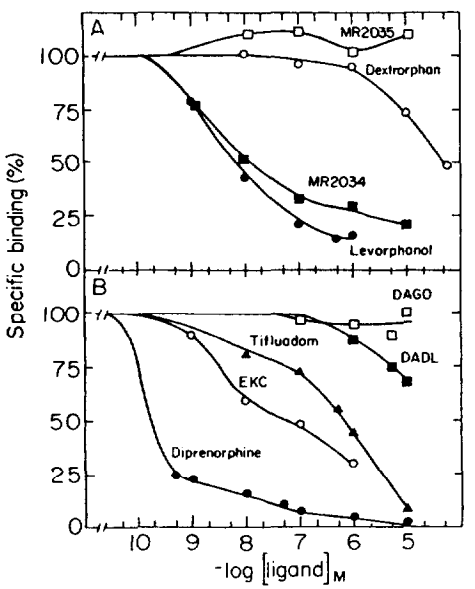

FIGURE 1. Pharmacology of K opiate binding sites in SC-DG culturess. The pharmacological profile of various ligands was assessed by competition experiments. K receptors in membranes of 2 weeks old SC-DG cultures were labeled by [^{3}H]diprenorphine (1.5nM) in the presence of DAGO (0.1µM) and DADL (0.1µM). The data correspond to a typical experiment replicated two to three times with similar values.

TABLE 1. Regulation of opiate receptors upon chronic agonist or antagonist treatment

Treatment	SC-DG CELLS μ (% of control)	SC-DG CELLS κ (% of control)	NG108-15 CELLS δ (% of control)
Etorphine 1 μM, 1 day	47±2****	96±7	0±0****
Etorphine 1 μM, 3 days	N D	80±1 *	
U50488 10 μM, 1 day	N D	95±11	30±11**
Naloxone 10 μM, 5 days	261±17***	164±3****	123±3***

Regulation of μ and κ receptors was assayed in 2 week old SC-DG cultures and that of δ sites in NG108-15 cells. Results are expressed as percent of control binding to untreated paired cultures. Values were deduced from saturation experiments; the control binding values for μ, κ and δ receptors are 48±3, 187±36 and 435±21 fmoles/mg protein, respectively (n=3). Significance of data was calculated by the unpaired student t test (*p<0.05, **p<0.02, ***p<0.01, ****p<0.001). ND (not determined).

Displacement experiments revealed that the binding to K sites was stereospecific since MR 2034 and levorphanol were potent competitors, while their respective inactive enantianers, MR2035 and dextrorphan were ineffective (figure 1A). K selective ligands such as MR2034, ethylketocyclazocine and tifluadom were able to compete with [^{3}H]diprenorphine binding at low concentrations, whereas μ or δ ligands were shown to be very weak displacers (figure 1B). The affinity of the K agonist U50488 decreased significantly in the presence of 100μM GTP and 100mM NaCl (data not shown). Following chronic exposure to the non-selective opiate agonist etorphine (1μM, 24h), K receptors did not down-regulate, whereas under the same experimental conditions, 53% of μ sites down-regulated (table 1). The same results were obtained by using the K selective agonist U50488 (10 μM, 24h). In contrast, we found that in NG10815 cells which contain predominantly δ sites, one day treatment with 1 μM etorphine or 10 μM U50488, a concentration with also occupies δ receptors induced δ receptor down-regulation by more than 90% and 70%, respectively. Prolonging the time of agonist exposure(etmphine 1 μM, 3 days), we observed a weak but significant down-regulation of κ receptors which amounted now to 20% (table 1). Followinq chronic antagonist treatment (naloxone 10 μM, 5 days), μ receptors strongly up-regulated (261% of control), while κ sites exhibited a weaker up-regulation (164% of control). Similar experiments performed in NG108-15 cells showed that δ receptors slightly up-regulated (125% of control).

DISCUSSION

In agreement with previous studies showing that the adult spinal cord contains high amounts of κ receptors (Attali et al. 1982; Czlonkowski et al. 1983), our data now demonstrate that after neuronal differentiation in vitro, κ opiate receptors are expressed at high concentrations (more than 70% of total receptors) in SC-DC cultures. The opioid nature of these binding sites is demonstrated by the saturability, high affinity and stereospecificity of ligand binding. The κ properties of the binding sites is revealed by the pharmacological

profile of various compounds in competition experiments. κ selective ligands were good displacers, while μ and δ ligands were relatively ineffective. The regulation of K agonist binding (U50488) in cultured cells by GTP and Na^+ correlates well with previous studies showing a similar regulation in adult rat spinal cord (Kelly et al. 1982), which suggests a possible couplinq of K receptors to adenylate cyclase (see also Attali and Vogel this volume). Our data concerning K receptor regulation reveal striking differences with μ and δ receptors. Contrary to μ receptors in SC-DG cells or δ receptors in NG10815 cells, K receptors were not down-regulated upon chronic exposure of SC-DG cultures to etorphine or U50488. Only after 3 days of agonist exposure, a slight down-regulation of K sites is observed (20%). This lack of apparent down-regulation for K receptors suggests that the regulation upon chronic activation might occur at a different level such as post-receptor binding regulatory compnents (Attali and Vogel this volume). Upon chronic antagonist treatment, K receptor regulation, exhibited again a different feature when cmpared to μ receptors. However, although K sites displayed a weaker up-regulation, the significant increase in K opiate binding might be the result of several mechanisms. These may include either increased receptor synthesis, lowered degradation, unmasking of active receptor molecules, or even conversion of newly synthesized non-binding receptors to active receptors. It is interesting to note that antagonist-induced μ receptor up-regulation in fetal mouse spinal cord-ganglion explants does not require the synthesis of new receptor molecules (Tempel et al. 1986). Taken together, these data reveal that contrary to μ or δ receptirs, K binding sites are less susceptible to modulation upon chronic agonist or antagonist treatment, suggesting that they are regulated by different control mechanisms.

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SENSITIVITY OF ANTINOCICEPTIVE TESTS TO OPIOID AGONISTS AND PARTIAL AGONISTS

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ABSTRACT

There is considerable variation in the potency of opioids across different animal models of antinociceptive activity. In the less sensitive tests the partial agonist analgesics behave as antagonists. The activity of opioids in antinociceptive tests appears to be dependent on both the intensity of the noxious stimulus and the intrinsic activity of the drug.

INTRODUCTION

It has long been recognised that the potencies of analgesic agents exhibit considerable variation between different animal models. For example, the ED_{50} for morphine in the hot plate test has been reported to be 30 times greater than in the abdominal constriction model (Ward and Takemori 1983). In addition, several drugs (eg. nalorphine) which display potent antfnociceptive activity in the abdominal constriction test are inactive in tests employing heat as the noxious stimulus (Tyers 1980). It has been suggested that the neural pathways involved in the transmission of heat and non-heat stimuli may possess different types of opioid receptor, and that drugs which are ineffective against heat stimuli act predominantly at k-sites.

However, this hypothesis does not explain why the potencies of drugs such as morphine vary between tests. Neither does it explain the observation made by several groups (Zimet et al. 1986. Sewell and Spencer 1976, Luttinger 1985) that heat tests can be made to detect the agonist-antagonist analgesics by lowering the temperature of the stimulus.

The present study was designed in an attempt to clarify the relative contribution of receptor selectivity, intrinsic activity and test methodology to the analgesic efficacy of opioids.

METHODS

Dose responses to each of the test drugs were performed on 4 analgesic tests employing different noxious stimuli : 55° hot plate (mouse), acetic acid induced abdominal constriction (mouse), paw pressure (rat) and tail flick (rat). The animals used were either female mice of the Alderley Park strain weighing 18-20g or newly weaned female Alderley Park strain rats weighing 40-60g. In all studies the test drug was administered subcutaneously 30 minutes prior to test.

Mouse hot plate

Reaction times of mice placed on a hot plate maintained at 55°C were determined. Any drugs devoid of activity were tested as antagonists of the k-agonist tifluadom. In this situation dose responses to tifluadom were performed in the presence and absence of a constant dose of antagonist. Groups of at least 6 mice were used in all hot plate studies.

Acetic acid induced abdominal constriction

Acetic acid (0.4 ml) was administered intraperitoneally 30 minutes after the test drug. Animals were left for 2 minutes before counting the cumulative number of responses over the next 15 minute period. A minimum group size of 10 was employed. In the first part of the study the acetic acid concentration was 0.4%, but in later experiments 0.25% and 0.6% concentrations were also used.

Paw pressure

Thresholds for each hind paw were measured using a modified "Analgesimeter" (Ugo Basile, Milan) with the endpoint being taken as the first struggle or vocalisation by the rat.

Tail flick

The method used was essentially the same as that described by D'Amour and Smith (1941). The rat's tail was placed over a focussed light beam and the latency to flick recorded. A cut-off time of 20 seconds was imposed. In both rat studies at least 10 animals were used per group.

RESULTS AND DISCUSSION

The activity of each of the drugs varied according to the test situation (table 1) with only the full agonists and high intrinsic activity partial agonists displaying full dose response curves across the complete range of tests. U50488H produced a flattened dose response curve on the hot plate test but complete dose responses on the rat paw pressure and tail flick tests and the mouse abdominal constriction test. The lower intrinsic activity partial agonists xorphanol, nalbuphine, bremazocine, levallorphan and nalorphlne were effective only on the abdominal constriction test.

The potencies of the drugs varied across the range of tests making it possible to rank the assays in order of decreasing sensitivity as follows: abdominal constriction > tail flick = paw pressure > hot plate. These findings prompt the suggestion that each model provides a different noxious stimulus intensity with hot plate providing the highest and abdominal constriction test the lowest. If, by increasing the stimulus intensity, the apparent number of "spare" receptors is reduced, then it is possible to explain the current observations. In the hot plate test, which provides the highest stimulus intensity as reflected by the low potencies of the test drugs. only the full agonists fentanyl and tifluadom and the high

intrinsic activity partial agonist morphine have sufficient efficacy to generate a full dose response. U50488H is less efficacious and this is reflected by a flattened dose response on the hot plate test although full dose responses were produced against the other lower intensity stimuli. The low intrinsic activity partial agonists have sufficient efficacy to be effective against only the lowest stimulus.

TABLE 1 Antinociceptive potencies of a range of opiolds

	ED_{50} (mg/kg s.c.)			
	HOT PLATE	TAIL FLICK	PAW PRESSURE	ABDOMINAL CONSTRICTION
Morphine	3.4 (3.3 – 3.9)	1.6 (1.3 – 1.9)	1.6 (1.4 – 1.8)	0.41 (0.28 – 0.62)
Fentanyl	0.04 (0.03–0.07)	0.013 (0.007–0.02)	0.01 (0.009–0.029)	0.003 (0.001–0.008)
Tifluadom	3.4 (2.9 – 3.9)	1.75 (1.0 – 2.7)	2.2 (1.8 – 2.6)	0.3 (0.23 – 0.45)
U–50488H	Shallow dose response	13.9 (9.8 –19.6)	16.9 (12.0–23.8)	1.1 (0.63 – 2.2)
Xorphanol	>30	>10	>10	0.08 (0.04 –0.19)
Nalbuphine	>30	>30	>30	1.4 (0.58 – 1.4)
Bremazocine	>30	>30	>30	0.01 (0.003–0.07)
Levallorphan	>30	>100	>100	1.1 (0.34 –0.71)
Nalorphine	>100	>100	>100	0.77 (0.1 – 3.4)

If this explanation is correct, the lower efficacy drugs should antagonise the effects of agonists acting at receptors for which the partial agonists have affinity. This hypothesis was tested by determining the effects of nalorphine, levallorphan and bremazocine on the dose response to tifluadom in the hot plate test. The tifluadom dose response was shifted to the right in a parallel manner in the presence of each of these partial agonists (results not shown) thus providing support for the hypothesis.

These findings do not support the suggestion of Tyers (1980) that μ-agonists are equipotent against heat and non-heat stimuli whereas k-agonists are less effective against heat. In the light of the current findings it is obvious that μ and k-drugs with sufficient efficacy are equally effective against heat and non-heat stimuli as demonstrated by their similar potencies in the paw pressure and tail flick assays, employing mechanical and thermal stimuli respectively. The most likely explanation for this discrepancy is that many drugs regarded as k-agonists are, in fact, partial agonists (Miller et al. 1985). The current data suggest that it is the efficacy of the drug rather than its receptor selectivity which determines its profile against different noxious stimuli.

TABLE 2 Effect of acetic acid concentration on potency

	Acetic Acid Concentration		
	0.25%	0.4%	0.6%
Morphine	0.13 (0.04–0.34)	0.41 (0.28–0.62)	0.55 (0.25–0.98)
Tifluadom	0.28 (0.18–0.48)	0.30 (0.23–0.45)	0.31 (0.17–0.55)
U-50488H	0.43 (0.13–1.25)	1.1 (0.63–2.2)	1.1 (0.66–2.2)
Levallorphan	0.02(0.002–0.16)	1.1 (0.34–7.1)	*
Nalorphine	0.12 (0.01–13.2)	0.77 (0.09–3.4)	3.9 *
Nalbuphine	0.42 (0.2 –1.1)	1.4 (0.58–1.39	2.0 *

* Shallow dose response – difficult to estimate ED_{50}.

Further support for the hypothesis is provided by the second part of the current study which examined the effects of changing the stimulus intensity within one assay - the mouse abdominal constriction test (table 2). Responses to the μ- and k-agonists, morphine and tifluadom were not significantly influenced by the acetic acid concentration. In contrast the k-partial agonists xorphanol, nalorphine and levallorphan, and the p-partial agonist nalbuphine exhibited lower potency and flattened dose response curves when tested against the higher acetic acid concentrations.

In conculsion, antinociceptive tests exhibit a wide range of sensitivity to opioids. Some models such as the hot plate assay are only sensitive to high doses of full agonists, whilst partial agonists display antagonist properties. The more sensitive assays such as the abdominal constriction test appear to have a high receptor reserve in the abdominal constriction model. These findings highlight the need for caution when comparing data from different tests, or even from the same test performed under slightly different conditions.

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EVIDENCE FOR DELTA RECEPTOR MEDIATION OF [D-PEN^2,D-PEN^5]-ENKEPHALIN (DPDPE) ANALGESIA IN MICE

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ABSTRACT

Possible involvement of cerebral delta opioid receptors in antinociceptive processes was studied in a test utilizing heat as the noxious thermal stimulus. The investigation focused on selective agonists and antagonists for mu and delta opioid receptors. Morphine and [D-Ala^2,NMPhe^4,Gly-ol]enkephalin (DAGO) were used as agonists for the mu receptorwhile [D-Pen^2,D-Pen^5]enkephalin (DPDPE) was the agonist for the delta receptor. Two approaches were employed: first, the intracerebroventricular (i.c.v.) analgesic activity of the agonists was determined in the absence, and in the presence of graded i.c.v. doses of the selective delta antagonist, ICI 174,864 (N, N diallyl-Tyr-Aib-Aib-Phe-Leu-OH)(where Aib is alpha-aminoisobutyric acid); second, acute tolerance to morphine was produced and the possible presence of acute cross-tolerance between subcutaneous (s.c.) morphine and the delta agonist investigated. ICI 174,864 antagonized the analgesia produced by DPDPE, but not that resulting from morphine or DAGO. biorphine pretreatment resulted in the development of acute tolerance to i.c.v. morphine, and acute cross-tolerance to i.c.v. DAGO, but not to i.c.v. DPDPE. These results provide evidence that both cerebral delta and mu opioid receptors are responsible for the mediation of analgesia in tests utilizing heat as the nociceptive stimulus.

INTRODUCTION

Traditional views regarding the involvement of supraspinal opioid receptorsintests ofanalgesiawhere heat is employed as the nociceptive stimulus have focused on the exclusive involvement of mu, rather than delta and kappa, receptors (Audigier et al. 1980; Chaillet et al. 1984; Changetal. 1982). While the analgesic effects of i.c.v. kappa agonists are generally difficult to detect, both mu and delta agonists produce effective analgesia by this route (Porreca et al. 1984). Thus, it is of interest to determine whether even highly delta selective agonists such as DPDPE (Mosberg et al. 1983) produce supraspinal analgesia in thermal tests by activation of mu receptors, or whether cerebral delta receptors are also involved in this effect. The present investigation attempts to determine the possible involvement of cerebral delta opioid receptors in thermal analgesic tests using direct antagonism with the highly selective delta antagonist, ICI 174,864 (Cotton et al. 1984) as well as the determination of possible cross-tolerance between morphine and the selective delta agonist, DPDPE.

MATERIALS AND METHODS

Analgesic assay: Male, ICR mice (20-25 g) were tested forthelatencytoa

rapid flick of the tail following immersion into warm (55°C) water. The mice then received graded i.c.v. doses of agonist and distilled water, or agonist and antagonist. The i.c.v. injection was made following the induction oflightether anesthesia by an incision in the scalp, location of bregma and injection directly into the lateral ventricle using a microliter syringe in a volume of 5 µl. Twenty min after the i.c.v. injection, a time determined to be close to the peak analgesic effect of the agonists, the mice were retested for the latency to tail-flick. A cut-off time of 15 sec was employed in order to minimize damage to the tail. Post-drug latency was comparedtotheindividual pre-drug latency and percent analgesia determined.

Acute tolerance: The procedure used was that described by Vaught and Takemori (1979). Mice received a single s.c. injection of morphine sulfate (100 mg/kg) or water 5 hr prior to testing. The morphine pretreatmentnolongerhadanyagonisteffectatthis time. Graded doses of morphine, DAGO and DPDPE were then given i.c.v. andtestingtook place after 10 min.

RESULTS

Co-administration of ICI 174,864 (at 1 or 3 µg, i.c.v.) with i.c.v. DPDPE resulted in a dose-related antagonism of the analgesic effect (fig. 1). In contrast, ICI 174,864 (3 µg) failedtoantagonizethemorphine (fig.1) or DAGO (not shown) dose-response lines.

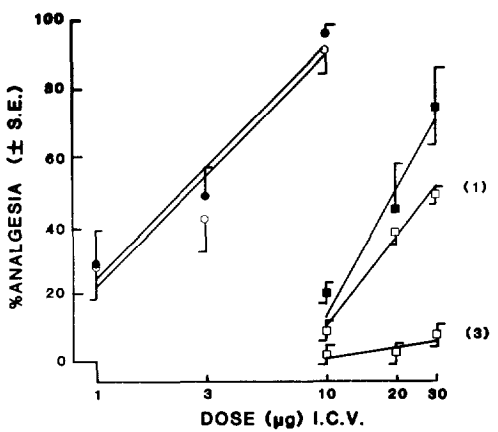

Figure 1. Analgesic dose-response lines for i.c.v. morphine (circles) or DPDPE (squares) in the absence (closed symbols) or presence of ICI 174,864 (open symbols) at 1 or 3 µg (DPDPE) or 3 µg (morphine).

Pretreatment with morphine (100 mg/kg, s.c. at -5 hr) did not result in any analgesic effect following the i.c.v. administration of water. The i.c.v. morphine dose-response line was displaced to the right by a factor of 6.6, indicating the developmentofacutemorphine tolerance (table 1).

In contrast, the i.c.v. DPDPE dose-response line was not affected (table 1), indicating a lack of i.c.v. acute cross-tolerance between morphine and the selective delta agonist.

TABLE 1. Analgesic A_{50} (and 95% C.L.'s)(µg/mouse) for 3 agonists given intracerebroventricularly to control or morphine pretreated mice (100 mg/kg, s.c. at -5 hr) in the warm-water tail-withdrawal test.

Agonist	Control	Morphine-pretreated	Potency
Morphine	0.25 (0.107 - 0.598)	1.65 (0.79 - 3.47)	6.6
DPDPE	5.71 (3.13 - 10.43)	7.84 (4.53 - 13.56)	1.37
DPLPE	6.77 (0.01 -2.8-16.4)	7.68 (5.17 - 11.41)	1.13

DISCUSSION

The present study has investigated the possible involvement of delta opioid receptors in the inhibition of responses to a noxious thermal stimulus. Both mu (morphine, DAGO) and delta (DPDPE) agonists produced full dose-response effects after i.c.v. administration, suggesting that both mu and delta receptorsmightbe responsible forthemediation of this effect. Previous studies, however, have suggested that all supraspinal analgesia determined in tests utilizing heat as the noxious stimulus relied on the sole activation of mu opioid receptors (Audigier et al. 1980; Chailletetal 1484; Chang etal. 1982). Ward and Takemori (1983) found that the naloxone pA_2 value against [D-Ala^2, D-Leu^5]enkephalin (DADLE) was slightly lower than that against morphine. Additionally, those investigators found that the i.c.v. analgesic effects of DADLE were antagonized by beta-funaltrexamine (beta-FNA)(Takemori et al. 1981), leading to the suggestion of involvement of both i.c.v. mu and delta opioid receptors in the tail-flick test. Our results using DPDPE, a highly selective delta agonist (Mosberg et al. 1983) together with ICI 174,864, a highly selective delta antagonist (Cotton et al. 1984), support this conclusion.

The co-administration of ICI 174,864 with DPDPE produced a dose-related antagonism of i.c.v. DPDPE, but not morphine or DAGO, analgesia. Administration of ICI 174,864 alone, at the doses studied, didnotaffect the tail-flick response. Differential antagonism has historically been taken as the strongest evidence fortheinvolvementofmultiple receptors inapharmacological effect. Thus, the present results strongly suggest that both cerebral mu (morphine, DAGO) and delta (DPDPE) receptors mediate analgesia in this test.

Determination of the possible presence or absence of acute cross-tolerance between morphine and DPDPE was also employed as a second approach in an attempttoconfirmthe results oftheexperiments using the

delta antagonist. While the presence of cross-tolerance suggests either activity at a common receptor or some common post-receptor pathway, the absence of cross-tolerance is reasonable evidence of activity of separate receptor types. Acute pretreatment with a bolus injection of morphine resulted in the development of acute tolerance as shown by the rightward displacementofthe i.c.v. morphine dose-response line. Similarly, morphine pretreatment displaced the i.c.v. DAGO dose-response line to the right demonstrating acute cross-tolerance. In contrast, however, the i.c.v. DPDPE dose-responselinewas not affected. The lack of i.c.v. cross-tolerance between DPDPE and morphine, while not establishing activity at separate receptors, supports the concept of involvement of two receptors in this effect.

The finding of a lack of i.c.v. acute cross-tolerance between DPDPE and morphine, when taken together with the antagonism of DPDPE, but not morphine or DAGO analgesia by ICI 174,864, provides strong and direct evidence of involvement of both cerebral delta, and mu opioid receptors in this analgesic test. This concept has been previously suggested on the basis of a lack of i.c.v. inhibition of gastrointestinal transit by DPDPE, butnotby mu agonists (Porreca et al. 1984). The involvement of kappa receptors in this thermally-initiated endpoint seems unlikely as kappa agonists do not produce reliable effects after i.c.v. administration. Thus, it appears thatbothmu and delta receptors in the brain participate in this analgesic effect; the identification of the spinal opioid receptors in this effect remains to be investigated.

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INTRAVENTRICULAR ADMINISTRATION OF BAM-18: ANTINOCICEPTIVE AND LOCOMOTOR ACTIVITY IN THE RAT

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ABSTRACT

BAM-18, a new endogenous opioid containing 18 amino acid residues, was tested in 3 behavioral paradigms. Tail-flick analgesia, a spinally mediated response, hot-plate analgesia, a centrally mediated response, and open-field locomotor activity. Rats were stereotaxically implanted with a unilateral cannula aimed at the lateral ventricle. Following recovery, each animal was tested in one of the paradigms after receiving an intraventricular injection of BAM-18, morphine or the Ringer's vehicle. BAM-18 produced significant tail-flick analgesia only at doses (50 ug) 50 times higher than those needed with morphine (1 ug). BAM-18 produced an extended hyperalgesia at lower doses (5 ug) that was also seen transiently at the high dose. The analgesia but not the hyperalgesia was reversed by naloxone (10 mg/kg, s.c.). BAM-18 produced significant naloxone-reversible hot-plate analgesia, but again it was less potent than morphine (50 ug for BAM-18 vs. 5 ug for morphine). There was no evidence of hyperalgesia in this paradigm. Locomotor activity, following 50 ug of BAM-18, resembled control injections for the first 18 minutes, then became reduced in a manner similar to morphine (5 ug). This reduction in activity was completely reversed by naloxone. These data suggest that BAM-18 is indeed an opioid molecule but is at least 10 times less potent at altering behavior than morphine.

INTRODUCTION

BAM-18 is a new endogenous opioid molecule of 18 amino acid residues which has recently been isolated from bovine adrenal medulla (Evans et al. 1985). BAM-18 is a derivative of the pro-enkephalin A molecule (Pittius et al. 1984), and is found in high concentrations in many areas of the central nervous system including striatum, hypothalamus, pons/medulla and spinal cord (Evans et al. 1985). We have found that BAM-18 binds with high affinity to both u and k receptors (k_i-0.29 nM and 0.75 nM, respectively) and might therefore be expected to exhibit potent analgesic activity. After central administration of BAM-18 or morphine, we tested rats in 2 analgesia paradigms, tail-flick which is a spinally mediated response and hot-plate which is centrally mediated (Jensen and Yaksh 1985), and also assessed drug effect on spontaneous locomotor activity. All observed behavioral changes after BAM-18 administration were tested for naloxone reversibility.

MATERIALS AND METHODS

Seventy male Sprague-Dawley rats (Charles Rivers, Portage, Michigan) were unilaterally implanted with a 24 gauge stainless steel injection cannula aimed at the lateral ventricle (-1.0 mm from bregma, +1.7 mm from midline and -3.0 mm from dura). Following recovery, the animals were placed in one of the following experimental paradigms.

Experiment 1.

Animals were placed in the tail-flick restrainer and allowed to acclimate for 30 minutes. The tip of the tail was blackened to reduce variability of heat absorbtion. The intensity of the heat lamp was adjusted for each animal to yield a mean baseline tail-flick latency of 7-9 seconds. Following 3 baseline readings, BAM-18 or morphine, dissolved in 20 ul of modified Ringer's solution, or Ringer's alone, was administered through the cannula and tail-flick latencies were performed every 2 minutes for a total of 30 minutes. Naloxone reversibility was determined by administration of naloxone (10 mg/kg, s.c.) 15 minutes before icv BAM-18 or Ringer's injections.

Experiment 2.

The hot-plate testing apparatus consisted of a Corning hot-plate covered with a 0.25 inch aluminum plate for even heat distribution, and a plexiglass open-ended box on the plate. The temperature was maintained at 45° C and monitored continually with a YSI thermistor thermometer. Animals were allowed to habituate to the apparatus on a cold plate. Testing was performed by lowering the animal, by its tail, onto the hot-plate. When the hind paws touched the plate, timing began and was terminated when the animal licked a hind paw. Three readings were taken and averaged to yield the pre-drug baseline. BAM-18 or morphine, dissolved in 20 ul of Ringer's solution, or Ringer's alone was administered through the cannula, and latencies were performed every 5 minutes for a total of 30 minutes. Naloxone reversibility was determined by administration of naloxone (10 mg/kg, s.c.) 15 minutes before icv BAM-18 or Ringer's injections.

Experiment 3.

Spontaneous locomotor activity was recorded in an Opto-Verimex-Minor activity monitor (Columbus Instruments). Animals were injected with BAM-18 or morphine dissolved in 20 ul of Ringer's solution or Ringer's alone, as in previous experiments, and placed in the animal activity monitor. Locomotor activity was recorded every 6 minutes for a total of 60 minutes. Naloxone reversibility was assessed by administration of naloxone (10 mg/kg, s.c.) 15 minutes prior to icv BAM-18 or Ringer's solution injection.

RESULTS

BAM-18 produced a significant analgesic response in the tail-flick paradigm (figure 1a) similar to that seen with morphine. However, the dose of BAM-18 required was 50 times that needed with morphine (50 ug and 1 ug respectively). At lower doses of BAM-18 (5 ug), and

transiently at higher doses, a hyperalgesic response was observed. The analgesia but not the hyperalgesia was reversed by naloxone (10 mg/kg, s.c.)(figure 1b), unmasking the hyperalgesia at the 50 ug dose. A significant analgesia was also observed with BAM-18 in the hot-plate paradigm (figure 2a). The dose required was

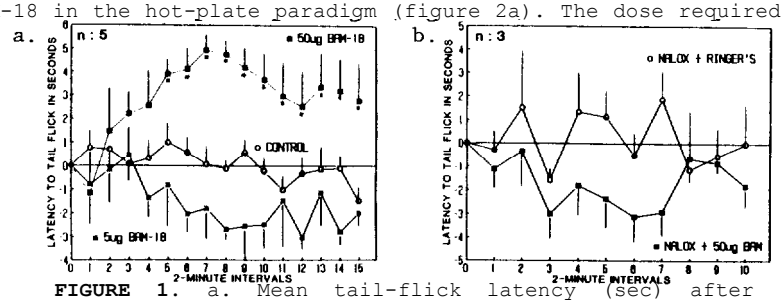
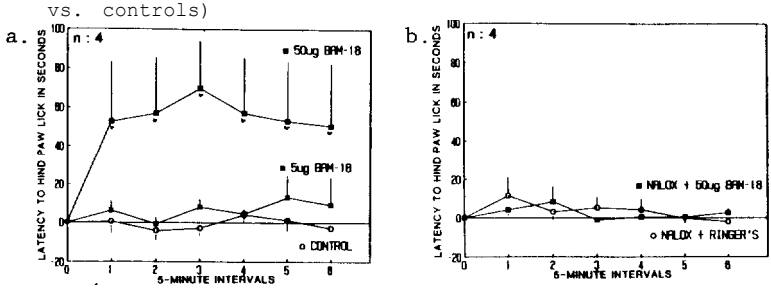


FIGURE 1. a. Mean tail-flick latency (sec) after intraventricular administration of 2 doses of BAM-18. b. Mean tail-flick latency (sec) after pretreatment with naloxone (10 mg/kg, s.c.) and icv administration of either BAM-18 or Ringer's solution. Scores represent change from baseline (drug score - baseline score). Bars are S.E.M.'s. (* $p < 0.05$, F-test, vs. controls)



administration of BAM-18. b. Mean hot-plate latency (sec) after pretreatment with naloxone (10 mg/kg, s.c.) and icv administration of either BAM-18 or Ringer's solution. Scores represent change from baseline (drug score - baseline score). Bars are S.E.M.'s. (* $p < 0.05$, F-test, vs. control)

10 times greater than an analgesic dose of morphine (50 ug BAM-18, 5 ug morphine). The BAM-18-induced analgesia was completely reversed by naloxone (10 mg/kg, s.c.)(figure 2b). BAM-18 was 50 times less potent than morphine in producing analgesia in the tail-flick test (a spinally mediated response) and 10 times less potent in the hot-plate test (a centrally mediated response). These data suggest that the degradation of BAM-18 plays a significant roll in determining its potency in the production of analgesia. BAM-18 (50 ug) did not affect locomotor activity during the first 18 minutes following injection (figure 3a). During the later time frames, BAM-18 caused a reduction in spontaneous activity similar to that seen with morphine, albeit at a larger dose than morphine (5 ug). The reduction in spontaneous activity caused by BAM-18 was completely reversed by naloxone (10 mg/kg s.c.)(figure 3b). Occasionally, seizure-like activity was seen with the 50 ug dose of BAM-18. The seizures were

of three types, wet-dog shakes, head and fore-paw shakes, and barrel-rolling. The barrel-rolling behavior was observed in one animal even after naloxone. Non-naloxone reversible barrel-rolling activity is routinely seen with 100 ug intraventricular injections of Dynorphin (1-13) (Herman et al. 1980) which has some sequence homology with BAM-18.

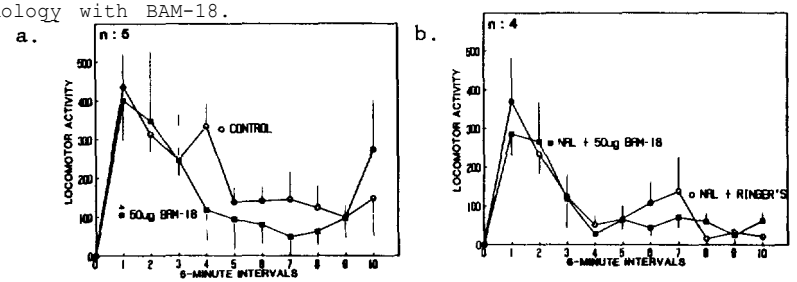


FIGURE 3: a. Mean open-field locomotor activity after icv administration of BAM-18. b. Mean open-field locomotor activity after pretreatment with naloxone (10 mg/kg, s.c.) and icv administration of either BAM-18 or Ringer's solution. Bars are S.E.M.'s. (* $p < 0.05$, F-test, vs. control)

In conclusion, BAM-18, when administered centrally, is capable of producing analgesia in 2 different behavioral tests. However, it is consistantly less potent than morphine. Reliable analgesia has been achieved with as little as 0.5 ug ivc in the tail-flick paradigm (McGregor et al. 1978) whereas in the present work a dose of 50 ug BAM-18 was required. Similarily, BAM-18 reduces spontaneous activity but, again, at a larger dose than is required for morphine. A hyperalgesia in the tail-flick paradigm was observed at low doses of BAM-18 and was unmasked by naloxone at higher doses. All observed effects of BAM-18 (except the hyperalgesia) were reversed by naloxone.

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The authors wish to thank Columbus Instruments International Corporation for their generous loan of the Opto-Verimex-Minor activity monitoring system. The naloxone was kindly supplied by Endo Laboratories Inc., Garden City, New York. Research was supported by AFOSR Grant 84-0325 and NIH Fellowship DA-05288-01.

A SINGLE icv INJECTION OF KCl INDUCES A SELECTIVE AND LONG-LASTING INHIBITION OF OPIOID ANALGESIA IN MICE

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ABSTRACT

The icv administration of potassium chloride a few min before the icv injection of opioids, antagonized the supraspinal analgesia induced by these substances in the mouse. The antagonistic effect of a single injection of KCl lasted for 3 to 4 days and was accompanied by opposite changes in 3H-DADLE binding to mesencephal ic (decrease) and spinal cord (increase) membranes.

INTRODUCTION

Potassium ion has usually been considered as an inert agent on properties of the opioid receptor in vitro (Pert and Snyder 1974). However, electrophysiological studies have recently shown that op io ids cause hyperpolarization of neurons by opening certain potassium channels in the neural membrane. This action of opioids was reduced by increasing the extraneuronal concentration of ion (Willians et al. 1982). On the basis of these findings, the influence of icv administration of KCl on the analgesia induced by opioids at supraspinal level in the mouse was investigated.

MATERIALS AND METHODS

Pept ides were obtained from Bachem (Bubendorf, Switzerland). Tritiated dihydromorphine (DMH) and D-Ala2,D-Leu5-enkephal in (DADLE) were purchased from Amersham Corporation and **New** England Nuclear respectively. Albino male mouse CFLP. 22-27 g were used throughout. Analgesia was evaluated by the tail-flick method in a water bath at 52° C. and expressed as a percent of the maximum possible effect after setting a cut off time of 10s. Binding experiments were carried out on crude P2 fractions at 25°C for 30 min in 2 ml of 50mM TRIS buffer pH 7.7 in the presence of several concentrations of labeled 1 igands. Samples were filtered through Whatman GF/B filters and counted in a scintillation counter Beckman LS-5801.

RESULTS

KCl or NaC (0.1-1 µmole/mouse,icv) given 30 min before the icv administration of opioids, did not modify the analgesia induced by morphine and related alkaloids (normorphine, levorphanol...), which agrees with previous reports (Chapman and Way 1980). Similarly, the ions did not alter the analgesic properties of DTLET and morphiceptin. However, potassium but not sodium (1 µmole/mouse), clearly diminished in a dose-dependent and non-competitive manner the analgesia induced by several opioid peptides of different receptor selectivities (table 1): µ, FK 33-824; δ, DADLE, DPLPE. In mice pretreated with KCl icv 0.3, 0.5 and 1 µmole/mouse, DADLE only reached 85, 60 and 20% of the maximum analgesic effect. KCl treatment also reduced the analgesia elicited by endogenous opioid peptides derived from the Proenkephalin precursor. In contrast, the analgesic action of ß-endorphin remained practically unchanged.

TABLE 1. Effect of KCl and NaCl on the analgesia induced by opioids in the mouse

	test time	NaCl	KCl	
Opioid	min	ED50	ED50	slope
Morphine	30	3.00±0.26	3.30±0.22	0.90
DADLE	15	0.10±0.02	> 30*	0.24
DPLPE	15	20.9±3.10	>200*	0.54
DTLET	15	1.14±0.17	1.44±1.15	1.15
FK 33-824	30	0.0027±0.0003	0.15±0.09*	0.51
Morphiceptin	5	6.91±0.75	7.22±0.60	1.06
Met-ENK-Arg-Phe	5	37.1±4.20	>300*	nd
Adrenorphin	15	28.1±2.53	> 100*	nd
Peptide E	30	1.51±0.72	> 10*	nd
ß-endorphin	30	0.042±0.004	0.070±0.006	0.80

Salts (1µmole/mouse) were given icv 30min prior to the opioids. Groups of 8 to 12 animals were used. Slopes and ED50 were computed from the Hill transformation of experimental data. ED50s are in nmoles/mouse. * Statistically different from the group treated with NaCl, Student's t test;p<0.05. Abbreviations,nd:non determinated; DPLPE: (D-Pen2, L-Pen5)-enkephalin; DTLET: (D-Thr2, Leu5)-enkephalin-Thr6; FK 33-824: (D-Ala2, N-Me-Phe4, Met-(o)5-ol)-enkephalin.

The antagonistic effect of a single icv injection of KCl (1 nmole/mouse) was found to be of a remarkable long duration. The analgesia was still diminished 3 days later and after a slow recovery phase the animals showed a normal response to opioids on the 5th day (table 2).

TABLE 2. Time-course of KCl antagonism on opioid-induced supraspinal analgesia

Opioid	Dose	Day:	Ratio Na/K 1	2	3	4	5
Morphine	3.0		0.86	0.89	1.12	1.15	0.88
DADLE	0.1		5.80*	3.20*	2.26*	1.67*	1.20
FK 33-824	0.003		2.80*	2.34*	2.14*	1.75*	1.37

Salts <1µmole/mouse,icv) were given once on day 1, 30 min prior to the opioids. Opioids were administered icv at ED50 (nmole/mouse) and the analgesic effect studied daily for 5 consecutive days. Ratios between the percent of the maximum analgesic effect induced by opioids in groups of mice (n=10) pretreated either with NaCl or KCl are shown. * Statistically different from the group treated with NaCl, Student's t test; $p<0.05$.

TABLE 3. Time-course of KCl effect on 3H-DADLE binding

Structure		Day:	Ratio Na/K 1	2	3	4	5
Mesencephalon	Kd		1.00	1.11	1.02	1.03	1.10
	Bmax		0.95	1.53*	1.35*	1.12	1.11
Spinal cord	Kd		1.05	1.00	0.95	0.95	0.98
	Bmax		1.03	0.75*	0.80*	0.96	1.10

Salts (1µmole/mouse.icv) were given once a day 1. Groups of mice were killed 30 min after the salt injection on day 1 and at the intervals above indicated. Ratios between parameters obtained from NaCl and KCl treated mice are shown. The experiment was repeated four times. Kd and Bmax for 3H-DADLE specific binding to mesencephalic and spinal cord membranes are respectively: 3.2 ± 0.2 nM, 115 ± 5 fmoIe/mg protein and 1.9 ± 0.1 nM, 56 ± 3 fmole/mg protein. * Statistically different from 1, Student's t test; $p<0.05$.

The specific binding of 3H-DHM and 3H-DADLE to mouse brain and spinal cord membranes was also monitored during the 5 days after the single icv injection of the salts (1 µmole/mouse). No change was shown when the whole brain was studied. However, in KCl treated mice, 3H-DADLE specific binding decreased in mesencephalon and increased in spinal cord. These changes were evident 24 h after the KCl injection and slowly returned to the initial values on day 4 (table 3). In contrast, 3H-DHM binding to these structures remained unchanged.

DISCUSSION

The apparent selectivity of the antagonism induced by KCl on the opioid analgesia and the changes found in the number of opioid receptors labeled by DADLE, agree with the proposed involvement of, multiple receptors in the supraspinal analgesia induced by opioids (Höllt et al. 1985). Assuming that hypothesis, the "potassium-sensitive" opioids would induce at least a part of the analgesic effect after binding to a receptor(s) whose function is susceptible of being reduced by that ion. The 'potassium-sensitive' receptor appears not to be the one preferred by either the opiate alkaloids, morphiceptin or DTLET to promote analgesia. Interestingly, DTLET and its analog DSLET seem to induce analgesia through the morphine receptor (Chaillet et al. 1984). On the other hand, it has been shown that morphine- and ß-endorphin-induced analgesia can be modulated without altering the antinociceptive properties of the δ ligand DADLE or the μ ligand FK 33-824 (Tulunay et al. 1981). In the present study, it was evidenced the antagonism by KCl of DADLE- and FK 33-824-evoked analgesia but not of morphine, thus confirming the possibility of modulating the analgesic effects of FK 33-824 and morphine independently. Of interest is a recent report describing the existence of an additional type of binding site to which μ- and d-selective enkephalin analogs display their highest affinities (Lutz et al. 1985). We find particularly interesting the antagonism by KCl of the analgesia elicited by the Proenkephalin-derived peptides. This result might indicate that a common mechanism underlies the effect of that family of endogenous opioids. Although further work is required to identify the opioid receptor whose function is decreased by potassium as well as the processes responsible for that antagonism, these results suggest a regulatory role for that ion on the function of the opioid receptor in vivo.

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INCREASE IN MET-ERKEPHALIN LEVEL AND ANTINOCICEPTIVE EFFECTS INDUCED BY KELATORPHAN IN THE RAT SPINAL CORD.

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ABSTRACT

Kelatorphan, a mixed inhibitor of "enkephalinases" is used for demonstrating the implication of endogenous enkephalins in nociceptive transmission at the spinal level.

INTRODUCTION

The high levels of enkephalins in the superficial dorsal horn of spinal cord, together with dense opiate receptor binding sites, may be related to a role in the modulation of nociceptive transmission at the spinal level. However, demonstration of this putative role is prevented by the rapid degradation of enkephalins by three peptidases : enkephalinase, aminopeptidase M and a dipeptidylamino-peptidase whose specific role is not clearly established. Mixed inhibitors of these three enzymes were developed and kelatorphan is the first of a new series of compounds exhibiting high analgesic properties by both i.c.v. and i.v. routes (Fournié-Zaluski et al. 1985). Consequently, kelatorphan was also studied for its ability to protect endogenous enkephalins (Bourgoin et al. 1986) and to depress the responses of nociceptive neurons in the spinal cord (Dickenson et al. 1986).

RESULTS AND DISCUSSION

Effect of kelatorphan on the in vitro outflow of endogenous met-enkephalin from rat spinal cord slices. Superfusion of dorsal horn slices with 2 and 20 µM kelatorphan, produces an increase of 82% and 175% in the spontaneous and 130% and 230% in the K^+evoked outflow of enkephalin. The association of bestatin (20 µM) and thiorphan (1 µM) is inactive on the spontaneous release.

Effect of kelatorphan on the in vivo release of met-enkephalin like material (MELM) from the rat spinal cord. The subarachnoidal space of anesthetized rats was perfused with artificial CSF, with or without, the various inhibitors. As shown in table 1, kelatorphan increases both the spontaneous outflow of MELM and the outflow evoked by K^+ depolarization or by a noxious treatment.

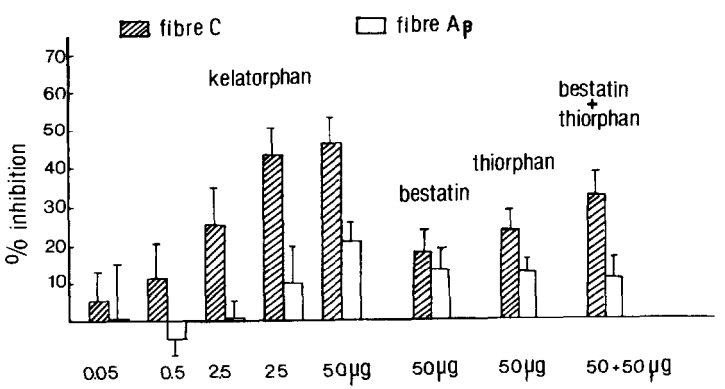
TABLE 1. Comparison of the effect of kelatorphan and the association bestatin + thiorphan on the in vivo release of MELM from the rat spinal cord.

Treatment	Control	Th(1µM)+B(20 µM)	Kelatorphan(20µM)
None	4.33 ± 0.4	9.47 ± 0.66	10.82 ± 1.38
K^+ (40mM)	10.97 ± 1.59*		23.65 ± 3.29*
Muzzle pinching	10.10 ± 1.26*	19.82 ± 2.42*	22.28 ± 4.60"

* $P < 0.05$.

Inhibition of nociceptive neurones in rat spinal cord. The inhibitors were applied at the indicated concentrations onto the surface of the dorsal horn of anaesthetized rats and the neurons were recorded and characterized by their responses to electrical stimulation of A_β and C fibres. As observed in figure 1, the effect of kelatorphan was significant with a dose as low as 2.5 µg and the inhibition of C fibres was clearly dose dependent. The maximal effects were seen with 25 and 50 µg.

FIGURE 1. Inhibition of the A_β evoked activity and C-fibre responses by bestatin, thiorphan and kelatorphan.



The A fibre responses were only significantly altered by the highest doses. The effects of kelatorphan were greater than those produced by the same doses of bestatin and thiorphan and were reversed by naloxone.

Analgesic effects of kelatorphan on hot plate and tail flick tests. The properties of kelatorphan were also studied on the tail flick test, a spinal model of analgesia and on the hot plate test, which is considered as a supraspinal model. As shown in table 2, kelatorphan induces high analgesic responses on the jump latency time but also on both the licking, and the tail flick tests for the highest doses. All these effects are reversed by naloxone.

TABLE 2. Effects of kelatorphan on analgesic tests in mice.

	Hot plate test		Tail flick test(s) [c]
	Licking time(s) [a]	Jump latency time(s) [b]	
control	5 ±0.3	50±5	7.3±0.3
10 µg	4.3±O.3 (NS)	82±8 **	NT
40 µg	6.1±0.7 (NS)	123±16 ***	7.6±0.5 (NS)
80 µg	8.4±0.8 *	188±1O ***	7.9±0.6 (NS)
114 µg	8.6±_0.9 **	200±16 ***	11.9±0.6 ***
200 µg	9.2±1.3 ***	206±15 ***	12.2±0.6 ***

a) cut off time, 30 s ; b) cut off time, 240 s ; c) cut off time, 24 s.

CONCLUSION

The experiments performed in both CNS and spinal cord clearly indicated that the use of mixed inhibitors of "enkephalinase" are the most efficient method for the in vivo study of physiological properties of enkephalin.

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MODULATORS OF PAIN IN THE SPINAL CORD

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ABSTRACT

Intrathecal injection of subanalgesic doses of morphine (7.5 nmol) and dynorphin-A-(1-13) (1.25 nmol) in combination resulted in a marked analgesic effect as assessed by tail flick latency in the rat. The analgesic effect of the composite of dynorphin/morphine was dose-dependent in serial dilutions and not accompanied by any signs of motor dysfunction. Synergism between dynorphin and morphine can also be demonstrated without motor dysfunction, when dynorphin is injected together intrathecally with a serial increasing dosages of morphine. Metenkephalin showed an analogous synergistic effect with dynorphin and also morphine in the spinal cord, although dynorphin (and also metenkephalin) antagonized morphine analgesia by the intraventricular route. The underlying mechanism of the interactions among different classes of opioid ligands at different levels of the central nervous system deserves further study.

INTRODUCTION

The analgesic effect of dynorphin upon intrathecal injection has been well documented (Han and Xie 1982, Piercey et al. 1982, Przewlocki et al. 1983); but large dose of intrathecal dynorphin often causes motor paralysis of hind limbs, which might interfere with the tail flick analgesic test. Intraventricular injection of dynorphin and also metenkephalin have been proved to have an inhibitory effect on morphine or beta-endorphin analgesia (Lee et al. 1980, Friedman et al. 1981). So it would be interesting to compare the interaction of dynorphin, metenkephalin, and morphine in the spinal cord and the brain. This may provide important clues about the pain modulatory effects of different types of opioid ligands and their interactions.

METHODS

Rats, 200-250 gm in body weight were used. Intrathecal injections were given via PE-10 catheter within 24-48 hours after its implantation into the spinal subarachnoid space (Yaksh and Rudy 1976). Intraventricular injections (ICV) were given to the lateral ventricles through a stainless steel cannula inserted along an appropriate guide anchored to the skull. Tail flick latencies (TFL) were assessed (Jen and Han 1979) prior to drug administration and thereafter; the percent change of pain threshold was determined by taking the pre-drug level as 100 %. Statistical significances between groups were assessed by Student's t-test.

RESULTS

I. Synergistic effect between dynorphin and morphine given intrathecally:

Different groups of rats, 8 to 11 in each group, were given 1.25 nmol of dynorphin-A-(1-13) or 7.5 nmol of morphine separately or in combination at a total volume of 10 µl. The combined effect was much stronger than the mathematical additive effect of the two components, which were both not significantly different from the saline control (fig. 1).

The combination of morphine and dynorphin gave rise to a marked increase in TFL over 100 % of the basal level 10 min after injection, which was maintained at a height between 104±15 % and 119±7 % for the whole observation period of 80 minutes (fig. 2). The effect was also dose-related upon serial dilution. None of the rats showed any signs of motor dysfunction when they were allowed to move freely out of the holders.

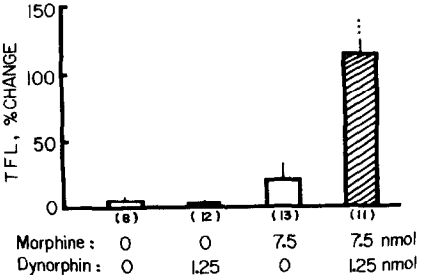


FIGURE 1. Analgesic effect of dynorphin-A-(1-13) 1.25 nmol, morphine 7.5 nmol and both drugs in combination. *** Indicates p <0.001 compared to either dynorphin or morphine alone.

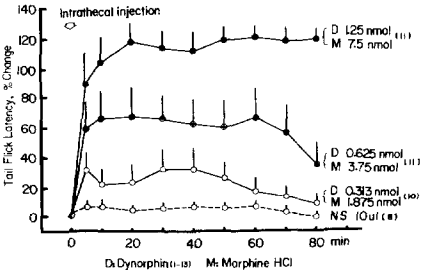


FIGURE 2. Dose-response effect of seriall dilutions of intrathecal dynorphine/morphine composite. Numerals in parentheses indicate number of animals.

Similar synergistic effect between dynorphin and morphine could also be observed when dynorphin was given on top of serial increasing dosages of morphine of 2, 4, 8, 16, and 32 nmol, given in succession every 10 min (fig. 3). Again, none of these animals showed any signs of motor dysfunction.

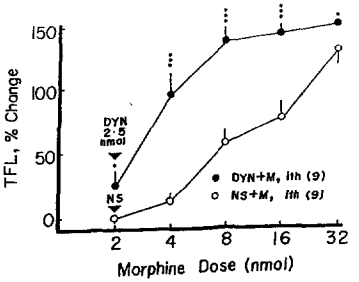


FIGURE 3. Synergistic effect between dynorphin and serial increasing dosages of morphine given intrathecally.

II. Synergistic effect between metenkephalin and morphine or dynorphin given intrathetally:

Analogous synergistic effect was observed between metenkephalin and serial increasing dosages of morphine or dynorphin given intrathecally (fig. 4 and 5). Subanalgesic dose of metenkephalin (100 nmol) was effective in potentiating morphine analgesia across various dose ranges of morphine or dynorphin.

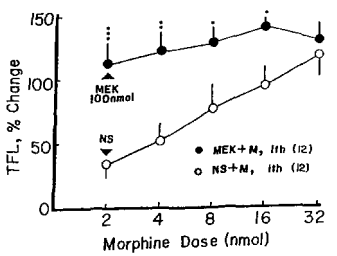


FIGURE 4. Synergistic effect between metenkephalin and serial increasing dosages of morphine given intrathecally.

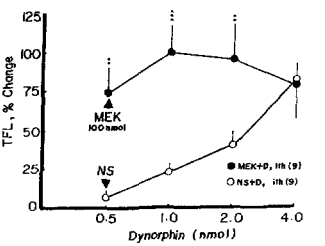


FIGURE 5. Synergistic effect between metenkephalin and serial increasing dosages of dynorphin given intrathecally.

III. Antagonism of morphine analgesia by dynorphin given intraventricularly:

Different groups of rats, 6-11 in each group, were given ICV injection of normal saline 8 µl, dynorphin-A-(1-13) 20 nmol or morphine 30, 60, and 120 nmol respectively. Saline and dynorphin did not cause any significant change in basal pain threshold. However, morphine, at different dose levels, produced a dose-dependent increase of 19±12, 75±18, and 139±7 % of basal pain threshold.

When dynorphin 10 and 20 nmol was given ICV together with 120 nmol of morphine in another 2 groups of rats, the analgesic effect of morphine was attenuated. A reduction of 88 % was noticed with 20 nmol of dynorphin and was statistically very significant ($p < 0.01$) from the group given morphine alone (fig. 6).

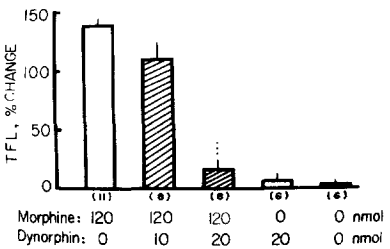
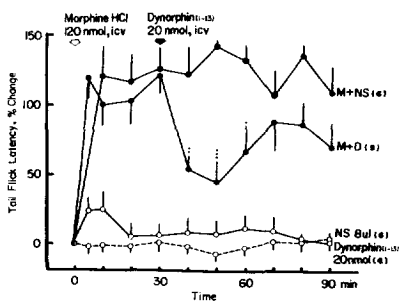


FIGURE 6. The effect of ICV injection of various doses of dynorphin-A-(1-13) and morphine in combination.

When dynorphin 20 nmol was given 30 min after 120 nmol of morphine was injected ICV, a drastic suppression of the analgesic effect of morphine was noticed within 20 min (68 % reversal, $p < 0.001$, as shown in fig. 7). Similar suppressibe effect was noticed when dynorphin 20 nmol was injected ICV together with 30, 60, and 120 nmol of morphine given in serial succession to rats (fig. 8). The differences in analgesic effect between the group given morphine and dynorphin in combination and the group given saline plus morphine were statistically significant or very significant across all dosages of morphine.



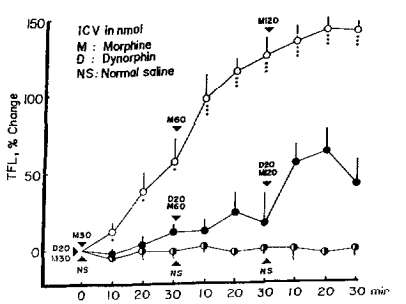


FIGURE 8. Antagonistic effect of dynorphin on increasing dosages of morphine given intraventricularly.

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DISCUSSION

Our data clearly showed that a subanalgesic dose of dynorphin-A-(1-13) produced a marked potentiation on morphine analgesia whereas none of the animals had any sign of motor dysfunction. Thus different effects of dynorphin-A on sensory and motor function may occur depending on the dose: a small amount. of exogenously applied or endogenously released (Han and Xie 1984) dynorphin-A primarily affects nociception. Motor activities are impaired only when a relatively large dose is administered or huge amount of dynorphin is released in case or cord injury (Faden et al. 1983). The supposition that dynorphin-A in spinal cord may be more involved in sensory rather than in motor function seems to be in line with the findings that the dynorphin-A content in the dorsal spinal cord is much higher than in the ventral cord (Botticelli et al. 1981).

Synergism between different classes of opioid ligands dynorphin, metenkephalin, and morphine at the spinal level implies that they may act' synergistically in the spinal cord and are important modulators of pain at that level. These findings strongly suggest that dynorphin-A-(1-13), morphine and metenkephalin are acting on different receptor populations or different binding sites of the receptor, resulting in a synergistic effect. Another interesting finding in our study is the sharp contrast between brain and spinal cord for dynorphin/morphine interaction. While dynorphin-A-(1-13) potentiated morphine analgesia in the spinal cord, it antagonizied morphine analgesia in the brain. This phenomenon was first reported in mice (Friedman et al. 1981, Tulunay et al. 1981) and is here confirmed in the rat. The same was true for the interaction between metenkephalin and morphine. The underlying mechanism of the intriguing interactions among different classes of opioid ligands at different levels of the central nervous system deserves further study.

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SIMILAR ACTIONS OF KAPPA AND MU AGONISTS ON SPINAL NOCICEPTIVE REFLEXES IN RATS AND THEIR REVERSIBILITY BY NALOXONE

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ABSTRACT

The mu agonist fentanyl and the kappa agonists U-50,488 and tifluadom were tested intravenously in electrophysiological experiments on spinal nociceptive reflexes in anaesthetised, spinalised rats. Responses of single motoneurones to thermal and mechanical stimuli were reduced to a similar degree by both mu and kappa agonists and these effects were reversed by low doses of naloxone.

INTRODUCTION

Most studies addressing the role of opiate receptor subtypes in the modulation of spinal nociceptive responses have used behavioural tests. Interestingly, systemic kappa agonists have been reported to be relatively ineffective against reflexes to thermal noxious stimuli in tail flick and hotplate tests, whilst having clear effects against reflexes to mechanical and visceral noxious stimuli (Tyers 1980, Upton et al. 1982, Ward and Takemori 1983). Several reports from Yaksh and his coworkers have indicated that this is also the case when kappa agonists are administered intrathecally, whereas mu agonists are effective against reflexes to thermal as well as to mechanical and other noxious stimuli (see Yaksh and Noueihed 1985). Other workers have reported that kappa opiates show less difference in effectiveness between thermal and non-thermal nociceptive tests using either systemic (VonVoigtlander et al. 1983) or intrathecal administration (Przewlocki et al. 1983).

We have been using an electrophysiological correlate of these behavioural studies so as to be able to record the responsiveness of single motoneurones to intravenously administered mu and kappa ligands. We have selected agonists on the basis that they show receptor selectivity, that they are suitable for systemic administration and that they are rapidly metabolised. We have used fentanyl as a mu agonist (see Magnan et al. 1982) and U-50,488 (VonVoigtlander et al. 1983) and tifluadom (Römer et al. 1982) for kappa receptors. We have used the opiate antagonist naloxone to test the opioid receptor specificity of the observed agonist effects.

MATERIALS AND METHODS

Experiments were performed on alpha-chloralose anaesthetised, spinalised rats. Using silver wire electrodes, recordings were made from the axons of single motoneurones in fine ventral root filaments. The motoneurones were excited by natural peripheral stimuli which were electronically controlled for duration, repetition rate and constancy of intensity. The opioids were tested for selectivity against responses to alternating thermal and mechanical noxious stimuli. Particular care was taken to adjust the stimulus intensities

so as to elicit similar action potential discharge rates in response to the different stimuli. Analysis was of the total number of spikes evoked by the stimulus.

All drugs were given intravenously in these spinalised preparations, both so as to expose the whole polysynaptic pathway to the motoneurone to even concentrations of the drug and so as to allow direct comparison with doses used in behavioural tests. Doses were augmented logarithmically; normally until the cumulative dose was sufficient to reduce the response to less than 25% of the control mean count of spikes per response. Tests were only considered acceptable if the response recovered by more than 50% of the reduction. More than one drug was tested on nearly all cells.

RESULTS

As predicted, fentanyl reduced thermal and mechanical nociceptive responses to a similar degree. In fact at 2 ug/kg on 23 motoneurones in 19 rats the thermal and mechanical nociceptive responses were reduced to means of 44% (±7 SEM) and 51% (±8) of control respectively. The kappa agonists U-50,488 and tifluadom showed a similar lack of selectivity when tested against alternating thermal and mechanical responses in 14 and 10 rats respectively. For example, at 2 mg/kg, U-50,488 reduced noxious heat responses of 23 cells to a mean of 45% (±7) and noxious pinch responses to 54% (±7). Tifluadom was more potent than U-50,488, 0.2 mg/kg being sufficient to reduce noxious heat responses of 10 cells to a mean of 47% (±12) and noxious pinch responses to 30% (±10) of control values. Such non-selectivity by the kappa agonists was however dependent on the neuronal firing rates during the two stimuli; this is illustrated in figure 1. The left side shows an apparently 'selective' reduction of the mechanical response by U-50,488. The right side shows that when firing rates of alternating responses of the same cell were matched by reducing the heat stimulus, this selectivity was no longer apparent.

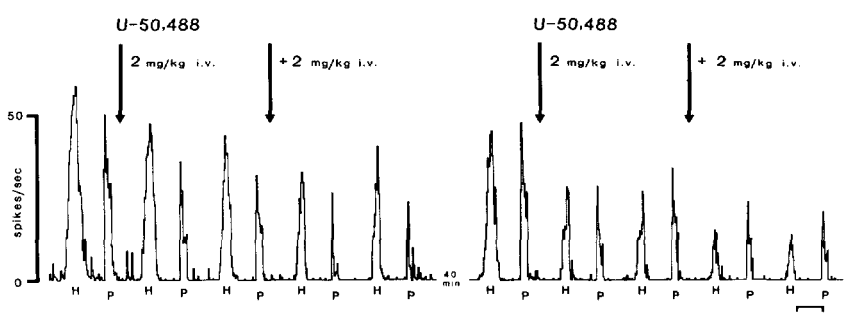

FIGURE 1. Responses of a motoneurone to noxious heat and pinch alternated in a regular 3 minute cycle. Alpha-chloralose anaesthetised, spinalised rat.

When tested in opiate naive rats, the opiate antagonist naloxone (0.1 to 10 mg/kg) had no effect on the responses of motoneurones to noxious heat or pinch stimuli. We were unable to distinguish between the effects of mu and kappa agonists with naloxone at 0.1 mg/kg i.v. since this dose effectively reversed the actions of fentanyl, U-50,488 and tifluadom. Thus, in 7 cells, naloxone at 0.1 mg/kg reversed the depression of heat responses by U-50,488 (1-16 mg/kg) from a mean of 16% (±7 SEM) to 81%(±12) of control. An example of such a result is illustrated in figure 2.

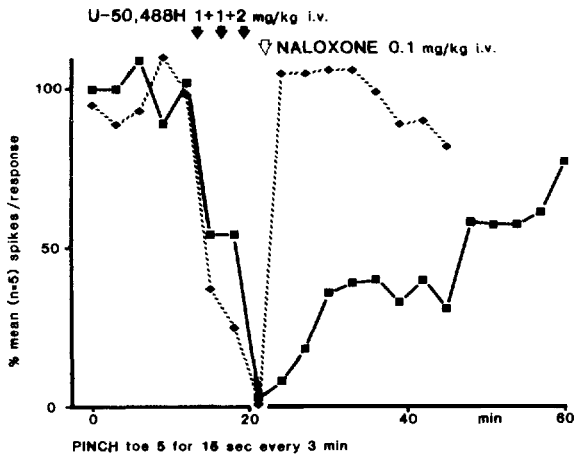

FIGURE 2. Responses of a motoneurone to noxious pinch stimuli applied every 3 minutes. The squares indicate responses to pinch during a test with U-50,488 and show the subsequent time course of recovery. The diamonds illustrate superimposed data from a subsequent, similar test on the same cell. In this case reduction of the pinch response by U-50,488 was rapidly reversed by a low dose of intravenous naloxone. Alpha-chloralose aneasthetised, spinalised rat.

DISCUSSION

Our electrophysiological experiments have shown that the kappa agonists U-50,488 and tifluadom reduce thermally-evoked reflexes to just as great a degree as mechanically-evoked reflexes. This non-selectivity contrasts with the results of most of the behavioural tests which have utilised spinal administration of opioids. This contrast between electrophysiological and behavioural tests can best be explained by two factors. Firstly, in our experiments great care was taken to adjust stimulus intensities so as to match the responses in terms of motoneuronal firing rate. The relative strength of reflexes in behavioural tests cannot be assessed in terms of neuronal firing rate and therefore cannot be matched in the same way. Figure 1 illustrates that the apparently selective depression by U-50,488 of a motoneuronal response to noxious pinch was entirely dependent on the

relative stimulus intensities. It is thus clear that stimulus intensity is a crucial factor in the assessment of the antinociceptive effects of opioids.

The second factor is the different receptor access by a drug between systemic and intrathecal or epidural administration. For most opioid drugs, the diffusion from intrathecal or epidural sites into the cord has not been assessed. For fentanyl it is apparently very poor, for the effective dose intrathecally can exceed the effective systemic dose (Durant and Yaksh 1986, Yaksh et al. 1986). In the absence of specific data for the kappa agonists it is thus possible that the relative ineffectiveness of topically administered kappa agonists in thermally-evoked reflexes is due largely to inadequate penetration of the cord to the appropriate opioid receptor sites. Systemically administered kappa agonists certainly can reduce such reflexes by an action restricted, via spinal transection, to the spinal cord.

The reversal of kappa opioid mediated effects by low doses of naloxone illustrates the problem of using this antagonist in attempts to distinguish between mu and kappa mediated effects in vivo.

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OPIOID ANALGESIA IN THE MOUSE: EVIDENCE FOR MULTIPLE RECEPTORS USING ß-FNA

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ABSTRACT

The icv injection of morphine or DADLE ED50 a few min before the alkylating agent ß-FNA resulted in complete protection of their respective analgesic effects when evaluated 24h later, although a 1 ittle cross-protection could be observed. The analgesia evoked by DADLE was partially protected using higher doses of morphine before R-FNA. However, higher doses of DADLE did not protect the analgesia induced by morphine. On the other hand, the antagonistic action of KCl on opioid analgesia was found to be dependent on the opioid utilized to protect the opioid receptor against the effect of R-FNA. These results are discussed in terms of multiple receptors mediating opioid analgesia at supraspinal level in the mouse.

INTRODUCTION

Frequently it has been assumed that opioids induce their antinociceptive effect at the supraspinal level after binding to the µ class of opioid receptor. However, several laboratories have evoked the mediation of multiple receptors in that phenomenon (see Höllt et al. 1985). With respect to that concept, we have recently reported (Garzón and Sánchez-Blázquez, this volume) that a single icv injection of KCl antagonized the supraspinal analgesia elicited by certain opioids. In the present study, we have investigated further the issue by analyzing the analgesic effect of different opioids on mice previously exposed to the effect of ß-FNA in the presence of various protecting opioids. Moreover, the antagonistic action of KCl on those animals treated with ß-FNA was studied.

MATERIALS AND METHODS

Albino male mouse CFLP, 22-27 g were used throughout. Opioids were obtained from commercial sources. ß-funaltrexamine from N.I.D.A.. Analgesia was evaluated by the tail flick test using a water bath at 52° C as noxious stimulus. Latencies were measured both before and after any given treatment and analgesia expressed as a percent of the maximum possible effect after setting a cut off time of 10s.

RESULTS AND DISCUSSION

The ß-fumarate methylester of nal tre xone (ß-FNA) given icv 0.2 nmole/mouse, antagonized the analgesia induced 24 h later by icv injection of morphine or DADLE ED50s (fig 1). Morphine or DADLE ED50s given a few min prior to ß-FNA produced a good protect ion of their respective analgesic effect, but little cross-protection could be observed. This result agrees with the proposed multiplicity of opioid receptor mediating analgesia at supraspinal level (see Höllt et al. 1985). Therefore, these two opioids at ED50 seem to mainly act on each selective receptor in order to induce analgesia. Although higher doses of morphine induced a good protection of DADLE ED50 analgesic effect, in contrast, DADLE at ED90 did not improve the analgesic action of morphine ED50 24h after the ß-FNA challenge. Thus indicating a low cross-binding of that opioid peptide to the morphine receptor. The μ ligand FK 33-824 and the δ ligand DTLET protected to a similar extent the analgesia elicited by morphine and DADLE ED50s (fig 1). This result suggests that both enkephal in derivatives might have access to morphine and DADLE analgesic receptors.

As previously described (Garzón and Sánchez-Blázquez, this volume) a single icv injection of KCl, but not NaCl, (1 μmole/mouse) reduced the analgesic activity of DADLE and FK 33-824 in naive mice (table 1). That treatment did not alter significantly the analgesia evoked by either morphine or DTLET, confirming that this pept ide induces analgesia through the same receptor used by the alkaloid (Chaillet et al. 1984). In mice treated with morphine ED90, 30 min before ß-FNA, it could be observed that the analgesia induced by these four opioids was much less sensitive to KCl. The 13-FNA mainly blocked the "potassium-sensitive" opioid receptors. Conversely, with DADLE ED90 given 15 min be fore ß-FNA, the analgesic effect of those opioids 24h later was now reduced by KCl administration. Thus, it is 1 ikely that opioids displaying a better affinity to the morphine receptor will induce analgesia insensitive to KCl antagonism. *On* the other hand, a simultaneous binding to both receptors will result in a slight decrease of analgesic potency which will be difficult to detect experimentally. That might be the case for DTLET. Therefore, the possibility of the "potassium-sensitive" receptor being an additional type of binding site to which μ- and d-selective enkephalin derivatives display a high affinity appears to be very attractive (Lutz et al. 1985).

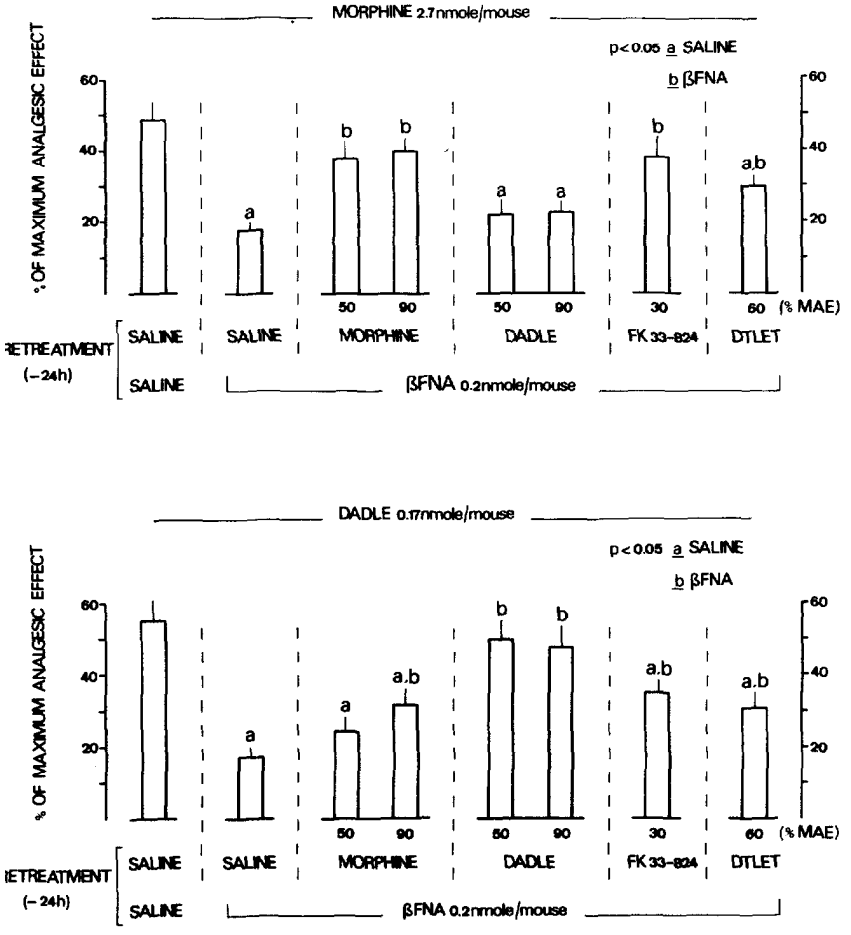

FIGURE 1. Protection against the antagonistic effect of 13-FNA on the supraspinal analgesia elicited by opioids in the mouse. Protecting opioids at doses inducing various degrees of analgesia were administered 30 min (morphine and FK 33-824) or 15 min (DADLE and DTLET) be fore R-FNA respectively. Analgesia induced by morphine and DADLE ED50s was evaluated 24h later. All substances were injected icv in a final volume of 4 µl. Values are the MEAN±SEM of percent of maximum analgesic effect from various groups each of 10 mice. Student's t test was applied for statistical analysis. a and b refer to saline and ß-FNA treated groups respectively.

TABLE 1. Effect of ß-FNA and KCl administration on the analgesia elicited by opioids in the mouse

					ß-FNA			
			Control		Morphine ED90		DADLE ED90	
Opioid	dose	test time	-	+	+		+KCl	
Morphine	27.0	30	90±14	87±4	76±6	74±5	50±4	25±3*
DADLE	1.7	15	89±5	33±2*	55±4	45±3	80±5	33±3*
FK 33-824	0.03	30	90±3	43±2*	71±6	65±6	90±3	39±3*
DTLET	1.5	15	61±3	50±2	45±4	46±3	58±3	29±2*

Morphine and DADLE ED90s (27 and 1.7 nmole/mouse) were given 30 or 15 min before ß-FNA (0.2 nmole/mouse) respectively. Opioid doses are in nmole/mouse. Analgesia was evaluated 24h after ß-FNA. KCl (1 umole/mouse) was administered 24h after ß-FNA and 30min before the opioids. All substances were injected icv in a final volume of 4 µl. Values are the MEAN±SEM of percent of maximum analgesic effect from various groups each of 10 mice. * Statistically different from the group not receiving KCl. Student's t test; $p<0.05$.
Abbreviations:
DADLE : (D-Ala2, D-Leu5)-enkephalin.
DTLET : (D-Thr2, Leu5)-enkephalin-Thr6.
FK 33-824: (D-Ala2, N-Me-Phe4, Met-(o)5-ol)-enkephalin.

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HYPERALGESIA PRODUCED BY INTRATHECAL OPIOID ANTAGONISTS DEPENDS ON RECEPTOR SELECTIVITY AND NOXIOUS STIMULUS

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ABSTRACT

Opioid antagonists selective for δ-, k- and μ-receptor subtypes were administered intrathecally in rats prior to determination of response thresholds to noxious heat, pressure and chemical visceral stimulation. All antagonists induced hyperalqesia differentially with two or more stimuli but δ- and μ-blockade failed to alter writhing activity. Thus, the extent of involvement of an opioid receptor subtype in antinociception depends on the type of noxious stimulation.

INTRODUCTION

The multiplicity of endogenous opioid systems is reflected in the fact that there are at least three families of endogenous opioid peptides (EOPs) and at least three different opioid receptors are widely recoqnised (Höllt et al. 1983). Among the questions raised by this complexity are: does any one EOP modulate pain processes and are they mediated by a specific receptor? If EOPs have physiological roles in antinociception and these are expressed through activation of specific receptors, antagonists selective for those receptors should produce altered nociceptive responding. Previously we studied opiate antagonists in rats never before exposed to exogenous opioids and showed that systemic administration of k-selective agents induced hyperalqesia to pressure without affecting nociceptive heat thresholds, whereas naloxone was equipotent in producing hyperalqesia with both stimuli (Pilcher and Browne 1983). In the CNS the recognised receptor subtypes are differentially distributed; k-agonists are ineffective analgesics when given intracerebrally (Wood et al. 1981) but they are active on intrathecal injection, as are δ- and μ-opioids (Przewlocki et al. 1983). Since it appears that spinal populations of δ-, k- and μ- receptors participate in modulation of pain we have now extended our studies to examine the effects of their antagonists applied directly to the spinal cord. For selective antagonism at δ-, k- and μ-receptors ICI 174,864 (Traynor et al. 1985), Mr 1452 (Merz personal commun.) and naloxone (Vonvoiqtlander et al. 1983) were used respectively.

MATERIALS AND METHODS

Male hooded rats were used. They were housed at about 22°C in a light-dark cycle with lights on 06.00 - 18.00 h. Food and water were freely available. On attaining a body weight of 300-350 g they were implanted with a polyethylene cannula in the subarachnoid lumbar sac according to Schmauss and Yaksh (1984). Injections of drugs and

analgesic tests were carried out 48 h after surgery. Drugs were injected in a volume of 10 µl followed by a flush with 10 µl isotonic saline over a period of 45 sec. Naloxone hydrochloride and ICI 174,864 ($Allyl_2$-Tyr-Aib-Aib-Phe-Leu-OH) were dissolved in isotonic saline. Mr 1452 ((-)-N(3-Furylmethyl)-∝-normetazocine methanesulphonate) was first dissolved in distilled water and diluted 1:10 with isotonic saline. Doses are expressed as the weights of the salts.

Analgesic Testing

Analgesic testing was carried out between 09.00 and 12.00 h. In the paw pinch and tail immersion tests baseline (control) thresholds were obtained 15 min before drug injections.

Paw pinch Initial nociceptive pressure thresholds of non-inflamed hind paws were determined using an 'Analqesy-Meter' (Uqo Basile, Milan).

Tail immersion Tail imnersion tests were carried out at 50 (±0.2)°C. Latencies to the nociceptive response, a flicking or withdrawing of the tail from the water, were determined.

Writhing test The number of writhing responses made during the 5 min immediately following an intraperitoneal injection of 4% sodium chloride was determined.

In all tests results were expressed as a percentage of the control value. Statistical analyses were performed using analysis of variance and post hoc t-tests.

RESULTS

All three antagonists produced their maximal effects in the tail inmersion and paw pressure tests within 1-4 min after cessation of injections. ICI 174,864 produced a significant reduction in paw pressure thresholds ($p<0.01$), which was maximal at 25% with 0.1 µg after only 1 min. The dose-response curve was almost flat over the range examined (0.01-1.0 µg) and doses above 2.0 µg caused hind limb flaccidity followed by severe rigidity. In the tail immersion test ICI 174,864 produced a significantly greater reduction in threshold (37%) than with pressure ($p<0.01$). In contrast to these hyperalgesic effects with heat and pressure, at 0.1 µg this antagonist had no effect on writhing scores.

The hyperalgesia induced by Mr 1452 was maximal at 1.0 µq and that obtained with pressure (49%) was significantly greater ($p<0.01$) than with heat (33%). This antagonist increased writhing responses by 18% but the effect was not significant. The hyperalqesic effect of Mr 1452 was greater than that of either of the other two antagonists with pressure and visoeral stimulation. Naloxone also gave peak effects at 1.0 µg and was most effective in the tail immersion test, when latencies were reduced by 70%. Naloxone produced significantly greater ($p<0.01$) reductions in tail flick latencies than the other antagonists. Marked hyperalqesia was also seen with the pressure stimulus (42%) and a weak, insignificant effect occurred in the

writhing test. Thus the order of hyperalgesic effectiveness of receptor blockade in the three tests was: tail imüersion, p > δ > k; paw pressure, k > μ > δ; writhing, k > μ > δ = 0.

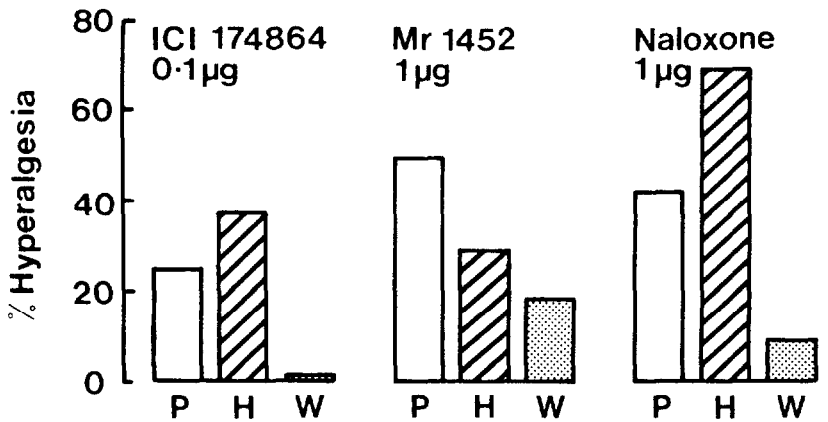

FIGURE 1. Hypóralqesic effects of opioid antagonists selective for δ-, k- and μ-receptors with different stimuli. P= paw pressure: H=heat; W=writhing. (n=5-8)

DISCUSSION

The results reported herein demonstrate that opioid antagonists administered to the spinal cord of opioid-naive rats produce hyperalgesia to different kinds of noxious stimuli. This heightened sensitivity is interpreted as resulting from prevention of antinociceptive modulation by endogenous ligands. It is also clear that the magnitude of the hyperalqesia depended not only on the antagonist's selectivity for a receptor subtype, but also on the nature of the noxious stimulus. Moreover, the time-course of an antagonist's action in the same animal was not identical for all stimuli. These differences in time-courses and effectiveness support two related notions: i) that neural systems concerned with nociception are distinguishable on the basis of their receptor subtype, ii) that the extent to which spinal δ-, k- and μ- receptors participate in modulation of pain processing depends on the qualitative nature of the stimulus. The present findings support our earlier work in which, using systemic administration of naloxone and the k-antagonists Mr 1452 and Mr 2266, it was shown that the latter are 10 to 15-fold more potent in producing hyperalgesia with pressure than heat.

In general, the above results are compatible with those of studies using selective agonists. Martin et al. (1976) first showed that the prototypic k-agonist, ethylketocyclazocine, was twice as potent as morphine, the p-prototype, in suppressing the mild pressure-evoked flexor reflex in spinal dogs. It was later reported that k-agonists were analgesic against noxious visceral stimulation and pressure but not heat, whereas μ-agonists were effective against all three

stimuli (Tyers 1980). When administered intrathecally, μ- and k-agonists inhibited responses in the writhing test but δ-agonists were ineffective (Schmauss and Yaksh 1984). In the hot plate and tail flick tests μ- and δ-but not k-agonists were analgesic. If these differential analgesic actions of exogenous agonists reflect reliably the physiological activation of receptors by their respective endogenous liqands, then the corollary, that selective antagonists will produce hyperalqesia differentially, should hold true. The results presented here support this proposition.

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IN VIVO STUDIES ON DELTA OPIOID RECEPTORS

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ABSTRACT

[D-Pen^2, D-Pen^5]enkephalin (DPDPE), a selective agonist at delta opioid receptors, causes excessive vertical rearing when given icv to rats or s.c. to mice. Tolerance develops to this behaviour. Rats do not rear excessively when injected icv with the following prototype agonists at opioid receptors: OAGO, dynorphin A, U-50488H or SK&F 10047. The incidence of OPDPE-induced rearing is reduced when rats are pretreated s.c. with ICI 174864 (a selective antagonist at delta opioid receptors) (A_{50}=0.09 mg/kg) but not by ICI 178173 (an inactive analogue of ICI 174864); this finding suggests that delta binding sites mediate the behaviour. Pretreatment with naloxone attenuates rearing but the antagonism is unimpressive over the dose range tested (0.05-1 mg/kg, s.c.). Low doses of haloperidol (A_{50}=0.05 mg/kg, s.c.) antagonize the rearing. Dopamine may therefore mediate the behaviour through delta receptor modulation of dopamine release. The practical gain from this study is as follows: a simple, discriminating test is now available for evaluating novel delta agonists and antagonists in vivo.

INTRODUCTION

[D-Pen^2, D-Pen^5]enkephalin (DPDPE) (Mosberg et al. 1983) is a selective agonist and ICI 174864 (Cotton et al. 1984) is a selective antagonist at delta opioid receptors. Initial experiments in vivo (Cowan et al. 1985) have shown that the icv administration of DPDPE to rats causes a marked increase in vertical rearing. This behaviour was studied in the present work as a possible in vivo index of delta agonist activity.

We used the following approaches to clarify the pharmacological basis of DPDPE-induced rearing: (a) interaction studies between DPDPE and ICI 174864, ICI 178173, naloxone and haloperidol, respectively! (b) tolerance experiments involving multiple icv injections of DPDPE, and (c) comparative behavioural studies with agonists selective for mu (DAGO), kappa (dynorphin A, U-50488H) and sigma (SK&F 10047) opioid binding sites, respectively.

MATERIALS AND METHODS

Male albino Sprague Dawley rats (180-200 g; n=4-8) were each stereotaxically implanted with an icv cannula. Five days later, the animals were placed singly in perspex boxes (25 cm long; 20 cm wide; 30 cm high) for an hour then challenged with test agent (5 µl icv). Animals were

observed and scored for behavioural changes for a period of 50 min after agonist administration. Potential antagonists were given s.c. 5 min before a standard dose of DPDPE (20 µg).

Female mice (20-25 g; ICI strain; n=6) were injected s.c. with DPDPE (3-30 mg/kg) and observed regularly over the following hour. The number of rears displayed by each mouse was counted at +10 min for 1 min.

In a tolerance study! DPDPE (20 µg) [Group 1] or saline [Group 2] was given icv to rats twice daily (at 9 a.m. and 5 p.m.). Animals in Group 1 were tested for 50 min in observation boxes after the 1st, 7th and 13th (final) injection. Animals in Group 2 were similarly challenged (with 20 µg of DPDPE) after the 7th and 13th injection.

The following compounds were used: [D-Ala^2, MePhe^5, Gly-ol^5] enkephalin (DAGO), DPDPE and dynorphin A (all from Peninsula); ICI 174864 (Diallyl-Tyr-Aib-Aib-Phe-Leu-OH, Aib is α-aminoisobutyric acid; ICI); ICI 178173 (Diallyl-Tyr-Aib-D-Ala-Phe-Leu-OMe; ICI); naloxone hydrochloride (Endo); N-allylnormetazocine (SK&F 10047; Sterling Winthrop) and trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyll-benzeneacetamide methanesulphonate (U-50488H; Upjohn).

RESULTS

In rats, the DPDPE dose-response relation for rearing was curvilinear with the 20 µg dose being most effective (fig. 1). Behavioural depression was associated with 50 µg of DPDPE; lower doses caused increased yawning, licking of penis, sniffing and biting/licking (forepaws, wood chips and faecal pellets). Behavioural activation was essentially over by +50 min. After s.c. injection to mice, DPDPE provoked increased rearing (table 1), grooming and restlessness for about 30 min.

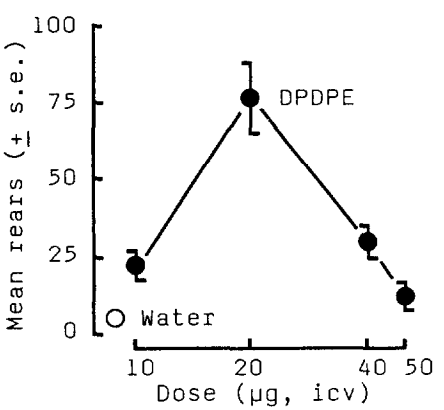

FIGURE 1. Curvilinear dose-response curve for DPDPE-induced rearing in rats.

TABLE 1. DPDPE-induced rearing in mice.

Dose (mg/kg, s.c.)	Rears during 10th min (mean ± s.e.)
Saline	7.33 ± 1.17
3	10.83 ± 0.83*
10	14.33 ± 1.17**
30	13.17 ± 2.24*

*P<0.05; **P<0.01 (Dunnett's test).

TABLE 2. Antagonism of DPDPE-induced rearing in rats.

Test agent	*A_{50} (mg/kg, s.c.)
Haloperidol	0,053 (0.022-0.13)
ICI 174864	0.087 (0.031-0.24)
ICI 178173	Inactive at 1 mg/kg
Naloxone	Flat dose-response curve

*Tallarida and Murray (1981).

ICI 174864 caused an unusual behaviour in rats - hindleg stretching. When the animals were given ICI 174864 (0.01, 0.10 and 1 mg/kg, s.c.) 5 min before the standard dose of DPDPE (20 µg), rearing was antagonized in a dose-dependent manner (table 2). ICI 178173 (an inactive analogue of ICI 174864) (1 mg/kg, s.c.) did not antagonize DPDPE-induced rearing. Pretreatment with naloxone (0.05-1 mg/kg, s.c.) attenuated rearing but the antagonism (35% at 1 mg/kg) was unimpressive.

Tolerance developed to DPDPE-induced rearing by the 7th injection (49±8 rears from 91±12 rears) and had fully developed by the 13th injection (25±16 rears). Control (saline-injected) rats reared excessively when challenged with DPDPE (20 µg) on the 7th and 13th injections.

When rats were injected icv with DAGO (0.125 and 0.50 µg), dynorphin A (0.50 and 10 µg), U-50488H (7.5 and 100 µg) and SK&F 10047 (5 and 50 µg) increased rearing was not observed.

DISCUSSION

Early behavioural experiments with [D-Ala^2, Met^5]enkephalin (amide) [DALA] revealed that this opioid peptide, when injected into the nucleus accumbens (Pert and Sivit 1977) or ventral tegmental area (Broekkamp et al. 1979) of rats, increases locomotor activity and vertical rearing. These brain areas, part of the mesolimbic dopamine system, play an important role in regulating exploratory and locomotor behaviours. Kalivas et al. (1983) have recently summarized evidence that favours enkephalinergic modulation of the mesolimbic dopamine system.

DPDPE, the selective delta agonist (Mosberg et al. 1983),

is behaviourally active when given icv to rats, and evokes a rich array of signs that are reminiscent of those described for amphetamine and apomorphine. Perhaps the most notable behaviour is repetitive vertical rearing, which can also be observed in mice after s.c. DPDPE. This behaviour has been the focus of the present communication. Our results with ICI 174864, ICI 178173, naloxone and haloperidol lead to the conclusion that DPDPE-induced rearing is controlled by delta receptor modulation of dopamine release. Future studies with newly available dopamine receptor antagonists will no doubt sharpen our views on the dopaminergic basis of DPDPE-induced rearing.

Follow-up experiments with ICI 174864 should be started so as to unravel the in vivo pharmacology of this interesting agent. For example, a high dose of naloxone (10 mg/kg) (Haber et al. 1978) attenuates amphetamine-induced rearing in rats (Gupta and Gregory 1967; Bryan and Ellison 1975). What can we expect with low doses of ICI 174864 in this paradigm?

Two practical gains result from our study. First, *if the icv route is used,* DPDPE-like compounds can be discriminated from agents with less selectivity for delta binding sites, e.g, DALA (Kalivas et al. 1983), as well as prototype agonists at mu, kappa and sigma recognition sites. Second, DPDPE-induced rearing provides a simple in vivo endpoint against which the next generation of potential delta antagonists may be tested and compared.

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EFFECTS OF NALOXONE AND NEUROTENSIN ON EXCESSIVE GROOMING BEHAVIOR OF RATS INDUCED BY BOMBESIN, β-ENDORPHIN AND ACTH

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ABSTRACT

Bombesin, β-endorphin and ACTH-(1-24) induce excessive grooming behavior in rats. Whereas ACTH increases the frequency of all components of grooming behavior (head washing, bodily grooming and paw licking), the most pronounced element of bombesin- and β-endorphin-induced excessive grooming is scratching. Naloxone counteracts peptide-induced grooming and in particular excessive scratching is reduced by this opiate antagonist. Also neurotensin suppresses peptide-induced grooming behavior and in particular scratching. It is concluded that the element scratching is the component of grooming behavior which is mainly displayed by activation of opiate receptors and that neurotensin is able to interfere with opiate receptor mediated behavior.

INTRODUCTION

ACTH and MSH are the first peptides reported to induce excessive grooming (Ferrari 1958; Ferrari et al. 1963). Later also other peptides appeared to be active in this respect. Neurohypophyseal hormones (Delanoy et al. 1978; Rees et al. 1976; Drago et al. 1986), prolactin (Drago and Bohus 1981; Drago et al. 1981), substance P (Katz 1979), bombesin (Gmerek and Cowan 1983; Katz 1980; Kulkosky et al. 1982; Van Wimersma Greidanus et al. 1985a), dynorphin and β-endorphin (Aloyo et al. 1983) have recently been shown to induce excessive grooming behavior. Interestingly, the various grooming-inducing peptides have been reported to induce structurally different grooming bouts. ACTH and related peptides produce excessive grooming, which is as far as the composition of the grooming behavior is concerned, comparable to normally occurring grooming behavior (Gispen and Isaacson 1981; Van Wimersma Greidanus et al. 1985a, 1986), whereas grooming induced by β-endorphin and bombesin is characterized by the predominant display of scratching (Gispen and Isaacson 1981; Gmerek and Cowan 1983; Van Wimersma Greidanus et al. 1985a). Naloxone, haloperidol and neurotensin have been shown to suppress the excessive grooming induced by ACTH (Van Wimersma Greidanus and Rinkel 1983; Van Wimersma Greidanus et al. 1986) and by bombesin (Van Wimersma Greidanus et al. 1985b), and it has been suggested that opioid and dopaminergic systems are involved in peptide-induced excessive grooming (Wiegant et al. 1977a, 1977b; Spruijt et al. 1986; Cools et al. 1978; Dunn et al. 1981; Gispen and Wiegant 1976). The present study deals with the nature of the excessive grooming induced by ACTH, β-endorphin and bombesin and with the effects of neurotensin and naloxone on thus induced excessive grooming. In particular the suppressive effects of neurotensin and naloxone on the various components of the excessive grooming are discussed.

MATERIAL AND METHODS

Male rats (150 g) of an inbred Wistar strain, equipped with a canula in the right lateral ventricle, were used. Bombesin was intracerebroventricularly (i.c.v.) injected in doses grading from 10 ng, 30 ng to 100 ng, β-endorphin in doses of 10 ng, 30 ng, 100 ng, 300 ng, 1 µg and 3 µg, whereas ACTH was administered in a dose of 1 µg. Pretreatment with naloxone was performed at 20 min prior to the i.c.v. injection of peptides, using a dose of 1 mg /kg. Pretreatment with neurotensin was performed immediately before peptide administration in a dose of 1 µg i.c.v. For observation of grooming behavior rats were placed into perspex boxes (26 x 20 x 13 cm). Using a 15 sec sampling time, the display of grooming elements (head washing: H; bodily grooming: B; sexual (= anogenital) grooming: A; paw licking: P; scratching: S) was recorded for a period of 50 min, starting 10 min after peptide administration and placing of the animals into the observation boxes.

RESULTS AND DISCUSSION

Bombesin induces a dose dependent increase of grooming behavior, with scratching as the most pronounced element. Even administration of the relatively low dose of 10 ng results in excessive grooming and in particular excessive scratching. Naloxone suppresses significantly the excessive grooming induced by bombesin. This suppression of bombesin-induced excessive grooming by naloxone is mainly due to a reduction in scratching and, to a lesser extent, bodily grooming. As a result of this differential suppression of the various elements of bombesin-induced excessive grooming by naloxone, a strong shift in the relative distribution of grooming elements occurs in bombesin-treated rats following pretreatment with this opiate antagonist. From these results it is concluded that excessive scratching as induced by bombesin is the component of grooming behavior which is mainly displayed by activation of opiate receptor systems, Neurotensin also suppresses bombesin-induced excessive grooming, mainly by reducing the element scratching. Consequently neurotensin induces a change in the relative distribution of grooming elements displayed by bombesin-treated rats, which is more or less similar as the one induced by naloxone. Thus, it is tempting to assume that neurotensin interferes with opiate receptor systems, at least with one of the behavioral results of such a system, i.e. scratching.

Administration of β-endorphin induces excessive grooming behavior as well. However, the β-endorphin induced excessive grooming is less pronounced and the frequency of grooming is quite lower than observed following similar doses of bombesin. Doses of β-endorphin exceeding 200 ng are even less powerful than doses of 30 ng and 100 ng and a dose of 3 µg does not exert excessive grooming at all during the observation period of 50 min. Time course studies reveal that this bell-shaped dose-response curve for the grooming inducing effect of β-endorphin is due to the β-endorphin induced immobility of the animals during the first half of the observation period. Doses of 30 ng and 100 ng β-endorphin induce excessive grooming with scratching as one of the predominant elements. This confirms the idea that excessive scratching is mainly due to activation of opiate receptor systems. Both naloxone and neurotensin suppress the β-endorphin induced ex-

cessive grooming, mainly by reduction of the element scratching. This differential suppression of the various components of β-endorphin-induced excessive grooming by naloxone and neurotensin again results in a shift in the relative distribution of the various grooming elements.

Administration of ACTH results in excessive grooming behavior in which all elements are more or less equally involved. In this respect ACTH-induced excessive grooming differs markedly from the excessive grooming behavior displayed by rats treated with bombesin or β-endorphin. Interestingly both naloxone and neurotensin suppress ACTH-induced excessive grooming without affecting the relative distribution of the various grooming elements.

From these data it is concluded that different neuroanatomical and/or neurophysiological systems in the brain are involved in excessive grooming induced by bombesin, β-endorphin or ACTH. In addition it appears that scratching is the behavioral element which is mainly displayed by activation of opiate receptor systems. Moreover, it seems likely that neurotensin interferes with opiate receptors.

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ENKEPHALIN CONTAINED IN DENTATE GRANULE CELLS IS IMPORTANT FOR KAINIC ACID-INDUCED WET DOG SHAKES.

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ABSTRACT

Kainic acid (KA) caused an initial decrease and a subsequent rebound in hippocampal enkephalin (ENK) in rats exhibiting wet dog shakes (WDS) without behavioral convulsions. Antibody to methionine-enkephalin but not dynorphin A(1-8) injected into lateral ventricles attenuated KA-induced WDS, as did naloxone. Granule-cell destroying injections of colchicine into ventral but not dorsal hippocampus caused a 60% reduction of hippocampal ENK and a complete elimination of KA-induced WDS. These studies suggest that release of granule cell ENK, which is 3 times more concentrated in ventral than dorsal hippocampus, plays an important role in KA-induced WDS.

INTRODUCTION

In the hippocampal formation, opioid peptides of the ENK family are contained in: a) projections from lateral entorhinal cortex to dentate gyrus and Amman's horn, b) dentate granule cells, and c) scattered neurons throughout hippocampus (Gall et al. 1981). Concentrations of ENK are several times higher in ventral than in dorsal hippocampus (Hong and Schmid 1981). The dynorphin (DYN) family of opioid peptides is contained in dentate granule cells (McGinty et al. 1983). Concentrations of DYN A(1-8) in ventral hippocampus are twice those in dorsal hippocampus (Kanamatsu et al., in press). KA causes an initial seizure-associated depletion of hippocampal ENK and DYN and a subsequent rebound at 48 hr that corresponds to changes in ENK and DYN immunostaining of the mossy fiber axons of the dentate granule cells (Kanamatsu et al. in press). The purpose of the present studies was to determine whether ENK or DYN contained in dentate granule cells is important for KA-induced WDS or behavioral convulsions. Studies were designed to determine : a) whether KA-induced changes in hippocampal ENK or DYN are associated with WDS as well as with seizures, and b) whether the effects of icv injections of antibody to methionine enkephalin (ME) or DYN, sc injections of naloxone, or intrahippocampal injections of colchicine that destroy dentate granule cells (Goldschmidt and Steward 1981) have an effect on KA-induced WDS or seizure behavior.

METHODS

Male Fischer-344 rats between 12 and 15 weeks of age were used in these studies. KA was administered sc (8 mg/kg), and rats were observed for WDS and limbic seizures (rearing, clonus, hypersalivation, and falling) for 2.5 hr. At this time, rats were anesthetized with pentobarbital (50 mg/kg) to prevent seizure-related mortality. Data were analyzed with analysis of variance significant main effects were followed with post hoc Fisher's LSD tests.

Study I. Rats (n=4) were injected with KA and were

with a radioimmunoassay described previously (Hong et al. 1978) in hippocampal tissue that had been frozen at -70

Stydy II. Rats (n=40, 10/group) were injected with 0, 2.5, 5.0, and 10.0 mg/kg of naloxone (NAL) sc 5 min prior to injection of KA.

Study III. Rats (n-38) were injected with 20 ul of antibody to ME, DYN A(1-8), or normal rabbit serum into the lateral ventricles 6 min prior to injection of KA. The ME antiserum crossreactivity was: ME, 100%; leucine enkephalin (LE), 0.47%; DYN A (1-8) < 0.06; ME -Arg-Phe (MEAP), < 4.0% and B-endorphin (BE), < 0.2%. The DYN A (1-8) antiserum crossreactivity was: DYN A (1-81, 100%; DYN A (1-13), 0.02%; DYN A (1-17), 0.01%; ME, 0.005%; and LE, 0.005%. For the ME antiserum, the IC-50 was 644 fmoles, and for antiserum to DYN A (1-8), it was 25.5 fmoles.

Study IV. Rats (n=86) were bilaterally injected with colchicine (COL) (2.5 µg/site) into dorsal and ventral hippocampus, dorsal hippocampus alone, or ventral hippocampus alone. After a 2 week recovery period, rats were injected with KA and killed 48 hr later. Rats (n-40) injected with COL into the dorsal or ventral hippocampus alone were killed by perfusion with 4% formalin, and brains were processed for cresyl violet and Luxol fast blue staining. Rats injected with COL into dorsal and ventral hippocampus were perfused with 1.2% sodium sulfide followed by 4% paraformaldehyde (n-19) for Timms staining of zinc and immunocytochemical staining of ME and DYN or were killed by decapitation for radioimmunoassay of ME and DYN (n-27):

RESULTS

Study I. Rats included in data analysis were those that exhibited WDS but not behavioral convulsions following systemic injection of KA. Those killed 1-2 hr post injection had an averaae of 45 WDS and those killed 48 post injection had an average of 55 WDS across the 2.5 hr observation period. Hippocampal ME was decreased by 23%

at the early time point and was increased by 40% at the later time point. DYN was initially decreased by 50% and returned to baseline at 48 hr.

Study II. NAL caused a dose and time-dependent decrease in KA-induced WDS. The maximal effect (59% reduction) was achieved with a dose of 5.0 mg/kg. This effect was observed from the onset of WDS 30 min post KA injection to approximately 90 min post injection.

Study III. Pretreatment with antibody to ME but not DYN A (1-8) caused a 30% reduction in KA-induced WDS when compared to rats receiving only control serum.

Study IV. COL injections into ventral hippocampus alone or combined injections into dorsal and ventral hippocampus caused a virtually complete elimination of KA-induced WDS. COL injections into dorsal hippocampus did not attenuate KA-induced WDS. None of the injections had an effect on the onset or topography of behavioral convulsions. COL caused a 60% depletion of ME and a 100% depletion of DYN in dorsal and ventral hippocampus. ME in ventral hippocampus is approximately 3.7 times that in dorsal hippocampus; therefore the absolute reduction of ME in ventral hippocampus was greater than that in dorsal hippocampus. COL blocked the KA-induced increase in hippocampal ME observed in saline-injected controls. Timms and Nissl staining combined with immunostaining for ME and DYN revealed severe (>75%) destruction of granule cells and their mossy fiber axons.

DISCUSSION

Several lines of evidence suggest that release of ENK within the hippocampal formation plays an important role in KA-induced WDS. WDS follow KA-induced epileptiform bursting of hippocampal neurons (Lothman and Collins 1981); electrical stimulation of dentate granule cells sufficient to cause a population spike also leads to WDS (Damiano and Connor 1984). Lesions of the CA3 region of hippocampus attenuate electrically-induced (Damiano and Connor, 1984) and KA-induced WDS (Isaacson and Lanthorn 1981). These studies suggest that release of a transmitter from granule cells onto CA3 pyramidal neurons plays an important role in KA- or electrically-induced WDS. Injections of ENK into the lateral ventricles cause naloxone-reversible WDS (Drust and Connor 1983), suggesting that this behavior involves actions at an opiate receptor. The granule cells of the dentate gyrus contain two families of opioid peptides, ENK and DYN (See Introduction) either of which could play an important role in the initiation of KA-induced WDS.

In these studies, we found that SC injections of KA that lead to WDS but not behavioral seizures cause initial depletions of ME and DYN and 48 hr later a rebound in ME. We also found that pretreatment with NAL attenuated

KA-induced WDS. These results suggest that release of an opioid peptide, possibly ME or DYN could play a role in KA-induced WDS. Therefore, we injected antibody to ME or DYN A(1-8) into the lateral ventricles prior to SC injection of KA and found that antibody to ME but not DYN attenuated KA-induced WDS. Selective COL lesions of the dentate granule cells of the hippocampal formation were performed to determine whether ME contained in these neurons was important for KA-induced WDS. We found that COL lesions of ventral but not dorsal hippocampus were sufficient to eliminate KA-induced WDS. Because systemic KA causes greater increases in metabolic activity in ventral than in dorsal hippocampus (Lothman and Collins 1981), we propose that release of ENK from dentate granule cells in ventral hippocampus onto neurons of the CA3 region plays an important role in the generation of KA-induced WDS but does not appear to be essential for subsequent behavioral convulsions. [5-T32-ES-07126]

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OPIATE RECEPTOR BINDING AND BEHAVIORAL EFFECTS OF MORPHINE IN RHA/VERH AND RLA/VERH RATS

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ABSTRACT

Large baseline differences were found in open field activity and rearing behavior between 2 psychogenetically selected rat lines, as well as differences in the effects of peripherally administered morphine (2,4 and 10 mg/kg) on those behaviors and on body temperature. The 2 lines also showed different capacities of tritiated DACO binding to preparations of striatal membranes.

INTRODUCTION

Roman high-avoidance (RHA/Verh) *rats* are selectec and bred for the rapid acquisition of two-way active avoidance, whereas Roman low-avoidance (RLA/Verh) rats are selected and bred for the non-acquisition of that response. Considerable literature has accumulated documenting neurochemical correlates of behavior in these rats, and profound differences exist between them in baseline activity and in stress situations, in which RHA/Verh rats become hyperactive and RLA/Verh rats tend to 'freeze' (Driscoll and Battig 1982, Driscoll 1986). Genetic differences in opioid peptide systems, in relation to known differences in behavior, have been extensively studied in several species (e.g. Neil et al. 1986, Frischknecht et al. 1986), and it is likely that genetically related behavioral modification, through selective breeding, can also lead secondarily to opiate changes. We have therefore compared the effects of morphine on open field behavior, nocieption and body temperature in RHA/Verh and RLA/Verh rats, and also compared both lines in regard to opioid receptor binding to preparations of crude membranes of striatum and hippocampus.

MATERIALS AND METHODS

28 male RHA/Verh (mean body weight 340 g) and 28 male RLA/Verh rats (332 g) were divided into groups of 7 and injected i.p. with either saline or morphine (2,4 or 10 mg/kg). 30 min later, the numbers of squares crossed and rearing behavior were monitored, individually, during 5 min in an open field (60 x 60x 30 cm, divided into 9 equal squares). 40 min after injection, the nocieptive response was measured with a tail-flick apparatus. The intensity of the heat beam was adjusted to obtain baseline response latencies of about 2 sec. A cut-off time of 15 sec was used to prevent tail damage. Rectal body temperature was measured 90 min after injection with a telethermometer probe. Statistical analyses were performed by 2-way ANOVAs followed by Duncan's multiple range test.

The brains of other groups of RHA/Verh and RLA/Verh male rats of the same age were removed, following decapitation, and striata and hippocampi were extracted and weighed. 4 strata were pooled for each replication. The tissue was homogenized in Tris buffer (pH 7.4 at 2°C), centrifuged at 39,000g for 15 min, the pellet suspended in Tris buffer and incubated at 37°C for 45 min and centrifuged again. The resulting pellet was suspended in Tris buffer at 25°C. For the assay, .8 ml final homogenate was added to .1 ml of the labeled ligand solution and .1 ml of Tris buffer of cold displacer ligand solution. The final tissue concentration was 5.5 mg fresh tissue/ml, corresponding to .43 mg protein/ml. The mixture was incubated for 2.5 hr at 25°C, filtered through a Whatman GF/B filter disc and washed 3 times with 5 ml ice-cold Tris buffer. The filters were dried and, after the addition of 10 ml scintillation cocktail (toulene: Triton X-100 at 2:1, and Butyl-PBD 5g/l_3), were cou ted at an efficiency of 42-45%. The ligands used were H-(D-Ala^2, MePhe^4, Gly-ol^5) enkephalin (tritiated DAGO) for mu-binding determination, and ^{3}H-(D-Ala^2, D-Leu^5) enkephalin (tritiated DADLE), added with cold DACO (7nM/nM DADLE) for delta binding. Cold DAGO was added to suppress the mu-fraction of tritiated DADLE binding. Non-specific binding was defined as the bound radioactivity in the presence of cold etorphine at a constant ratio of 1000x the concentration of labeled ligand. Scatchard analyses (striatum only) were performed by the use of LIGAND program (Dr. Munson, NIH, Bethesda, MD, USA).

RESULTS

In regard to both open field activity and rearing, it was found that RHA/Verh rats were more active than RLA/Verh rats ($p < .001$). Perhaps as a result of the large baseline (NaCI injection) differences, morphine administration did not significantly decrease behavior in RLA/Verh rats, while most dosages significantly diminished both horizontal and vertical activities in RHA/Verh rats. The analysis of tail-flick response latencies showed no significant genetic differences, only a pronounced drug effect ($p < .0001$). A significant

TABLE 1

Effects of morphine on open field behavior, nociception and body temperature in RHA/Verh and RLA/Verh rats (Results are expressed as the mean 2 s.e.m.; n=7 per treatment group)

		Number of Squares crossed	Number of Rearings	Tail-flick Latency (sec)	Body Temperature (°C)
RHA	NaCl	63.3±6.2	43.4±5.4	1.88±0.40	37.85±0.14
	2mg/kg	46.3±7.8	28.4±4.4	4.28±1.88	38.35±0.13
	4mg/kg	40.0±12.1	21.6±4.6	10.10±2.15	38.72±0.21
	10mg/kg	33.8±8.2	9.0±2.2	15.00±0.00	39.14±0.14
RLA	NaCl	19.7±5.2	11.8±3.2	2.48±0.67	37.74±0.16
	2mg/kg	14.0±4.9	11.6±1.9	7.91±1.92	37.98±0.16
	4mg/kg	17.7±4.0	8.3±2.2	9.09±1.82	38.02±0.17
	10mg/kg	6.6±3.1	23.1±1.8	13.46±1.54	37.52±0.29

antinociception was found in RHA/Verh rats with 4mg/kg, but already at 2mg/kg in RLA/Verh rats. In addition, a significant increase in body temperature after morphine, as compared to the NaCl controls, was found only in RHA/Verh rats (4mg/kg: $p < .005$, 10mg/kg: $p < .001$).

The 2 lines also differed significantly in tritiated DAGO binding to preparations of striatal membranes. As visualized in figure 1 A, the binding capacities differed significantly, Bmax being 1.72x greater in RHA/Verh than in RLA/Verh rats. The affinities did not differ (Student-t-test). No difference was found in tritiated DADLE binding to the same preparation (figure 1B). Curve-fitting by LIGAND program revealed a single binding site saturated by tritiated DAGO and 2 binding sites saturated by tritiated DADLE. In addition to table 2, the second binding site of tritiated DADLE was characterized by a Kd of 7.1±2.5 and 4.9±2.1 and a Bmax of 11.9±1.0 and 11.6±0.9 for RHA/Verh and RLA/Verh rats, respectively. The meaning of this

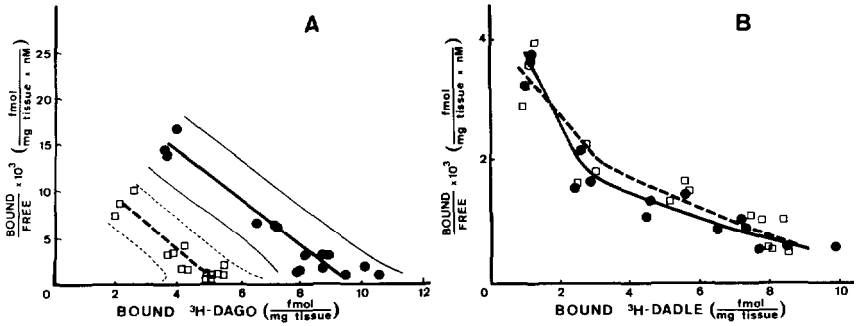

FIGURE 1

Scatchard plot of specific binding of tritiated DAGO (A) and tritiated DADLE (6) to preparations of striatal membranes of RHA/Verh (o) and RLA/Verh ( ) rats. Each point represents the mean of a triplicate incubation. Concentration ranges of labeled ligands were 0.3-12nM for tritiated DAGO and 0.3-17nM for tritiated DADLE. The thin lines in figure IA represent the 95%-condidence limits (±2 sigma) of the means.

TABLE 2

Binding results of tritiated DAGO and tritiated DADLE to preparations of striatal membranes of RHA/Verh and RLA/Verh rats, expressed as mean value (± s.e.m.) of 3 separate experiments. 4 striata were pooled for each experiment. Kd is expressed as nM and Bmax as fmol/mg of tissue.

	^{3}H-DAGO		^{3}H-DADLE		
	Kd	Bmax	Kd	Bmax	mu/delta
RHA	0.39±0.40	9.6±0.44	0.06±0.14	4.19±0.4.3	2.3
RLA	0.36±0.08	5.56±0.35	1.02±0.24	4.62±0.77	1.2

second binding site is not yet known. The first fraction of the Scatchard was therefore considered to define delta binding. Single-point binding of tritiated DAGO (5nM) and tritiated DADLE (1.5 nM) to preparations of hippocampal membranes from RHA/Verh and RLA/Verh rats did not differ significantly.

DISCUSSION

The genetic differences seen here in regard to the behavioral and thermoregulatory effects of morphine, as well as in the striatal mu-receptor binding capacities, provide valuable information which not only adds to the steadily increasing state of knowledge regarding this type psychogenetic selection, but also which indicates some potentially interesting directions for future research. First of all, opioids are believed to exert a neuromodulatory role, particularly *in* regard to the striatum and locomotor behavior, where they have long been known to participate in the regulation of dopaminergic and cholinergic transmission (Cheney et al. 1974, Frischknecht et al. 1986). Morphine also affects the production and metabolism of brain serotonin (Perez-Cruet et al. 1975). All 3 of these neurotransmitter systems (as well as locomotor activity) are known to differ in RHA/Verh and RLA/Verh rats in that the former have a more active striatal dopamine metabolism, a more active serotonergic system in several brain areas, and have lower levels of hippocampal and, possibly, striatal choline acetyltransferase activity than do the latter (Driscoll and Battig 1982, Driscoll 1986, Scatton et al. unpublished results). Therefore, a more detailed analysis of endogenous opioid peptides, as well as additional opioid receptors and regions, would definitely be of interest in these lines of rats.

Secondly, the mu opiate receptor, particularly in the striatum, appears to be involved in the initiation of acute opiate dependence (Eisenberg 1985, Neil et al. 1986) and, as genetic background appears to be important with respect to predisposition to dependence (Jacob and Ramabadran 1981). The finding in the present study that the Bmax of mu binding differs by almost 2 to 1 between RHA/Verh and RLA/Verh rats would seem to make a comparsion of these 2 rat lines, in regard to opiate dependence studies, an undertaking of the utmost interest, with clinical implications.

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ACTION OF NALTREXONE ON THE SEXUAL IMPAIRMENT OF OBESE CAFETERIA RATS

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ABSTRACT

Two experiments were performed to explore 1) the sexual behavior of male obese cafeteria rats, 2) a possible role of endogenous opiates in the regulation of their sexual behavior. In obese cafeteria rats the proportion of ejaculators and the number of ejaculations per hour were significantly lower compared to controls. Naltrexone provoked an increase in the number of ejaculations/h both in control and cafeteria rats due to an induction of copulatory behavior in sexually inactive rats. Thus nutritional obesity provokes an impairment of sexual behavior and an injection of naltrexone (2.5 mg/Kg IP) lifts this inhibition in a similar way as in inactive, control rats.

INTRODUCTION

Two kinds of obesity in rodents, the genetic obesity of Zucker fa/fa rats and the obesity nutritionally induced by a choice of highly palatable foods, include hyperphagias ; both these hyperphagias are suppressed by opiate antagonists, naloxone or naltrexone (Margules et al. 1978, Mandenoff et al. 1982). It is known that opiate antagonists enhance sexual behavior in non obese rats (Hetta 1977, Myers and Baum 1979) ; agonists inhibit it and antagonists lift this inhibition (Gessa et al. 1979, Hetta 1977, McIntosh et al. 1979, Mumford and Kumar 1979, Myerson and Terenius 1977, Pellegrini et al. 1978). Therefore we made the hypothesis that the decrease in sexual performances could be linked to a hyperendorphinism in nutritional obesity as well as in genetic obesity. In genetic obesity a sexual impairment is documented (Edmonds and Withyachumnarnkul 1980), and it has been recently discovered that sexuality and fertility are restored by an injection of naloxone (personal communication : J. Walter, D. Margules). However no information is available concerning the sexual behavior during nutritional obesity. The present work has two aims : to evaluate sexual behavior in cafeteria obese rats and to determine the effect of an opiate antagonist, naltrexone, on this behavior.

MATERIALS AND METHODS

Animals

56 male Wistar rats weighing between 200 and 250g were housed in groups of 4. They were maintained at 24°C with a 12 hour light/dark cycle. They were distributed in two groups : a group "control" (n=28) received ordinary laboratory chow (Extra Labo M25) ad lib; a group "cafeteria" (n=28) was given 4 palatable foods in addition to chow. The 4 supplementary items were changed each day on a weekly rotation. The animals were weighed regularly. At week 9 the mean body weight of "cafeteria" rats was 514.25±6.09g vs 438.69±5.22g for the controls (mean ± sem $\overline{P}$ < 0.001). The first sexual behavior test was then performed. One week later control and cafeteria rats were divided in two subgroups: one IP injected with NaCl (1 ml/Kg of body weight), the other with naltrexone (NTX 2.5 mg/Kg), The sexual behavior tests were conducted 30 mn after the injections.

Sexual behavior tests

The tests were carried out during light period (between 02.00 and 06.00 pm) over one hour. 3 males were placed in a cage. Ten minutes later four 3 month old female rats, made sexually receptive by an IN injection of oestradiol benzoate (10µg/rat) 48 hours and 24 hours prior, were introduced. During the sexual test, some rats were sexually active while others were inactive. The parameters observed were:-intromission latency (latency of the occurence of the first intromission after the introduction of the females in the cage) ; -pre-ejaculatory latency (latency from the first intromission to ejaculation), - refractory period (post-ejaculatory period between an ejaculation and the next intromission) - number of ejaculations per hour (Nb ejac/h).

Statistical Analysis

The proportions of sexually active and inactive control and cafeteria rats were compared by X2 test. The parameters studied in control and cafeteria rats were compared with the Student T test. Effects of naltrexone on the different parameters were analyzed with the T paired test.

RESULTS

Table 1 shows that at week nine the proportion of active cafeteria rats is significantly smaller than that of active control rats (*P< 0.01). The number of ejaculations/h in the cafeteria group is smaller than in the control group (** P< 0.025).

Table 1	Control	Cafeteria
Active n	14	5
%	50%	17.8%*
Inact n	14	23
%	50%	82.2%
Act + Inact n	28	28
Nb ejac/h (mean + sem)	1.96±0.42	0.67±.31*”

When the active cafeteria rats are considered alone, the number of ejaculations/h and the parameters characterizing the sexual behavior are not significantly different from those of the active control rats ("control" (n=14) vs “cafeteria” (n=5): intromission latency 12.33±2.15 vs 10.22±3.02, pre ejaculatory latency 19.71±4.37 vs 18.4±4.50, refractory period 4.38±0.71 vs 5.0±0.71, expressed in minutes + sem, intromission frequency 3.92±0.33 vs - 3.8±0.667.
Tabie 2 shows that naltrexone increases the percentage of active rats both in cafeteria and control qroups, though this increase is not statistically siqnifi - cant. Naltrexone significantly increases the global number of ejaculations in both groups. But if the sexually active rats are considered alone the number of ejaculations is not significantly increased by naltrexone (4.57±0.97 for controls and 5±1.41 for cafeteria), nor are any of the other parameters studied.

Table 2	Control		Cafeteria	
	NaCl	NTX	NaCl	NTX
Active	7		2	5
%	50%	78.6%	14.3%	35.7%
Inact	7	3	12	
%	50%	21.4%	85.7%	6493%
Act+Inact n	14	14	14	14
Nb ejac/h (mean+sem)	1.92±2.2	3.5±2.17**	0.5±1.29	1.28±1.98*

NTX vs NaCl *P< 0.05, ** P < 0.025

DISCUSSION

The first finding was quite expected: a nine week cafeteria diet inhibits the sexual behavior since out of 28 rats only 5 ejaculate during a one hour sexual test. Thus an impairment of sexual behavior occurs in various types of obesity, both genetic and nutritional, suggesting an effect of obesity itself. The results concerning the involvement of the opiate system in such an impairment are less clearcut. In control rats naltrexone does not modify sexual behavior of active rats, but does induce sexual activity in inactive rats. This result is quite

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consistent with those of the literature : it has been reported that in sexually active rats, small or medium doses of opiate antagonists do not modify the sexual behavior (Gessa et al. 1979, McIntosh et al. 1979, Myerson and Terenius 1977), while high doses facilitate it (McIntosh et al. 1977, Myers and Baum 1979). In inactive rats high (McIntosh et al. 1979) and even low doses (Gessa et al. 1979) induced mounting and ejaculations. In inactive cafeteria rats, naltrexone increases sexual performances, however the amplitude of such an increase is not significantly different from that observed in control rats. The dose of 2.5 mg/Kg of naltrexone used to test an eventual hypersensitivity to opiate antagonists in sexual behavior of cafeteria rats could appear to be high since we previously showed that a dose as low as 0.5 mq/Kg is efficient for suppressing hyperphaqia induced by cafeteria diet without modifying food intake in chow fed rats (Mandenoff et al. 1982). However 0.5 mq/Kq of naltrexone did not provoke any change in sexual performances either in cafeteria or in control rats (unpublished results). Thus there is no experimental evidence that in terms of sexual behavior cafeteria rats are more sensitive to naltrexone than control rats. In conclusion, like genetic obesity, nutritional obesity provokes an impairment in sexual behavior and an opiate abtaqonist induces the copulatory behavior in cafeteria inactive rats as well as in controls. Although Naltrexone lifts the inhibition of sexual behavior induced by the cafeteria diet, it is not proved that the impairment of sexual behavior in cafeteria obese rats is related to an hyperendorphinism.

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OPIATES STIMULATE FOOD CONSUMPTON IN THE LAND SNAIL HELIX ASPERSA

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ABSTRACT

Morphine stimulates food consumption in the land snail Helix aspersa. This stimulation of food consumption can be blocked by naloxone, the potent opiate antagonist. In addition, this opiate-induced food consumption exhibits tolerance by the sixth day of treatment. This study further highlights opioid mechanisms in relatively simple organisms and also suggests that these mechanisms are "ancient" signal systems.

INTRODUCTION

Opioid substances have been shown to have diverse functions that transcend their original proposed role in nociceptive/analgesic systems. The importance of this family of compounds is highlighted even more with their discovery in invertebrates. Recently we have reported the isolation and identification of Met-enkephalin, Leu-enkephalin and Met-enkephalin Arg^6-Phe^7 in Mytilus pedal ganglia (Leung & Stefano 1984; Stefano and Leung 1984). The same neural tissue also has been shown to contain opioid receptors that are similar to those reported to exist in mammals (Kream et al. 1980; Stefano et al. 1980). As a result, it is *no* longer surprising to find opiate functions in various invertebrates. In mammals opiate mechanisms have been shown to be involved in the regulation of normal ingestive activities (Morley et al. 1983). Opiates have been shown to not only stimulate food consumption but various ingestive behaviors as well (Sanger 1981; Sanger and McCarthy 1981; Morley et al. 1983). Naloxone, the potent opiate antagonist, can block these morphine-induced activities (Brands et al. 1979) as well as decrease feeding in food deprived mammals (Brown and Holtzman 1979). In an earlier study Kavaliers et al. (1984) demonstrated that opiates could induce feeding in the slug Limaz maximus. Thus, this study complements the earlier study in that morphine also stimulates this activity in Helix aspersa.

MATERIALS AND METHODS

Helix aspersa were obtained from the Pacific Biomarine Company in California. The animals were maintained in the laboratory at 21± 1°C under a 12-hr light and 12-hr dark cycle. They were housed prior to experimentation in a large Styrofoam box containing moistened paper towels, lettuce, carrots and rat chow. The organisms were divided into four groups and placed in individual 1L beakers containing moistened paper toweling clinging to the

side and bottom. For each experiment there was a minimum of five groups of organisms. A substrate of lettuce and carrots were pureed in a blender and poured into a 5 cm petri dish. This was then weighed before and after a 3-hr feeding period and corrected for evaporation. Animals receiving drug treatments were injected through the epidermis in a region superior to the anterior ganglionic complex. The organisms received either drug, vehicle or no injection.

Table 1. The effects of opiates on food consumption in the land snail Helix aspersa

OPIATE	DOSE(M)	%CONSUMED	OPIATE	DOSE(M)	%CONSUMED
vehicle	----	100	naloxine	10^{-7}	100
morphine	10^{-7}	100	naloxone	10^{-6}	90
morphine	10^{-6}	124[a]	naloxone	10^{-5}	50[b]
morphine	10^{-5}	140[b]	naloxone	10^{-4}	70[b]
morphine	10^{-4}	135[b]			
etorphine	10^{-4}	138[b]			

Morphine 10^{-5} and Naloxone 10^{-5} 95% Consumed

Animals were maintained in groups of four for a 3-hour interval. Each group consumed .7g of food. The variation between groups was no greater than 4% Statistical analysis was by Student's t-test, a = $P<0.05$, b = $P< 0.0.1$ Vehicle and control values were identical (.7g/3 hr).

Table 2. Demonstration of tolerance to morphine in reference to its induced stimulation of food ingestion.

DAYS	MORPHINE DOSE (10^{-6}M)	%CONSUMED
1	AS NOTED	125
2	AS NOTED	125
3	AS NOTED	120
4	AS NOTED	113
5	AS NOTED	99
6	10^{-5}	130

Experiments were as noted in table 1. These animals were injected with morpine 4x/day for the indicated number of days. Statistical analysis was by Student's-t-test, a = $P<0.05$, b = $P< 0.01$.

RESULTS

Vehicle and control organisms ate 0.7g/3 hr of the prepared food. Morphine treated organisms exhibited a significant dose-dependent increase in food consumption (table 1). This increase in food consumption occurred within the first 1.5 hr following morphine injection. Interestingly, organisms observed for 24-hr (vehicle

and morphine treated) were found to consume the same amount of food, indicating that the morphine groups' food consumption decreased following the initial morphine-induced stimulation of ingestion. Naloxone, an opiate antagonist, when administered alone was found to decrease food ingestion in a dose dependent manner (table 1). In addition, when co-injected with morphine it blocked the opiate induced stimulation of food consumption. Again, the maximal effects of naloxone occurred during the first 1.5 hr of treatment.

In another sec of experiments, organisms treated with morphine (10 M) 4x/24 hrs) for 5 days gradually lost the morphine induced stimulation of food consumption. However, increasing the dose of morphine on the 6th day brought it back (table 2). This may serve to indicate that this behavior was made tolerant to morphine.

DISCUSSION

The present report suggests that an endogenous opioid system may be present in Helix aspersa and that it may be involved with the organisms feeding responses and food intake. The same type of regulatory role of opioids in regard to food consumption has been reported in Limax maximus by Kavaliers et al. (1984). In both studies, naloxone was able to antagonize the opiate stimulation of food ingestion, indicating the presence of specific opiate receptors. In addition, in both organisms naloxone alone was able to reduce food intake, strongly suggesting the presence of an endogenous opioid system regulating feeding behaviors. Interestingly, this system can be made tolerant to chronic morphine treatments, a finding which clearly demonstrates the cellular complexity of invertebrate opioid activities.

In general the growing body of evidence for the presence of endogenous opioid regulatory systems in invertebrates is increasing (for review, Leung & Stefano 1986; Stefano 1986). The data to date suggests that opioid evolvement had an early development and that this "old" status highlights the importance of these particular signal molecules.

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EFFECT OF ELECTROLYTIC AND CHEMICAL VENTROMEDIAL HYPOTHALAMIC LESIONS ON FOOD INTAKE, BODY WEIGHT, ANALGESIA AND THE CNS OPIOID PEPTIDES IN RATS AND MICE.

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ABSTRACT

The hyperphagia and obesity induced by ventromedial hypothalamic (VMH) electrolytic lesions in female rats were asscciatedwitha 70-94% decrease in the level of ß-endorphin (ß-E) in the hypothalamus and other regions of brain, but not in the pituitary. Dynorphin (Dyn) and methionine-enkephalin (ME) levels were also decreased. Rats with VMH lesions were less sensitive to the inhibitory effect of naloxone on their food-intake. Mice injected with gold thioglucose (GIG) also showed a decrease inthehypothalamiccontent of ß-E and Dyn and exhibited 30% less analgesia compared to control mice after cold swim stress.

INTRODUCTION

Lesions in the VMH region of brain cause hyperphagia, obesity and several other behavioral and physiologic abnormalities (Hetherington and Ranson 1940; Powley 1977; Bray and York 1979). In view of the role of opioid peptides in appetite control (Morley et al. 1983), the objectives of this study were to examine the effects of VMH lesions on the CNS opioid peptides and correlate these changes with specific behavioral characteristics such as food intake and analgesia.

MATERIALS AND METHODS

Female Sprague-Hawley rats (240-260 g) were obtained from lab Supply (Indianapolis, IN) and were maintained as described earlier (Vaswani et al.1983). The details of the stereotaxic VMH lesioning technique are described by Richard III, 1984. Briefly, the rats were anesthetized with sodium pentobarbital (40 mg/kg) and were positioned in a Kopf 1404 research stereotaxic instrument. Prophylactic antibiotics (ampicillin 70/mg/kg i.m., and gentamicin, 5 mg/kg i.m.), were administered immediately and 24 hr after surgery. The electrode was positioned 0.2 mm posterior to bregma, ± 0.7 mm lateral to the mid-sagittal suture and 10 mm below the surface of the skull. Bilateral VMH lesions were produced by passing an anodal current between the 0.5 mm uninsulated tip of an epoxy coated stainless steel electrode (0.11 mm diameter) and a rectal cathode for 20 sec (2 ma x 20 sec current = 40 millicoulombs). control rats were subjected to sham operation. Sold thioglucose (GIG), 800 mg/kg, was given i.p. to female outbred CD-1 mice (Charles River), 21-25 g, to produce bilateral VMH lesions. For histological verification of the extent of VMH and GTG lesions, animals were perfused

with 10% formaldehyde solution in saline. The brains were removed, blocked and sectioned in 20p sections, cut at -20°C on a cryostat and picked up on gelatin coated slides. Slides were stained with cresyl violet and an anatomical comparison and verification of lesion placement was done. Upon gross inspection, the median eminence was found intact in all VMH-lesioned rats. Opioid peptides were measured by RIA as reported previously (Tejwani et al. 1985). Analgesia in mice was measured by using a hot plate at 55°C and the movement of the hind limb was used as the criterion of acute exposure. Measurement of opiate receptor (μ-receptor) birding was done by using [15,16-^{3}H]-etorphine, according to the method of Pert and Snyder (1973) as modified by Bardo et al (1982) and Young et al. (1983) .

RESULTS AND DISCUSSION

Since ithasbeenreportedthatweightgain in animals with VMH lesions is accentuated by a high fat diet (Sawchenko et al. 1981) rats in our study were maintained on a high fat diet (Vaswani et al. 1983).

Daily consumption of a high fat diet increased by 136% in rats with VMH lesions (n=22, P<0.001). Fifteen days later these rats weighed 35% more than sham operated animals (P<0.001, data not shown) . VMH lesions in rats (n=9) significantly reduced B-E levels in the hypothalamus (-85%), striatum (-94%), midbrain/thalamus (-88%), medulla/pons (-97%), hippocampus (-74%) and cortex (-70%) but not in the pituitary (fig. 1A). Dynorphin level decreased (fig. 1B) in the hypothalamus (-54%), pituitary (-66%) and hippocampus (-28%). Brain ß-E cell bodies are confined to the medial basal hypothalamus and arcuate nucleus. Destruction of cell bodies or fibers eminating from this area would conceivably reduce brain ß-E content. Watson et al. (1978) observed that unilateral arcuate lesions produced a 40% decrease in D-E in the central gray area, while Kerdelhue et al. (1982) showed that mediobasal hypothalamus (MBH) lesioned rats had an 89% decrease in ß-E in the central gray area. Milan et al.(1980) have found that small discrete lesions of the arcuate nucleus produced a marked reduction in the hypothalamic ß-E and Dyn levels. In the present study the reduction in pituitary Dyn most probably represents a destruction of neurointermediate lobe Dyn fibers originatihg in hypothalamus. The reduction in hippocampal Dyn may represent a yet unidentified hypothalamic-hippocampal pathway. We observed (fig. 2A) a decrease in ME level in the hypothalamus (-37%) and hippocampus (-38%). In spite of a decrease in the level of opioid peptides, lesioned rats were still sensitive to the effect of naloxone, although to a lesser extent. Naloxone decreased the food intake in rats with VMB lesions by 33%, compared to 67% in control rats (fig. 2B).

Eleven weeks after GTG administration, mice weighed 32% more than control (fig. 3A). GTG lesions significantly reduced hypothalamic ß-E (-80%) and Dyn (-19%) but did not affect ME level. Mice with GTG lesions had no statistically different sensitivity to naloxone or levorphanol but showed 30% less analgesia compared to control mice after 1.5 min of cold swim stress (fig. 3B). We

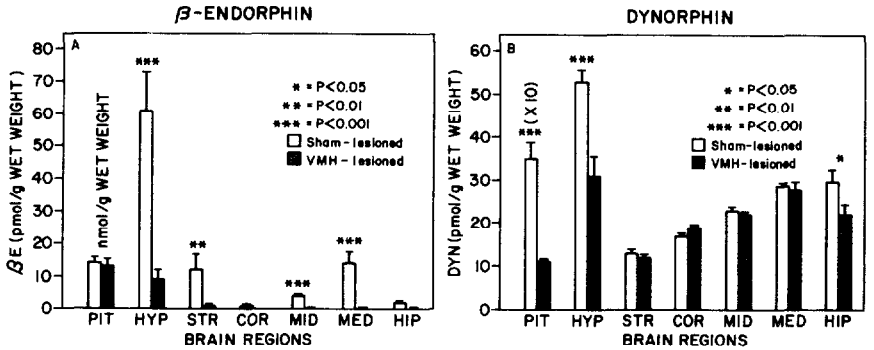

FIGURE 1 Effect of VMH lesions on ß-endorphin$^{1-31}$ (A) and dynorphin$^{1-13}$ (B) levels in female rats 12 days after electrolytic lesions (n=9) orshamlesions (n=7).

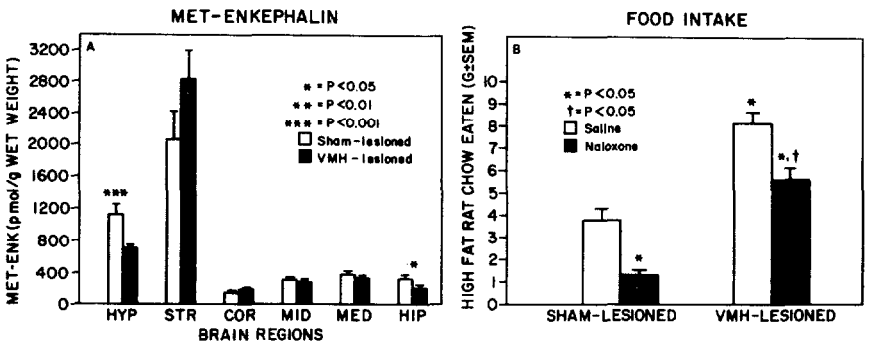

FIGURE 2 A. Effect of VMH lesions on methionine-enkephalin level. B. Effect of naloxone on food-intake. Rats were tested 9 days after lesioning, after 12 hr of food-deprivation. Naloxone (1 mg/kg) was given i.p. 30 min before testing. Food-intake was measured for 1hr.

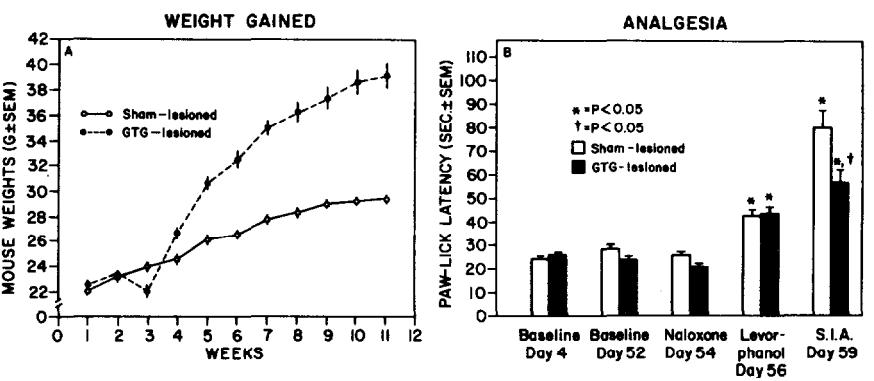

FIGURE 3 A. Effect of GTG lesions on weight gain in female CD-1 mice. B. Analgesic response of mice with GIG lesions to 1 mg/kg, i.p. injection of naloxone or levorphanol or stress-induced analgesia (1.5 min swim in H_2O at 4°C).

conclude that electrolytic or GTG lesions in VMH decrease CNS opioid peptide levels which is reflected by an alteration in the feeding behavior and analgesia in these animals.

Compensatory up-regulation and down-regulation of neurotransmitter receptors have been shown to occur with chronic neurotransmitter decreases and increases, respectively. Compensation of initially perturbed behaviors has been postulated to occur in parallel with changes in receptor affinity or number. In our study, examination of ^{3}H-etorphine receptor binding in VMH and GTG-lesioned animals revealed no change in the opioid receptor affinity or number in any of the brain regions where a decrease in the opioid level was observed (data not shown).

ACKNOWLEDGEMENTS

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BOTH ADRENAL OPIOIDS AND GLUCOCORTICOIDS INVOLVED IN THE INCORPORATION OF INFORMATION INTO MEMORY POST-STRESS

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Various behavioral paradigms have clearly shown that glucocorticoids and opiates influence the acquisition and maintenance of several behaviors in the rat. Recently, in an experimental paradigm originally designed to screen for antidepressants (4), we have shown that manipulation of steroid and opioid levels profoundly influence the maintenance of an immobile floating response. This response develops when rats are swum in a restricted space from which they cannot escape. Rats are placed in a narrow plexiglass cylinder for 15 minutes; initially they swim vigorously, but progressively spend more and more time relatively immobile. Normal, naive rats are immobile for ~70% of the last 5 minutes of the test period, and for a similar percentage of a 5 minute retest period 24 hrs later.

FIGURE 1: Rat showing characteristic immobile posture.

We have shown that rats have no difficulty in acquiring the immobility response after adrenalectomy, hypophysectomy or concurrent

hypophysectomy and adrenalectomy (adrx-hypox). As is shown in figure 2, hypophysectomized animals showed levels of immobility similar to intact animals on retest. In contrast, adrenalectomized rats (or adrx-hypox) showed a significant reduction in immobility, remaining immobile for only 28%-30% of the time.

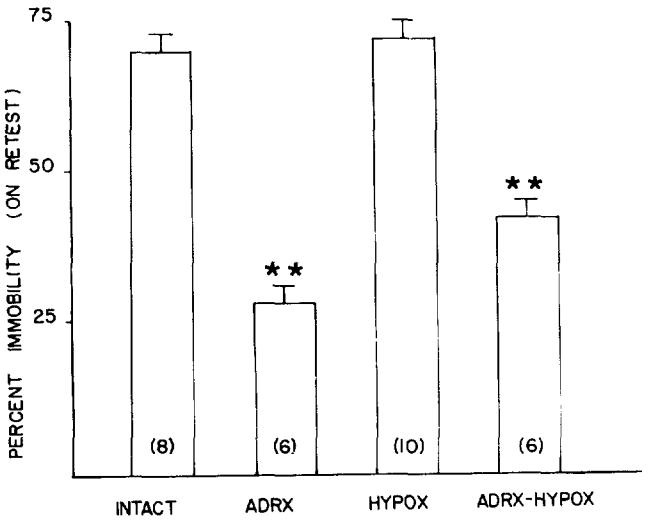

FIGURE 2: Percent immobility during the 5 minute retest by intact, adrenalectomized, hypophysectomized and hypophysectomized-adrenalectomized rats. Columns represent mean ± SEM values, the number of animals in each group is shown within each column. Significant differences from control are shown. **P <0.01.

This behavioral effect of adrenalectomy was reversed by the glucocorticoids corticosterone or dexamethasone (DM), but not by the mineralocorticoid deoxycorticosterone, administered within an hour- of the initial test period. The catecholamines adrenaline and noradrenaline were shown to be without effect. The non-specific opiate agonist D[Ala^2-Met^5]enkephalinamide (DAMEA), at 5 or 50 µg, reversed the behavioral effect, with animals remaining immobile for 68% of the retest period (3).

On the basis of these findings we suggested that maintenance of immobility was dependent on glucocorticoids and/or adrenal opioid peptides, and that in vivo these mechanisms may have a coordinate effect.

To verify the specificity of the opiate effect we administered naloxone (0.1-10 mg/kg) to adrx rats with the DAMEA, and as a control tested the effect of equivalent doses of naloxone on the dexamethasone reversal. With increasing doses of naloxone there was a progressive fall in immobility on retest in both DAMEA and DM treated animals. At

10 mg/mg naloxone, DAMEA treated animals were immobile for 31% and DM treated animals for 36% of the retest period. We therefore tested naloxone at similar doses in the intact animal. Over the dose range used, naloxone lowered immobility equivalently in intact and in adrx rats treated with DAMEA or DM; with 10 mg/kg intact animals were immobile for only 36% of the 5 minute retest.

This inhibitory action of naloxone was seen only when it was given within 1 hour of the initial swimming exposure. That the opioidergic mechanisms involved in the maintenance of the immobility were within the CNS was strongly indicated by the inability of MRZ2593, a quaternary naloxone analogue which does not cross the blood-brain barrier, to affect the swimming response (2).

On the basis of these studies, we proposed a model of maintenance of immobility involving at least two opioidergic pathways, and that the integrity of the second of these pathways is an absolute requirement for the response to occur. In this model there are sensors for peripheral opioids putatively outside the CNS, and sensors for adrenal corticosteroids either central or peripheral. Both of these relay onto a common effector which is itself central and opioidergic. Activation of either pathway is sufficient to stimulate the common effector and facilitate maintenance of the immobile response.

DAMEA is a relatively non-specific opiate agonist, and naloxone though with a higher affinity for mu receptors than kappa or delta receptors - is by definition a non-specific opiate receptor antagonist. Accordingly we have undertaken studies to delineate the receptor specificity of the opioidergic pathways involved in the reversal of the effect of adrenalectomy. Equimolar doses of morphine sulphate (mu-selective), [D-Ala^2-D-Leu^5]enkephalin (delta-selective) and ketocyclazocine, dynorphin 1-8, dynorphin 1-17 and the heptapeptide [Met^5]enkephalin [Arg^6-Phe^7], all kappa-selective agonists, were administered to adrenalectomized rats and the behavioral effect observed. Reversal of the effect of adrenalectomy occurred with ketocyclazocine, dynorphin 1-17 and [Met^5]enkephalin [Arg^6-Phe^7], but not with 10-fold higher doses of the mu- or delta-selective ligands. The reversal of the effect of adrenalectomy by ketocyclazocine was antagonized by MR2266, a kappa-selective partial agonist/predominant antagonist (1).

That it is the peripheral opioidergic pathway which is kappa-selective was shown by studies in which equivalent doses of MR2266 were without effect in intact rats, or in adrx rats treated with DM.

Further evidence for glucocorticoids and opioids of adrenal medullary origin being involved in the maintenance of the immobile behavior was shown when intact animals were treated with the anti-glucocorticoid RU38486 (3.75 mg/rat) and/or the nicotinic antagonist mecamylamine (10 mg/kg) - Administered alone, they were without effect; administered together immobility was significantly reduced, with animals remaining immobile for 40% of the 5 minute retest period. Under these condititons both adrenal medullary secretion and glucocorticoid action is blocked. RU38486 and MR2266, simultaneously administered to the intact animal, similarly impaired maintenance of the immobile response.

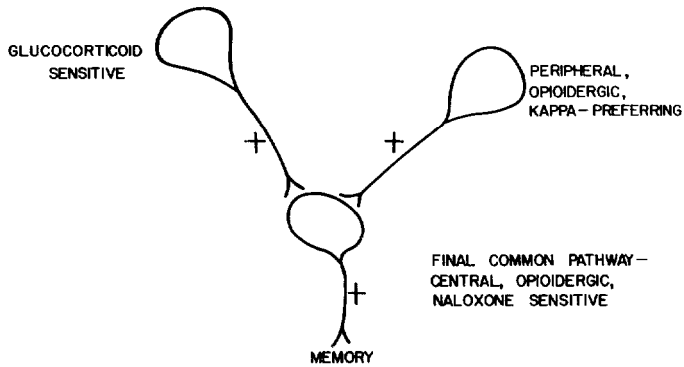

FIGURE 3: Hypothetical model showing pathways for maintenance of immobility.

Additional evidence in support of our hypothesis was the finding that hypophysectomized animals given mecamylamine showed similar levels of immobility on retest as found in adrenalectomized animals; MR2266 administered to hypophysectomized rats equivalently inhibited the maintenance of immobility. These findings strongly support the hypothesis that when animals are swum under conditions of moderate stress, the immobile response which is acquired is maintained by either glucocorticoids or adrenal medullary kappa-selective opioids, with both of these endocrine effects being mediated through a final, common, non-kappa selective opioidergic pathway.

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EFFECT OF CHRONIC EXERCISE ON β-ENDORPHIN RECEPTOR LEVELS IN RATS

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ABSTRACT

A radioreceptor assay using $[^3H]_2$-β-endorphin (B-EP) was used to examine binding in the brains of rats that had been exercised on a treadmill for 5 months. The binding of $[^3H]_2$ -β-EP to brain homogenates gave an average Kd of 0.830 ± 0.055 nM for the controls and 0.826 ± 0.022 nM for the runners, and respective Bmax's of 166 ± 20 pM and 159 ± 27 pM.

INTRODUCTION

There has been a great deal of press on the effects of "endorphins" on "runners high", "exercise withdrawal syndrome", and "depression" connected with cessation of running. It has been well documented that exercise increases plasma β-endorphin levels in man (Colt et al. 1981 and Gambert et al. 1981) and that an up-regulation of opiate receptors is known to occur when the opiate antagonist naltrexone is chronically implanted in rats (Zukin et al. 1982). These facts prompted us to examine the effect of chronic running on opiate receptor behavior in rats. The results of this study are presented.

MATERIALS AND METHODS

Thirty six male Fisher 344 (Simonsen) rats weighing from 150-170 gms were run on a treadmill. To habituate the rats to the treadmill they were run for 3 weeks (10-20 min). Thirty of the most consistent runners were randomly divided into 3 groups of 10, two groups were designated as runners and one as the control. Dry ice was placed at the back of each runway to keep the animals running. Electric shock, initially used, was more stressful and injurous to the animals than dry ice. The experimental groups were run daily for 22 weeks, gradually working up to 1.4 km/hr for 60-70 min. The control group was placed on the other half of the treadmill but not run. At the end of the training period the animals were weighed, decapitated and the following tissues were removed: brain, pituitary, soleus muscle and plasma. The levels of β-endorphin in plasma and pituitary was determined by RIA as described earlier (Watson et al. 1982). The soleus muscles were assayed for the amount of citrate synthase activity (Srere 1982) as an indicator of each rats conditioning level (Lewis et al. 1980).

Radioreceptor assays (RRA) with $[^3H]_2$ -β-EP were performed on brain homogenate prepared as described previously (Ferrara et al. 1979). The final homogenate was resuspended at a concentration of 10 mg wet-weight of brain per ml of cold Tris buffer. Binding experiments

were performed on 0.666 ml aliquots of membranes (0.5mg protein). Membranes were incubated with Tris buffer or unlabeled β-EP (500 nM) and then with varying amounts of $[^3H]_2$-β-EP (0.3 - 3.0 nM) for 60 min at 22°C then 10 min at 0°C in an ice water bath. The incubation was terminated by rapid filtration. Receptor-ligand interaction was examined by Scatchard analysis (Scatchard 19491. Results were analyzed for significance by Student's T-test.

RESULTS AND DISCUSSION

The biochemistry of the changes which occur due to chronic physiological conditions, such as aging, stress, excessive food intake, habitual exercise and the major chronic functional psychoses are poorly understood. Although ill defined, there appears to be a relationship between the central and peripheral opioid peptides and/or their receptors in many age related disorders.

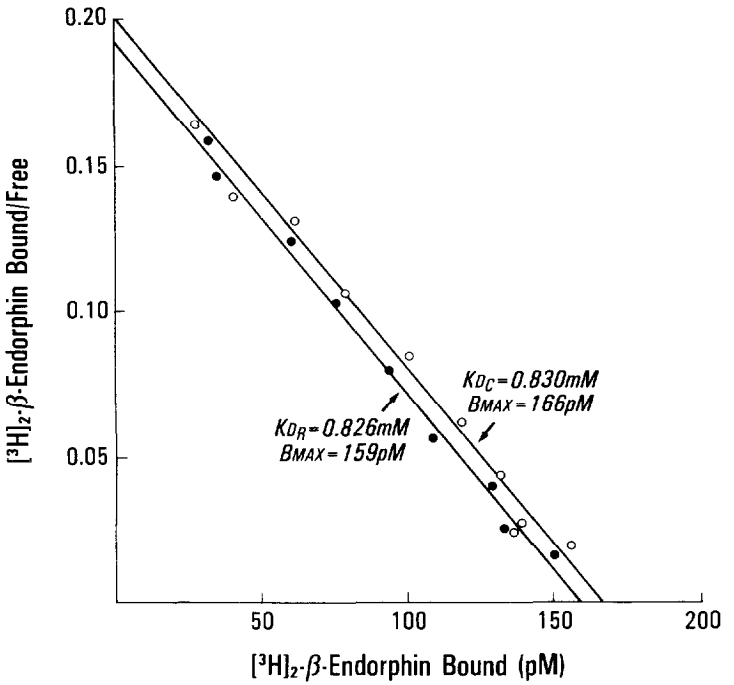
While increases in β-endorphin levels in plasma drop to normal in 30-60 minutes after cessation of exercise (Gambert et al. 1981), any changes in the receptor number for β-endorphin would be expected to return to normal over a period of five to seven days (Zukin et al. 1982). This decrease in receptor number, with a concomittant periodic increase in opioid peptide level could possibly explain the "addiction" felt by runners, and may also be one factor in a number of compulsive behavior patterns or the general phenomenon of addiction. Very little work has been carried out which addresses the possibility of change in the receptor systems in chronic physiological states.

Our research began with a working hypothesis that receptor dynamics occur for a variety of physiological changes, and very likely occur differently for the various opiate receptors and brain areas. These changes may therefore effect physiological/psychological variables such as mood, sleep, sexual behaviors, etc. to different degrees at different times.

A convenient natural means for inducing receptor changes was needed, and since there has been much press on the effects of the endogenous opioid peptides (usually lumped together as "endorphins") on "runner's high" or "addiction to running", it was decided to examine the possible changes in the receptor behavior in chronically exercised versus sedentary rats. The rat has long been used as a model for physical fitness studies (Harpur 1980). The RRA for β-endorphin was selected because it has been used extensively in this laboratory (Ferrera et al. 1979). The exercise regimen used, is known to give substantial exercise conditioning to heart and leg muscle in the rat (Baldwin et al. 1972). When the receptor assay for β-endorphin was carried out using 9 runners and 9 sedentary controls and their Scatchard plots examined, it was found that an apparent small decrease of receptor number for the runners (Bmax = 159 pM versus Bmax = 166 pM) had occurred with no change in affinity of binding (Fig. 1). The differences were consistent throughout the nine points of the Scatchard analysis, although the individual points reached 95% significance by Student's T test in only three instances. While the difference in receptor number was small, the decrease for

the runners is in the direction one might expect if an agonist down-regulation were operative (Zukin et al. 1982). A loss of body weight of approximately 10% was found for the running rats, the effect of which will be examined in subsequent experiments.

Figure 1



Scatchard plot of $[^3H]_2$-β-endorphin binding to rat brain homogenates of the running group (o) and the control group (o). Each point represents the mean of values obtained from nine animals.

Our results also indicated a substantial increase in B-endorphin levels in plasma for runners versus controls, but no apparent difference in levels in the whole pituitary (Table I). Since it is known that β-endorphfn levels increase in some brain areas in rats (Wardlaw and Frantz 19801, while decreasing in others (Gambert et al. 1980), the small decrease in receptor number found for the runners versus the controls indicates that regional brain areas need to be examined for both differences in receptor numbers and β-endorphin levels. The difference in β-endorphin levels in anterior and posterior pituitary versus whole pituitary also need to be examined. These studies are in progress.

Table I

Plasma, β-EP levels in Pituitary, Citrate syntase in Soleus and Body weight.

	Runners	Controls
Plasma β-EP (fmoles/ml)	14.5 ± 3.56	2.95 ± 0.55
Pituitary β-EP (pmoles)	1020 ± 85	865 ± 63
Soleus-citrate syntase (μM/g/min)	13.04 ± 0.32	5.16 ± 0.14
Body weight (g)	311.0 ± 2.95	355.3 ± 2.60

Results are presented as means ± SEM.

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INTRATHECAL MORPHINE AND BLOOD PRESSURE IN PREECLAMPSIA

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ABSTRACT

Ten term parturients with moderate-severe preeclampsia received intrathecal morphine for labor analgesia. Significant reductions in blood pressure and heart rate, which were unrelated to analgesia, were observed.

INTRODUCTION

Preeclampsia is a serious disease of pregnancy which is associated with proteinuria, hypertension and edema. Toxemia is responsible for up to 20% of reported fetal and maternal deaths (Bonica 1965). For toxemic patients, the ideal technique of analgesia for labor and delivery does not exist. Each method has significant disadvantages (James and Wheeler 1982). In the case of normal parturients, it has been demonstrated that prolonged analgesia (8-11 hours) for labor can be achieved with intrathecal morphine (ITM) without any adverse effects to mother or newborn. No change in maternal heart rate or blood pressure was observed (Abboud et al. 1982, Baraka et al. 1981).

Anesthetic management of preeclamptic patients during the labor and delivery period remains controversial. It was of interest to determine whether ITM afforded any advantages as an analgesic method for parturients with severe toxemia.

MATERIALS AND METHODS

Ten parturients (average age, 20 years), greater than 34 weeks gestation, in early labor and undergoing oxytocin augmenation of labor were studied. Moderate to severe preeclampsia was defined by the following criteria: 1) systolic blood pressure >160 mm Hg or diastolic blood pressure >100-110 mm Hg; 2) proteinuria; 3) oliguria. Treatment consisted of intravenous hydration and continuous infusion magnesium therapy (prior to intrathecal morphine). Each patient received 1.0 - 1.2 mg of morphine sulfate (preservative free) in 2.0 cc of 10% dextrose solution via a 25 gauge spinal needle at the L2-3 interspace while in the sitting position. After

one minute, the patient was placed supine with the head elevated at 30 degrees and with a left lateral tilt by wedge. Oxygen (5 L/minute by face mask) was administered continuously. The patient's blood pressure (systolic, diastolic, mean) and heart rate were monitored continuously using an Ohio 2100 Adult Non-invasive Blood Pressure Monitor.

Progress of labor was estimated by continuous external cardiotocography and by hourly vaginal determination of cervical dilation. Oxytocin augmentation was used in each case. Pain level was evaluated by the patient using the visual linear analog scale (Revill et al. 1976). Also, an investigator independently assessed the intensity of pain and its relief, and time of onset (Writer et al. 1981). These assessments were made before ITM injection, every 15 minutes for one hour, every 1/2 hour for the next seven hours and then every hour until delivery. Maternal venous blood gases were obtained before ITM injection and every 4 hours afterwards up to 24 hours. Maternal arterial blood gases were determined at delivery. Fetal scalp pH determinations were obtained in one patient during labor. The condition of the infants was evaluated by Apgar scores at 1, 5, and 15 minutes, by measuring time to sustained respirations (TSR) and determining umbilical venous blood gases from doubly clamped cord at delivery. Maternal vital signs were monitored for a total of 24 hours post-intrathecal injection. Anesthesia for delivery was provided by pudendal block (1% lidocaine). One patient underwent caesarian section due to failure to progress. Maternal side effects attributed to morphine were rated absent, mild or severe every 60 minutes.

RESULTS AND DISCUSSION

We observed that 1.0 and 1.2 mg of morphine intrathecally administered provided prolonged and complete analgesia (up to 8 hours) during oxytocin stimulated labor in moderate and severely preeclamptic patients. The average diastolic blood pressure and mean arterial blood pressure were reduced 22% and 15% respectively. Heart rate averaged a 14% decrease. These autonomic changes and pain relief occurred within 60 minutes of ITM injection. The decrease in blood pressure and heart rate occurred prior to the onset of oxytocin-induced labor. Although magnesium sulfate was used, other antihypertensive therapy was not required. Fetal and maternal acid-base status remained normal during labor. Time to sustained respirations for the infants was less than 30 seconds. Apgar scores were greater than 8 at 5 minutes in all infants. In the post-partum period (up to 30 hours) no significant adverse effects in mother *or* newborn were noted. Maternal and neonatal blood gases

were normal at delivery. Maternal blood gases were unchanged up to 24 hours post-partum. No respiratory distress was observed. Several patients had loss of pin prick and cold sensation over the neck, chest, abdomen, legs and feet. Following ITM some patients complained of nausea, vomiting and itching.

Although Baraka et al. (1981) and Abboud et al. (1982) have demonstrated the efficacy of intrathecal morphine as an analgesic during labor, it is still considered an experimental technique. Dosage, as well as other factors, remain to be determined. In this small series of preeclamptic patients, we observed satisfactory analgesia for labor (up to 8 hours) and up to 30 hours post-cesarian section. No significant maternal or fetal/neonatoal side effects were seen. More important was our observation of a temporally related decrease in diastolic and mean blood pressures and heart rate in preeclamptic women during labor. This decrease was unrelated to pain relief since induction with oxytocin had not begun.

These findings suggest that ITM may be of therapeutic benefit to toxemic patients. Abouleish et al. (1981) observed moderate blood pressure decrease in monkeys receiving ITM (0.07 mg/kg). No such changes have been observed in normal human parturients. The mechanisms by which blood pressure and heart rate are lowered by ITM in preeclamptic patients remains speculative. Laubie et al. (1977) noted that morphine-like agents reduced sympathetic tone (lowered blood pressure and heart rate) by an action on the CNS. The clinical significance of these preliminary findings remains unknown, but appears promising.

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CENTRALLY ADMINISTERED LEUMORPHIN POSSESSES POTENT DEPRESSOR ACTIVITY IN CONSCIOUS RATS

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ABSTRACT

The effect of intracerebroventricular (i.c.v.) administration of leumorphin on basal blood pressure and angiotensin II (AII)-stimulated increase in blood pressure was examined in conscious unrestrained rats. The i.c.v. injection of leumorphin (0.06 and 0.6 nmol) elicited significant depressor effect. The i.c.v. administration of leumorphin with AI1 (0.1 nmol) significantly attenuated the AII-induced pressor response and, furthermore, lowered blood pressure below the basal level. These results suggest that leumorphin is involved in the central control of blood pressure.

INTRODUCTION

The possibility that opioid peptides are involved in the central modulation of blood pressure homeostasis stems from the cardiosuppressive effects of opiate alkaloids and is strengthened by the neuroanatomical findings that opioid peptides are distributed in brain regions especially in the areas involved in cardiovascular regulation (Khachaturian et al. 1985). Several experiments of central administration of opioid peptides suggest the integral role of opioids in blood pressure control, but no unanimous conclusion on their effects has been drawn, since their effects largely depend upon the state of consciousness of the experimental animals (Yukimura et al. 1981) or the sites of administration (Feuerstein et al. 1982).
The possible existence of leumorphin with 29 amino acids was predicted by the nucleotide sequence of preproenkephalin B (Kakidani et al. 1982) and we demonstrated its existence in the pituitary and brain using radioimmunoassay coupled with gel filtration and high performance liquid chromatography (Nakao et al. 1983, Suda et al. 1984, Suda et al. 1985). We also reported that leumorphin is a κ-ligand (Suda et al. 1983) and possesses potent actions on consummatory behaviors in rats (Imura et al. 1986). In the present study, we examined the effects of intracerebroventricular (i.c.v.) administration of leumorphin on blood pressure using conscious unrestrained rats.

MATERIALS AND METHODS

Animals Thirty-seven male Slc: Wistar rats (220-270 g) were used. A stainless steel cannula was stereotaxically implanted into the left

lateral ventricle 1-2 weeks before the experiments. One day before the experiment, a polyethylene catheter was placed in the abdominal aorta through the left femoral artery. For i.c.v. injection, the peptide dissolved in isotonic saline or saline as a vehicle was injected (5 µl) for 10 sec. Blood pressure (mean arterial pressure) was monitored while the animals were conscious and freely moving in their cage as previously reported (Itoh et al. 1986).

Experiment 1. Effect on basal blood pressure Twenty-one rats were divided into 4 groups, and rats in each group received one of the following administrations: saline, 6 nmol of leucine-enkephalin (Leu-enkephalin), 0.06 nmol or 0.6 nmol of leumorphin after a 30-min stabilization period. Blood pressure following the administration was monitored for 60 min and compared with the basal level.

Experiment 2. Effect on angiotensin II (AII)-induced pressor response Sixteen rats were divided into 3 groups and rats in each group received 2 i.c.v. injections with a minimal interval of 100 min: first, the administration of 0.1 nmol of AI1 alone (control), and second, the simultaneous administration of AI1 (0.1 nmol) and of saline (Group I), 0.06 nmol of leumorphin (Group II) or 0.6 nmol of leumorphin (Group III) (treated). Pressor responses to AI1 were compared between the first and second administrations.

Peptides AII and Leu-enkephalin were purchased from Protein Research Foundation (Osaka, Japan) and human leumorphin was synthesized by a conventional solid phase method.

Statistical analysis Results are expressed as means ± S.E. Corresponding values on the same animal were assessed by Student's paired t-test and values among groups were compared by Duncan's test following a one-way analysis of variance for multiple comparisons.

RESULTS

Experiment 1 The i.c.v. injection of leumorphin at the doses of 0.06 nmol and 0.6 nmol caused significant decreases in blood pressure, while 6 nmol of Leu-enkephalin or saline had no apparent effect on basal blood pressure (table 1). Two doses of leumorphin manifested the same magnitude of maximal depressor effects but the temporal profiles were different. The duration of significant suppression of blood pressure was 2 min in 0.06 nmol of leumorphin and 10 min in 0.6 nmol of leumorphin. There were no apparent changes in heart rate (2.8 ± 3.5 % for Leu-enkephalin, 4.4 ± 0.6 % for 0.06-nmol leumorphin, and 9.3 ± 2.2 for 0.6-nmol leumorphin).

Experiment 2 Hypotensive effects of leumorphin were more notable when blood pressure was elevated by centrally administered AII. As shown in table 2, pressor response to central AII was reduced by the two doses of leumorphin by 60-70 %. Both doses of leumorphin even lowered blood pressure below the basal level (-8.1 ± 3.1 mmHg for 0.06 nmol and -19.0 ± 1.8 mmHg for 0.6 nmol) and this depressor effect persisted more than 20 min.

DISCUSSION

The present study has demonstrated potent suppressive effects of i.c.v. administration of leumorphin on both basal blood pressure and pressor response to centrally administered AII in conscious rats. In our preliminary study, the depressor effects of leumorphin were partially blocked by the preadministration of naloxone, indicating

TABLE 1. Effects of i.c.v. injection of Leu-enkephalin and leumorphin on basal blood pressure in conscious unrestrained rats

Treatment Group			Baseline (mmHg)	Maximal Change (mmHg)
Saline			127.8 ± 2.6	0.9 ± 2.9
Leu-enkephalin	6	nmol	117.5 ± 4.4	2.5 ± 1.9
Leumorphin	0.06	nmol	116.8 ± 4.2	-6.5 ± 2.7†
Leumorphin	0.6	nmol	119.1 ± 3.5	-7.2 ± 1.7†

Values are the means ± S.E.
Significantly different from saline-control:
† = P<0.01, Duncan's test.

TABLE 2. Effect of i.c.v. injection of leumorphin on blood pressure increase induced by i.c.v. administration of angiotensin II in conscious unrestrained rats

Treatment group		Baseline (mmHg)	Maximal response (mmHg)	% reduction of maximal response (%)§	Recovery time (min)
Group I	control	121.7	22.9		68.4
(Saline)		±3.0	±2.7	2.2	±9.9
	treated	122.6	21.4	±6.0	53.1
		±2.6	±1.6		±6.4
Group II	control	129.8	18.1		51.0
(Leumorphin		±4.5	±1.9	65.3	±15.4
0.06 nmol)	treated	131.5	5.6	214.4†	2.1
		±4.6	±2.0*		±1.2*
Group III	control	120.8	24.0		58.6
(Leumorphin		±4.0	±2.8	±69.2	±9.6
0.6 nmol)	treated	125.2	7.8	±4.9†	2.5
		±4.0	±2.2***		±1.3**

Values are the means±S.E.
§: 100 x ((Maximal response) control - (Maximal response) treated) / (Maximal response) control.
Significantly different from control: *=P<0.05, **=P<0.05, ***=P<0.001, Student's paired t-test.
Significantly different from Group I: †=P<0.01, Duncan's test.

that the observed effects were mediated by opioid receptors. Little is known about the role of κ-ligand on blood pressure regulation. In anesthetized rats, the microinjection of κ-agonist, U50488H into the nucleus tractus solitarius was reported to increase blood pressure and decrease heart rate (Carter and Lightman 1985), while dynorphin(1-13) microinjected into the hypothalamic periventricular nucleus was found to cause hypotension and bradycardia (Feuerstein and Faden 1982). Our results using conscious rats further indicate the involvement of leumorphin, κ-agonist, on the maintenance of blood pressure. Sites of action or mechanisms of the depressor

effect of leumorphin cannot be ascertained from our present experiment since leumorphin administered intracerebroventricularly may affect multiple opioid sensitive circumventricular sites (Atweh and Kuhar 1977). However, we have observed the inhibition of vasopressin secretion induced by centrally administered AII by i.c.v. injection of leumorphin (Yamada et al. 1986). This finding together with our observation that leumorphin is present in the hypothalamus (Suda et al. 1985) suggests that the depressor effect of leumorphin is in part mediated by the inhibitory modulation of vasopressin secretion, as observed with other opioid peptides (Summy-Long et al. 1981).
The potent inhibitory effect of leumorphin on blood pressure demonstrated in this study and our previous works of its marked suppressive actions on water intake (Imura et al. 1985) and vasopressin secretion (Yamada et al. 1986) imply the involvement of leumorphin in body fluid and blood pressure homeostasis.

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RELATIONSHIP BETWEEN DIHYDROETORPHINE AND CHOLINERGIC SYSTEM IN THE INHIBITION OF RESPIRATION AND HEART RATE

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ABSTRACT

The respiratory inhibition caused by dihydroetorpine (DHE) was fully antagonized by pilocarpine or physostigmine, but the bradycardia was not. When atropine (icv) was combined with DHE, the respiratory inhibition was antagonized but the bradycardia persisted. However, intravenous atropine antagonized both the respiratory and cardiac inhibition caused by DHE.

Key Words : dihydroetorpine, atropine, pilocarpine, physostigmine, respiration, heart rate

INTRODUCTION

Dihydroetorpine (DHE) is a potent analgesic agent. When given intravenously, it causes significant respiratory and cardiac inhibition. B-7601, a *new* muscarinic blockade, antagonizes the bradycardia after DHE (Bian et al. 1985). Bethanechol, a cholinomimetic, acts on the respiratory center, causing initial excitation and subsequent inhibition, of which the latter can be antagonized by atropine, but not the former (Gonsalves and Borison 1980). Considering that opioids decrease the release of Ach and thus depress respiration (Pepeu 1973), we studied the effects of DHE, atropine and the cholinomimetics on respiration and heart rate to explore the relationship between DHE's inhibitory effects on respiration, heart and cholinergic system.

MATERIALS AND METHODS

Hybrid rabbits were used; body weight was 1.8-2.5 kg and six groups with 5-7 in each group.

Pilocarpine hydrochloride and physostigmine salicylate were purchased from the Medical Supply Station of Shanghai, and atropine sulfate from the Chengdou First Pharmaceutical Factory. DHE was given by the Academy of Military Medical Sciences of China. Respiration and ECG were recorded with RM-86 polygraph. The tidal volume was estimated according to the amplitude of respiration.

RESULTS AND DISCUSSION

Effects on respiration:

DHE (0.5 µg/kg, iw) inhibited rabbits' respiration, the effect being most apparent 5-10 min after injection. The frequency was decreased by 70±12% or more (mean ± SD, $p<0.01$) with no significant change in tidal volume and the change lasted over 40 min.

Pilocarpine (2.5 mg/kg, icv) accelerated respiration from 53±14 to 94±21 times/min ($p<0.01$) 10 min after its administration without significant change in tidal volume. When DHE was given at this point, respiration became slower but still more rapid than the control. After physostigmine (0.3 mg/kg, iv), the respiration increased markedly by 10 min (from 59±9 to 159±48 times/min, $p<0.01$); the tidal volume was decreased from 56±29 to 33±27 ml, but the ventilation volume remained higher than the control. When DHE was then given, the frequency became lower (still higher than the control) and the tidal volume matched that of the control (fig. 1). These results suggest both pilocarpine icv and physostigmine iv stimulate the respiratory center and antagonize the respiratory inhibition of DHE. This may be related to the possibility that DHE could resemble morphine in that it agonizes opiate receptors and retards the release of Ach (Pepeu 1973).

Atropine (0.1 mg/kg, icv) increased the frequency of respiration from 51±11 to 75±37 times/min ($p>0.05$) 5 min after injection, with no significant change in tidal volume. Five minutes after or before DHE, atropine antagonized the inhibitory effect of DHE on respiration, the changes being significant ($p<0.01$ and $p<0.05$ respectively). When a bigger dose of atropine (1 mg/kg) was given intravenously, its antagonistic effect also appeared, but rather slowly (30 min after injection). It is clear that atropine and DHE are antagonistic in modifying respiration. Gonselves demonstrated that bethanechol first stimulates and then inhibits respiration, and proposed the possibility that there are 2 subtypes of muscarinic cholinoreceptors in respiratory center, excitatory M_1 and inhibitory M_2, of which atropine blocks the latter selectively (Gonsalves and Borison 1980). We postulate that Ach is the excitatory transmitter for the respiratory center, while DHE agonizes opiate receptor, depressing the release of Ach in the CNS (Bian et al. 1985) and blocks M_2 receptor, it antagonizes the inhibitory effect of DHE on respiration.

TABLE 1. Antagonist effect of atropine on respiratory inhibition of DHE in rabbits (n-6 in each group)

Group	Change of respiration (%, mean±SD) minutes after DHE				
A	-70±12	-72± 11	-71±10	-65±10	-62±l2
B	51±77	46±55	36±54	40±40	37±44
C	-55±ll	-57±17	-49±5**	-38±10	-29±8
D	-54±12	-53±12	-4O±20*	-30±34	-23±8**
E	-60±16	-61±l5	-58±9	-50±11	-39±19**

compared with group A: *p<0.05; **p<0.01
A: DHE 0.5 μg/kg, iv. B: Atropine 0.1 mg/kg, icv;
C: Atropine 0.1 mg/kg, icv 5 min after DHE iv;
D: Atropine 0.1 mg/kg, icv 5 min before DHE iv;
E: Atropine 1 mg/kg, iv 10 min after DHE iv.

Effects on heart rate:

DHE (0.5 μg/kg, iv) as well as pilocarpine (2.5 mg/kg, icv) decreased the heart rate of rabbits from 262±26 to 187±32 beats/min and from 268±27 to 182±12 beats/min respectively (p<0.01). Subsequent DHE following pilocarpine further decreased the heart rate to 167±14 beats/min, showing no significant alteration (fig 2.). The bradycardia faded gradually and the heart rate remained slower than the control at 40 min. Physostigmine alone did not decrease heart rate significantly (p>0.05), nor did it affect the bradycardia following DHE significantly. The bradycardia following pilocarpine icv is secondary to the raised blood pressure, which is induced when pilocarpine agonizes the central M receptor, and therefore is reflex in nature (Henry and Rachel 1982).

Atropine (0.1 mg/kg, icv) made the heart rate decrease from 276±29 to 227±4 beats/min (p<0.05). Its addition after or before DHE showed no remarkable interaction between them. However, intravenous atropine (1 mg/kg) antagonized DHE's bradycardia almost completely. In view of the fact that the bradycardia of DHE was abated by atropine iv, but not icv, it may be inferred that atropine blocks the M receptors in the cardiac muscles and thus nullifies the elevated vagal tone over the heart, which is elicited by DHE through its stimulation of the μ or κ receptor in the cardiovascular center (Feldberg and Wei 1978; Xia 1985).

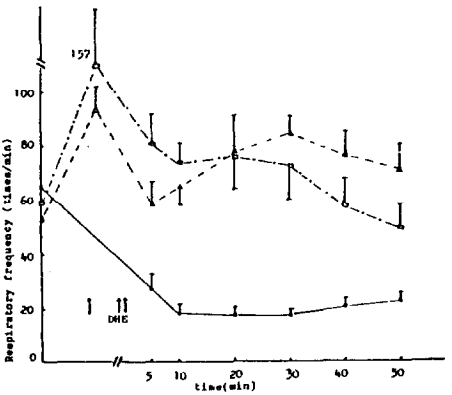

Figure 1. Antagonist effect of pilocarpine and physostigmine on respiratory inhibition by DHE in rabbits (n=6). ●---● DHE 0.5μg/kg,iv; △---△ pilocarpine 2.5 mg/kg,icv; □---□ physostigmine 0.3 mg/kg, iv. * pilocarpine or physostigmine

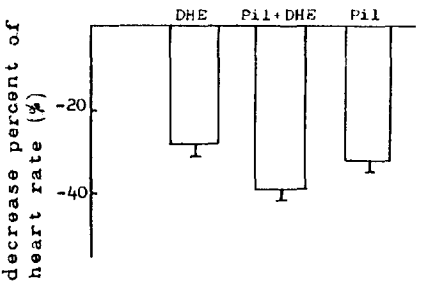

Figure 2. Effect of DHE and pilocarpine (pill on heart rate in rabbits (each group=6)

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THE ROLE OF μ- AND δ- OPIOID RECEPTORS ON THE INTESTINAL PROPULSION IN RATS

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ABSTRACT

The agonist effects on intestinal transit of relatively selective μ- and δ- ligands, administered intraperitoneally, and their antagonism by the preferentially μ- and δ- antagonists naloxone and ICI 174,864 were studied in rats 5 min after a charcoal meal. The dose-response curves of the preferential μ- ligands morphine and [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGO) were shifted by naloxone at low doses but not by ICI 174,864. The preferential δ-peptide [D-Pen^2, D-Pen^5] enkephalin (DPDPE) had no significant agonist activity. [D-Ala', D-Leu^5] enkephalin [DADLE] induced dose-related effects that were weakly antagonized by ICI 174,864 and partly by low-dose naloxone. Thus the inhibition of intestinal transit induced by opioids may depend mainly on the interaction of the agonists at the u-receptors, while the δ- receptors may play only a secondary role. DADLE agonist effects probably depend on interaction at μ-, δ- and at non-opioid receptors.

INTRODUCTION

Inhibition of intestinal transit in rats is a well-known opioid effect currently attributed to local and CNS-mediated mechanisms (Manara et al. in press). It has been reported that the CNS-mediated antipropulsive activity of opioids depends on their interaction at u-receptors or at μ- and δ- receptors when respectively supraspinal or only spinal mediations are involved (Porreca and Burks 1983; Galligan et al. 1984; Porreca et al. 1984).
To clarify the contributions of opioid receptor types in the local component of opioid-induced inhibition of intestinal transit, we showed that k-receptors probably play no major role (Gambino et al. 1983; Petrillo et al. 1984; Tavani et al. 1984; Petrillo et al. 1985). In this study, in order to determine the contribution of μ- and δ-receptors, we investigated the effects on intestinal propulsion of relatively selective μ- and δ- agonists and antagonists after intraperitoneal injection.

MATERIALS AND METHODS

Overnight fasted male CD-COBS rats (Charles River, Italy) 180-200 g, were tested for intestinal transit (Manara et al. in press). Five min after a charcoal meal (Tavani et al. 1980) rats were killed and the

distance travelled by the charcoal meal was recorded as a percentage of the total length of the small intestine (% intestinal transit). Drugs and peptides were dissolved in distilled water and injected intraperitoneally (i.p.). The following drugs and peptides were used: morphine HCl (Salars, Italy), naloxone HCl (gift from Endo Laboratories, USA), N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864)(gift from Dr. J.S. Shaw, ICI, U.K.), [D-Pen^2, D-Pen^5] enkephalin (peninsula, USA), [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGO) and [D-Ala^2, D-Leu^5] enkephalin (DADLE)(Bachem, Switzerland).

RESULTS AND DISCUSSION

The two relatively selective u-agonists morphine (Magnan et al. 1982) and DAGO (Handa et al. 1981) injected i.p. into rats at the peak time or a plateau of the effect (5 and 1 min before charcoal respectively, data not shown) induced dose-related inhibition of intestinal transit, fitting a straight regression line (Fig. 1). The doses reducing transit to 50% that of drug-free controls (ED_{50}) were about 15.2 and 6.2 µg/kg for morphine and DAGO respectively. Pretreatment with the preferential p-antagonist naloxone (Magnan et al. 1982)(50 µg/kg i.p. 6 min before charcoal) shifted the dose-response curves to the right and the ED_{50} were 239 µg/kg for morphine and 153 µg/kg for DAGO. The relatively δ-selective antagonist ICI 174,864 (Cotton et al. 1984; Corbett et al. 1984) at the dose of 1 mg/kg 6 min before charcoal, did not inhibit intestinal transit (110% of controls) and did not significantly shift the dose-response curves of the two agonists. This leads us to conclude that both morphine and DAGO i.p. inhibited intestinal transit in rats mainly through the u-type opioid receptor.

The relatively selective δ-agonist DPDPE (Mosberg et al. 1983, Corbett et al. 1984) at doses from 60 to 2000 µg/kg i.p. 5 min before charcoal did not reduce intestinal transit dose-dependently (Fig. 1). A very slight agonist effect was seen at a few doses but there was never any significant difference from controls. No stronger agonist effects were observed when DPDPE was injected 1 or 10 min before the meal (data not shown). Metabolic inactivation of the peptide in our conditions cannot be excluded, although it is unlikely as DPDPE is reported to be stable in the hamster vas deferens preparation (McKnight et al. 1985).

Conversely DADLE, which has been reported to act at δ-sites with cross reactivity at µ- sites (Magnan et al. 1982), injected i.p. 5 min before the meal, induced a dose-related inhibition of intestinal transit with an ED_{50} of about 23 µg/kg (Fig. 1). When 50 µg/kg of naloxone was injected 6 min before the meal, DADLE's effect was partly antagonized, but with no dose relation. A dose of 5 mg/kg of naloxone was necessary to antagonize the effect of 80 µg/kg of DADLE from 41% to 69% of control value. In the presence of 1 mg/kg of ICI 174,864 i.p. 6 min before charcoal the dose-response curve of DADLE showed a

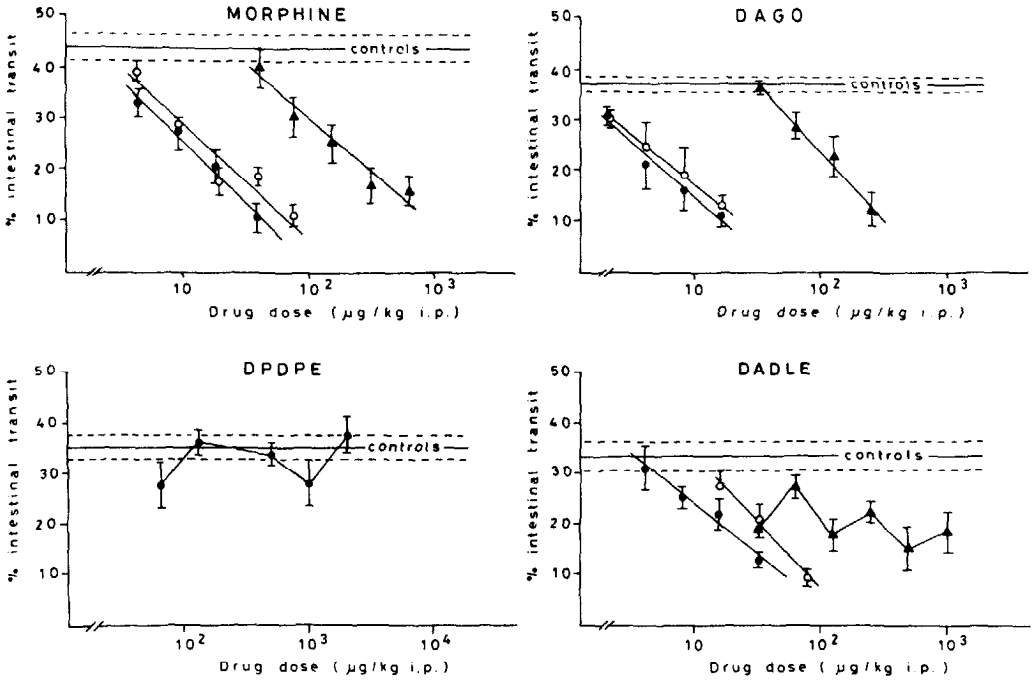

Fig. 1 - Dose-related inhibition of the intestinal transit induced by morphine, DAGO, DPDPE and DADLE. Overnight fasted rats were injected i.p. with water (●), naloxone (▲)(50 µg/kg) or ICI 174,864 (○)(1 mg/kg) 6 min before a charcoal meal, with morphine, DPDPE or DADLE 5 min before or with DAGO 1 min before the meal. Controls received water. % intestinal transit was measured 5 min after the meal. Mean and S.E. of 5-8 rats per group. All curves except naloxone + DADLE and DPDPE significantly fitted a straight line. Comparison of regression lines by analysis of variance showed that morphine vs ICI + morphine and DAGO vs ICI + DAGO were the same, whereas DADLE vs ICI + DADLE ($p<0.05$), morphine vs naloxone + morphine and DAGO vs naloxone + DAGO ($p<0.01$).

very slight but statistically significant shift to the right and the ED_{50} was 38.5 µg/kg. Because of the low solubility the highest dose of ICI 174,864 in our conditions was 4 mg/kg i.p. This dose itself had no antipropulsive effect and partly prevented the antipropulsive effects of 40 µg/kg i.p. of DADLE, reducing the inhibiting effects of the agonist from 41% to 64% of control value. The very weak antagonism of ICI 174,864 on DADLE-induced constipation and the slight tendency of some doses of DPDPE to inhibit intestinal transit suggest that the d-receptor probably plays a secondary role in the antipropulsive action of i.p. opioids. Thus DADLE's effect might also depend on its interaction at u-sites and probably at some other non-opioid sites not easily displaceable by opioid-antagonists.

In conclusion, the inhibition of intestinal transit in rats induced by i.p. opioids appears to depend mainly on the agonists' interactions at the p-receptor, while the δ-receptor seems to play only a secondary role. However more selective compounds are needed to determine the exact role of µ- and δ-receptors in locally elicited intestinal transit inhibition.

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INTRATHECAL DYNORPHIN A (1-13) AND (3-13) REDUCE SPINAL CORD BLOOD FLOW BY NON-OPIOID MECHANISMS

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ABSTRACT

Dynorphin A (Dyn A)-related peptides have been implicated in the pathophysiology of spinal cord injury in part because their intrathecal (i.t.) injection causes hindlimb paralysis. The effects of paralytic doses of i.t. Dyn A (1-13) and Dyn A (3-13) on spinal cord blood flow and cardiac output were examined in rats using radiolabeled microspheres. Both Dyn A (1-13) and Dyn A (3-13) significantly reduced blood flow in lumbosacral spinal cord without altering cardiac output. Pretreatment with naloxone failed to block these reductions in blood flow. Thus, the paralytic effects of Dyn A may result from non-opioid actions of Dyn A to reduce spinal cord perfusion.

INTRODUCTION

Endogenous opioids have been implicated in the pathophysiology of spinal cord injury based on evidence that the opioid antagonist naloxone improves spinal cord blood flow (Faden et al. 1981a; Young et al. 1981) and neurological function (Faden et al. 1981b) following experimental spinal trauma. Among the opioids found in spinal cord, dynorphin A (Dyn A)-related peptides have been specifically implicated as potential mediators in the pathogenesis of spinal cord injury for several reasons: 1) trauma-induced loss of motor function is reduced following treatment with the relatively κ selective opioid receptor antagonist WIN44,441-3 (Faden et al. 1986) suggesting involvement of an endogenous κ ligand such as Dyn A; 2) in contrast to other opioids, Dyn A immunoreactivity progressively increases at the injury site following trauma (Faden et al. 1985); and 3) Dyn A-related peptides are unique among opioids in producing a flaccid hindlimb paralysis following intrathecal (i.t.) injection in rats (Przewlocki et al. 1983; Faden and Jacobs 1984; Herman and Goldstein 1985; Long et al. 1985). Dyn A may promote injury mechanisms by altering spinal cord blood flow following trauma, and in turn, the therapeutic actions of naloxone may result from protection against Dyn A contributions to post-traumatic ischemia. Alternatively, the paralytic effects of i.t. Dyn A have been shown not to involve actions at opioid receptors (Faden and Jacobs 1984; Herman and Goldstein 1985; Long et al. 1985). Consequently, deleterious actions of Dyn A in injured spinal cord may result from the non-opioid actions of this peptide. Therefore, the present studies were conducted to determine 1) if i.t. Dyn A reduces spinal cord blood flow, and 2) whether Dyn A-induced blood flow alterations involve opioid or non-opioid mechanisms. Using radiolabeled microspheres, we examined the effects of Dyn A (1-13) and the non-opioid fragment Dyn A (3-13) on spinal cord blood flow. Additionally, we evaluated the ability of naloxone to block these actions of Dyn A (1-13).

MATERIALS AND METHODS

Male Sprague Dawley rats (300-350g) were anesthetized with ketamine hydrochloride (100 mg/kg, i.m.). Saline-filled polyethylene catheters (PE IO) were introduced into the spinal subarachnoid space through the atlanto-occipital membrane and advanced to the level of the lumbar enlargement (8.5 cm). On the following day, rats were again anesthetized with ketamine (100 mg/kg, i.m.). A PE 50 catheter was advanced through the carotid artery into the left ventricle of the heart for injection of microspheres, and a PE 50 catheter was inserted into the tail artery for mean arterial pressure recordings and reference blood sampling.

Approximately one hour following catheterization, a 0.5 ml microsphere suspension containing 150,000 to 250,000 microspheres labeled with either ^{141}Ce or ^{86}Sr was injected over a 15-20 second period, followed immediately by a 0.5 ml heparinized saline flush delivered over 15-20 seconds. Reference blood withdrawal was begun IO seconds prior to microsphere injection and was continued for a total of 75 seconds (withdrawal rate of 1.0 ml/min). Two microsphere injections (20 min preceding and 10 min following i.t. injections) provided cardiac output (CO) and blood flow data. lntrathecal injections of Dyn A (1-13) (25 nnmoles), Dyn A (3-13) (50 nmoles), or saline were made in a total volume of 18 µl (including catheter flush). Following the second microsphere injection, brains and cervical, thoracic, and lumbosacral portions of the spinal cord were removed and weighed, and the activity of each isotope was measured in a gamma counter. Cardiac output (ml min^{-1}) was calculated as total injected radioactivity times withdrawal rate divided by the radioactivity in the reference blood sample. Tissue blood flow (ml min^{-1} $100g^{-1}$) was calculated as tissue radioactivity times withdrawal rate divided by reference blood radioactivity.

Differences between post- and pre-treatment measurements in each rat were evaluated by analysis of variance. Significant differences among treatment group means were determined by the least significant difference test.

RESULTS AND DISCUSSION

Dyn A (1-13) significantly reduced blood flow to the lumbosacral spinal cord (49-66%) without altering cardiac output or blood flows to the brain or other spinal cord regions. These reductions in spinal cord perfusion were limited to those regions where direct exposure to the intrathecally injected Dyn A would be expected on the basis of the measured distribution of ^{125}I-BSA in the spinal cord following its i.t. injection (results not shown). Naloxone pretreatment 10 minutes prior to injection of Dyn A (1-13) failed to block the reductions in lumbosacral blood flow. Additionally, lumbosacral blood flow was significantly reduced by the non-opioid fragment Dyn A (3-13). Thus, the Dyn A effects on spinal cord perfusion appear to involve non-opioid mechanisms. Similarly, the dose-related hindlimb paralytic actions of Dyn A (1-13) are not blocked by naloxone, and are shared by Dyn A (3-13) (Przewlocki et al. 1983; Faden and Jacobs 1984; Herman and Goldstein 1985; Long et al. 1985). Consequently, the paralytic actions of Dyn A may result from non-opioid actions of Dyn A to reduce spinal cord perfusion. Assuming that intrathecally injected Dyn A (1-13) mimics the actions of endogenous Dyn A in injured spinal cord, it would appear likely that the therapeutic actions of the opioid antagonist naloxone in spinal cord injury (Faden et al. 1981a; Faden et al. 1981b; Young et al. 1981) do not result from protection against Dyn A-induced ischemia, since these effects appear to involve non-opioid mechanisms.

TABLE I. (A) Cardiac outputs' (CO) and (B) blood flows[2] in brain, cervical, thoracic, and lumbosacral spinal cord 20 min preceding (Pre) and 10 min following (Post) i.t injections of Dyn A (1-13) (25 nmoles), Dyn A (3-13) (50 nmoles), or saline. Rats treated with naloxone (10 mg/kg, s.c.) were injected 10 minutes prior to i.t. administration of either saline or Dyn A (1-13) (25 nmoles). Data are presented Ias means ± S.E.M. for groups of 8 rats. * $p < 0.05$, [1]ml min^{-1}, [2]ml min^{-1} $100g^{-1}$

	TREATMENT				
	SAL	Dyn (1-13)	Dyn(3-13)	Nal/Sal	Nal/Dyn
A. CARDIAC OUTPUT					
Pre	179±13	187±1.5	204±12	197±29	200±26
Post	176±30	149±11	160±17	203±25	193±26
B. BLOOD FLOW					
Brain					
Pre	211±35	245±30	280±35	208±25	287±25
Post	194±23	207±18	217±26	203±28	212±19
Cervical					
Pre	96±7	108±11	113± 11	107±15	118±11
Post	104±10	103±8	112±12	106±12	95±9
Thoracic					
Pre	76±4	96±11	95±7	82±8	92±5
Post	80±10	83±5	79±8	83±7	57±7*
Lumbosacral					
Pre	75±4	100±8	109±10	91±9	102±8
Post	82±10	51±12*	61±13*	100±14	34±11*

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ENDOGENOUS OPIOIDS, OPIATE RECEPTORS AND TRAUMATIC BRAIN INJURY

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ABSTRACT

The present study examined the role of endogenous opioid peptides in the pathophysiological sequelae of fluid percussion head injury in the cat. Two hours following injury, tissue concentrations of dynorphin-like immunoreactive material (ir-Dyn) were significantly elevated in specific brain regions where injury, as evidenced by histological examination, was most severe. Changes in ir-Dyn but not beta-endorphin-like immunoreactive material (ir-End) were significantly correlated with a fall in regional cerebral blood flow (CBF) that occurred 2 h following injury. Administration of the opiate antagonist WIN44,441-3 (with enhanced activity at kappa-receptors) stereospecifically increased cerebral blood flow to the injured regions.

INTRODUCTION

Endogenous opioids have been implicated as secondary pathophysiological factors in shock and trauma (Faden 1984a,b). Traumatic injury to the central nervous system (CNS) may cause functional deficits through the release of endogenous factors, including endogenous opioids. We have provided evidence that the kappa-receptor/dynorphin opioid system may play a pathophysiological role in traumatic spinal cord injury (Faden et al. 1983; 1985a,b). Although the non-selective opiate antagonist naloxone has been found to improve several physiological variables (mean arterial pressure, brain perfusion pressure) following fluid percussion head injury in the cat (Hayes *et al.* 1984), there is no information available regarding the role of specific opioid peptides in traumatic brain injury or of the effects of opiate antagonists on CBF. In the present study, we utilized a fluid percussion device to induce traumatic head injury in the anesthetized cat and measured regional concentrations of ir-Dyn and ir-End by radioimmunoassay at 2 h after injury. Beta-endorphin was chosen as a comparison neuropeptide because: (1) it is derived from a different prohormone system (Li 1981); (2) it has a different distribution in the brain from that of dynorphin (Khachaturian *et al.* 1985); and (3) it has been implicated in the pathophysiolagy of shock and trauma (Faden 1984a,b). Changes in the regional concentration of opioid peptides were compared with changes in regional CBF at 2 h following injury in order to gain insight into the possible involvement of endogenaus opioid systems in the pathophysiological sequelae of traumatic brain injury. We also examined the effectiveness of the opiate-receptor antaganist WIN44,441-3 with its dextrostereoisomer WIN44,441-2 (which is inactive at the opiate receptor) on local CBF after traumatic brain injury.

MATERIALS AND METHODS

Male and female cats (2.75 - 3.25 kg) were anesthetized with sodium pentobarbital (30 mg/kg). Animals were then intubated, paralyzed with pancuronium (0.6 mg/kg) and artificially ventilated with 70% N_2O/30% 02. A femoral cutdown was performed far placement of venous (drug administratian) and arterial (blood gas) catheters. A catheter was also inserted into the left atrium far administration of radiolabelled microspheres. A hallow injury screw was rigidly fixed to the skull aver an 8 mm craniectomy centered over the sagittal suture midway between lambda and bregma. Head injury was induced by infusing a rapid epidural saline bolus, produced by the fluid percussion device, through the central injury screw. At 2 h past-injury (3.2 - 3.6 atmospheres [atm]), one group of animals (n=6/group) were sacrificed, brains removed, dissected and assayed far regional concentrations of endogenous opioids using radioinmuno-assays as previously described (Ghazarossian et al. 1980; McIntosh et a?. 1985; Molineaux *et al.* 1982). Antisera for radioimnunoassay was generously supplied by Drs. A. Goldstein (dynorphin) and G. Mueller (beta-endorphin). A second group of animals (n=5/group) were assigned to treatment with an intravenous bolus of either WIN44,441-3 (-) (0.2 mg/kg) or its dextrostereoisomer WIN44:441-2 (+) (0.2 mg/kg) administered 1.5 min following fluid percussion injury (3.2 -3.6 atm). Sequential measurement of regional CBF using intra-atrial injections of radiolabelled microspheres as previously described (McIntosh *et al.* 1986) was performed 10 min prior to drug administration, 30 min, 1 h and 2 h following drug treatment. At 2 h following injury, animals were sacrificed for CBF measurements.

RESULTS AND DISCUSSION

Tissue concentration of ir-Dyn were significantly elevated in the caudate nucleus ($p < 0.05$), frontal cortex ($p < 0.05$) and medulla

TABLE 1

Endogenous opioid response 2 h after head injury.

	Dynorphin (fmol/mg protein)		*Beta-endorphin* (fmol/mg protein)	
	Baseline	Post-injury	Baseline	Past-injury
Anterior Pituitary	4917 ± 704	1043 ± 539**	6938 ± 947	3393 ± 1185
Frontal Cortex	10 ± 1	17 ± 2*	13 ± 6	24 ± 16
Caudate Nucleus	301 ± 20	521 ± 50*	61 ± 24	93 ± 23
Parietal Cortex	55 ± 12	68 ± 5	16 ± 6	30 ± 21
Midbrain/Thalamus	60 ± 26	64 ± 6	77 ± 4	33 ± 9*
Medulla	40 ± 10	69 ± 4*	9 ± 3	18 ± 14

** = $p < 0.05$; ** = $p < 0.01$*

($p < 0.05$) where injury, evidenced by histological examination, was most severe. Tissue concentrations of ir-End were not significantly altered following injury (table 1). A significant fall in whole brain and regional CBF to caudate nucleus, frontal cortex and pons ($p < 0.05$) was observed in WIN (+) animals at 2 h post-injury (table 2). The change in ir-Dyn but not ir-End was correlated with the fall in regional CBF that was observed. In addition, WIN (-) caused a significant increase in CBF to frontal cortex, caudate nucleus, midbrain/thalamus and medulla.

TABLE 2

The effects of WIN(-) an cerebral blood flaw (ml/100 g/min ± S.E.M.).

Region	Baseline	10 min Post-drug	30 min Post-drug	1 h Post-drug	2 h Post-drug
Frontal Cortex	60 ± 14	59 ± 15	82 ± 10	94 ± 18*	93 ± 12*
Caudate Nucleus	55 ± 15	54 ± 8	72 ± 15	77 ± 10	84 ± 10*
Parietal Cortex	34 ± 7	33 ± 1	41 ± 8	41 ± 6	46 ± 7
Midbrain/ Thalamus	41 ± 10	44 ± 11	52 ± 13	60 ± 13	65 ± 10*
Medulla	38 ± 8	47 ± 11	44 ± 10	46 ± 12	54 ± 8*
Whole Brain	46 ± 4	42 ± 4	49 ± 8	53 ± 9	56 ± 7

The effects of WIN(+) on cerebral blood flow (ml/100 g/min ± S.E.H.).

Region	Baseline	10 min Post-drug	30 min Post-drug	1 h Post-drug	2 h Post-drug
Frontal Cortex	59 ± 8	55 ± 15	54 ± 15	52 ± 12	43 ± 10*
Caudate Nucleus	69 ± 17	67 ± 18	64 ± 17	61 ± 14	49 ± 16*
Parietal Cortex	43 ± 18	39 ± 11	39 ± 8	36 ± 7	32 ± 7
Midbrain/ Thalamus	57 ± 16	54 ± 18	47 ± 10	49 ± 4	34 ± 9*
Medulla	46 ± 10	45 ± 14	40 ± 15	34 ± 8	33 ± 10
Whole Brain	54 ± 6	40 ± 13	44 ± 10	42 ± 9	37 ± 5*

** = $p < 0.05$ when ompared to baseline values.*

Our results suggest that tissue concentrations of dynorphin but not beta-endorphin are elevated in brain regions where the fall in CBF is most pronounced following injury. This fall in CBF observed at 2 h post-injury could be reversed by administration of the opiate antagonist WIN (-), which has enhanced activity at kappa-receptors (Michne et al. 1978), but not WIN (+) (table 2). The lack of effect shown by the dextro-stereoisomer in this model implies that the efficacy of WIN (-) is mediated through an action at opiate receptors. These results are consistent with the hypothesis that dynorphin, acting through specific opiate receptors, may contribute to the pathophysiology of secondary brain injury following head trauma. In addition, it appears that opiate antagonists with increased activity at kappa-receptor sites may be effective in the treatment of reduced cerebral blood flow often associated with acute head injury.

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TREATMENT OF EXPERIMENTAL STROKE WITH THE OPIATE ANTAGONIST WIN 44,441-3 EFFECTS ON NEUROLOGIC FUNCTION, INFARCT SIZE, AND SURVIVAL

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ABSTRACT

Cats were treated using a blinded protocol with either the dextrorotary or levorotary form of WIN 44,441-3 injected subcutaneously six hours after middle cerebral artery occlusion, followed by continuous subcutaneous infusion. The levorotary form acutely improved neurologic function in 90% of animals, whereas the dextrorotary form had no effect. Survival rate and infarct size were the same in both groups. These results further implicate the kappa receptor in the pathophysiology of cerebral ischemia.

INTRODUCTION

The results of our previous work indicate that endogenous opioid peptides may contribute to the pathophysiology of neurological deficits caused by cerebral ischemia. In humans, the opiate antagonist, naloxone, reversed neurological deficits secondary to cerebral ischemia, whereas morphine exacerbated them; in both situations, there was no change in vital signs or level of consciousness (Baskin and Hosobuchi 1981). The neurological deficits produced by unilateral carotid ligation in gerbils were reversed by the intraperitoneal administration of naloxone (Hosobuchi et al. 1982). In baboons, naloxone partially reversed and morphine exacerbated ischemic neurological deficits resulting from focal cerebral ischemia; the effect was not due to a change in systemic parameters (Baskin et al.1982, Baskin et al. 1984, Zabramski et al. 1984). In cats with focal ischemia, naloxone and naltrexone improved neurologic function and prolonged survival (Baskin et al. 1986); dynorphin 1-13 also prolonged survival, suggesting that the kappa receptor is involved in this phenomenon (Baskin et al. 1984). This study was designed to further elucidate the role of the kappa receptor in focal cerebral ischemia by using the relatively selective kappa antagonist, WIN 44,441-3 to treat cats with experimentally induced focal cerebral ischemia.

MATERIALS AND METHODS

20 adult male cats were randomly assigned in blinded fashion to one of two groups, and were treated with the dextrorotary or levorotary form of WIN 44,441-3. The cats were sedated with ketamine, and anesthesia was induced by a mixture of halothane, nitrous oxide, and oxygen, administered by mask. The trachea was then intubated, but the cat was allowed to breathe spontaneously. One million units of penicillin G was then administered intramuscularly. Transorbital occlusion of the right middle cerebral artery (MCA) was performed using the technique described in cats by O'Brien and Waltz (1973).

In all cases, the MCA was occluded proximal to the take-off of the lenticulostriate arteries.

An incision was made in the mid-lumbar region and a subcutaneous pocket was created for the later placement of an osmotic pump designed to deliver drug solution at a constant volume. The cat was allowed to awaken, and was examined 6 hours following MCA occlusion. Neurological function was assessed independently by two individuals who were unaware of the experimental protocol. Sensory and motor function, level of consciousness, and pupillary reaction were graded using an 18-point scale (Baskin et al. 1986).

Cats then received an intraperitoneal injection of 10 mg/kg of the dextrorotary or levorotary form of WIN 44,441-3. A repeat neurological assessment was performed 20 mintues later. The cat was then sedated and an ALZET osmotic pump was implanted sterilely in the subcutaneous pocket in the lumbar region to deliver either the dextrorotary *or* levorotary isomer at 0.5 mg/kg/Hr. The investigators were blinded as to which treatment was being administred, but the pumps were coded in such a way that each cat received the same drug in both the acute injection and the chronic infusion phases of the study.

Neurological assessments were performed daily for as long as the cats were alive, or until 7 days after occlusion, at which time all were sacrificed. One million units of penicillin G was administered intramuscularly daily along with subcutaneous injections of lactated Ringer's solution calculated to provide adequate daily fluid maintenance. Once a cat began to eat or drink, subcutaneous fluids *were* discontinued. If *a cat* was found dead, a craniectomy was performed, the brain removed, and a coronal section made at the level of the optic chiasm. At the end of 7 days, all surviving cats were sacrificed, the brains removed, and slices prepared. The slices were incubated in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 25 minutes according to the method of Han et al.(1983). A TTC solution has been used extensively to demonstrate the presence and extent of acute myocardial infarcts (Ramkisson 1966), and gives a vivid indication of cerebral infarction in the acute stage. The reaction product of TTC and viable tissue is a deep red fonnazan that stains normal gray matter, whereas normal white matter stains with less intensity. Infarcted tissue does not stain.

Color slides were then made of the stained brain slices. A neuropathologist who was unaware of the experimental protocol made tracings of the entire affected hemisphere and infarcted area from projected images of the slide, A digitizing pad was used to calculate the percentage of infarcted tissue relative to the entire hemisphere for both sections in each cat; this defined the infarct size.

The Wilcoxon signed rank analysis was performed to compare neurological scores obtained before the acute drug injection with those obtained 20 minutes after injection. A Kruskall-Wallis analysis was performed to compare neurological scores before and after injection between groups. A two-way analysis of variance was

used to compare infarct size in all groups of cats. Kaplan-Meier survival curves were constructed, and Gehan's statistic was used to compare survival rates among groups.

RESULTS

Following acute administration, the levorotary form of WIN 44,441-3 produced statistically significant improvement in motor function in all animals tested, whereas the dextrorotary form had no effect (Figure 1).

FIGURE 1

Median Motor Function in Cats Tested 6 Hrs. After Middle Cerebral Artery Occlusion

	(-) WIN 44,441-3	(+) WIN 44,441-3
BEFORE INJECTION	2	2
(25/75th Percentiles)	2/3	2/3
AFTER INJECTION	4	2
(25/75th Percentiles)	4/5	2/3

There was no effect of either drug on sensory function, level of consciousness, or pupillary reaction to light. There were no statistically significant differences in survival rates or size of infarction between the detrorotary or levorotary groups.

DISCUSSION

Evidence for the involvement of the kappa receptor in central nervous system dysfunction is increasing. Tang (1985) has shown that the kappa agonist U50,488 can improve survival and hyperactivity associated with global cerebral ischemia in the rat. Tortella et al. (1986) have demonstrated that U50,488 can increase our seizure threshold either secondary to electroshock or drugs with convulsant activities. Faden and Jacobs (1985) have shown that WIN 44,441-3 can improve motor function in unanesthestized rabbits with spinal cord ischemia secondary to temporary occlusion of the abdominal aorta.

In our previous work, we have shown that a variety of opioid compounds can influence either neurologic function and/or survival in experimental focal cerebral ischemia. Indeed, it is possible to see a dissociation of these effects: diprenorphine improved neurologic function without altering survival (Baskin et al. 1986), whereas dynorphin 1-13 improved survival without changing the neurologic deficit (Baskin et al.1984). The fact that such dissociation exists suggests that naloxone and other compounds that alter both survival and function may do so at either different receptor subtypes or perhaps in several different as yet unidentified ways.

We selected WIN 44,441-3 for these experiments because of our recent experience with dynorphin 1-13 and the dramatic improvement in survival associated with its use in focal cerebral ischemia (Baskin

et al. 1984). As there is much evidence that dynorphin 1-13 binds mainly at the kappa receptor, and that WIN 44,441-3 has significant antagonist properties at the kappa receptor (Michne et al. 1978), we chose this compound for testing.

Our results clearly show biologic significance of the effect of WIN 44.441-3 on neurologic function in cerebral ischemia, as only the levorotary form produced the change. The lack of an effect on survival may be due to an inadequate dose for this to occur, or because of a differentially selective effect on neurologic function alone. While WIN 44,441-3 does bind at the mu, delta, and kappa receptor subtypes, its relatively selective kappa antagonist activity (Michne et al.1978) provides additional supportive evidence for the role of the kappa receptor in cerebral ischemia. Further studv of the compound in focal cerebral ischemia including dose-response testing is in progress.

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CONVULSANT ACTION OF NALOXONE IN THE RAT AMYGDALA

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ABSTRACT

Increasing doses of naloxone hydrochloride (100-1000 nmol) were microinjected unilaterally into the rat amygdala and the behavioral, neuropathological and electrographic responses were studied. Microinjections of low doses of naloxone (100-250 nmol) produced staring, gustatory automatisms and wet shakes whereas higher doses additionaly resulted in motor limbic seizures and status epilepticus. The electroencephalogram showed a sequence of alterations characterised by high voltage fast activity, spiking, bursts of polyspiking, electrographic seizures and postictal depression which first appeared in the amygdala and rapidly spread to hippocampal and cortical areas. The neuropathological analysis of frontal forebrain sections by means of light microscopy revealed seizure-related brain damage in amygdala, olfactory cortex, thalamus, hippocampal formation, substantia nigra and neocortex. Diazepam, 10 mg/kg i.p., when given prior to the microinjection of naloxone into the amygdala, abolished the epileptogenic and neurotoxic effects of the drug. The results suggest that naloxone, when microinjected into rat amygdala elicits electrographic and motor limbic seizures followed by seizure-related brain damage.

INTRODUCTION

There is a fairly large body of evidence to suggest that derangements in the function of endogenous opioid mechanisms in the brain may play a role in experimental, and human epilepsy. Opiates and opioid peptides can modulate the threshold for experimentally induced seizures in a way which depends upon the art of convulsions, the route of drug application and the species used (Frenk 1983). Intracerebroventricular application of high doses of morphine produce electrographic and behavioral manifestation of limbic seizure activity (Frenk 1983). Interestingly, the opiate antagonist naloxone, when given systemically in high doses causes generalised clonic convulsions which resemble stage 5 kindled seizures and result in prominent increase of 2-deoxyglucose metabolism in several limbic forebrain structures (Chugani et al. 1984). The amygdaloid complex has been shown to play a pivotal role in activating epileptic limbic circuits (Watson et al. 1983). Recently we reported that intraamygdaloid microinjection of morphine results in motor limbic seizures and brain damage (Turski et al. 1983a). The present study provides an electroencephalographic, behavioral and morphological analysis of the effects produced by application of the opiate antagonist naloxone into the amygdaloid complex of the rat.

MATERIALS AND METHODS

For intraamygdaloid injections adult male Wistar rats weighing 200-220 g were used. Naloxone hydrochloride was supplied by Endo Labs Inc (Garden City, N.Y., USA) was dissolved in saline and administered at doses of 100-1000 nmol into the amygdala. Diazepam (Polfa, Kutno, Poland) was suspended in a 3% solution of Tween 81 (Loba, Vienna, Austria) and given i.p. in the dose of 10 mg/kg 15 min prior to intraamygdaloid injection of naloxone, 1000 nmol. The methods for stereotactic implantation, the electroencephalographic registration and the morphological examination of the brains are described elsewhere (Turski et al. 1985).

RESULTS

Microinjection of low doses of naloxone hydrochloride, 100 nmol (n=4), into the amygdala presented no abnormal behavior. Higher doses, 250-1000 nmol, produced hyperactivity, gustatory automatisms and occasionally scratching or myoclonic movements of fore- or hindlimbs and wet shakes. This pattern of behavior persisted up to 30-40 min in animals

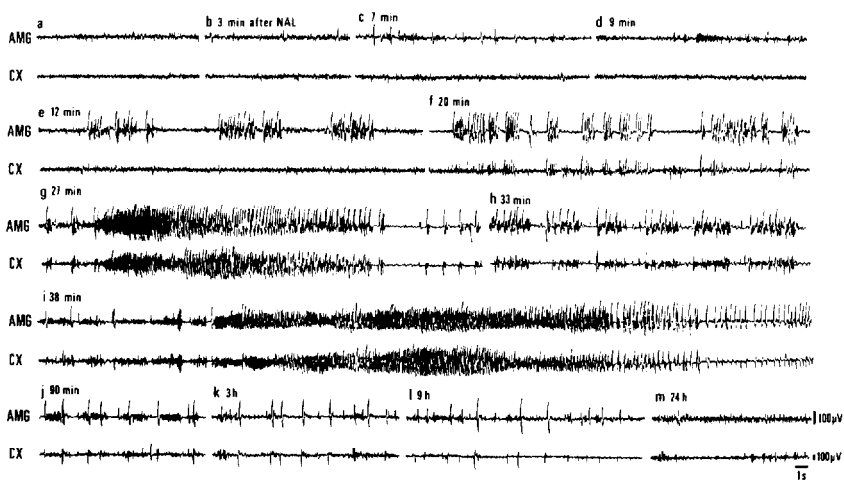

FIGURE 1. The evolution of electrographic activity elicited by unilateral injection of naloxone hydrochloride (NAL), 500 nmol, into the rat amygdala. (a) Pre-drug control recordings. (b-f) High voltage fast activity, spiking and bursts of polyspiking represent electrographic patterns registered in the amygdala 3-20 min after the injection of NAL. (g-i) Electrographic seizures dominate the EEG patterns within 20-90 min after NAL. (j-l) Progressive normalisation of electrographic activity is observed 2-12 h after the injection of NAL. (m) The EEG returns to the pre-drug pattern by 12-24 h post-injection. AMG, amygdala; CX, cortex.

receiving 250 nmol (n=6) of naloxone. With higher doses, 500 and 1000 nmol, the behavioral alterations increased and built up into seizures and status epilepticus. The ED_{50} (effective dose) of naloxone for generation of motor limbic seizures is 359.5 nmol (259.3-498.4)(n=24). Motor limbic seizures commenced after a mean delay of 17.2 ± 2.2 min following 1000 nmol (n=6) and 43.0 ± 12.5 min (n=5) following 500 nmol of naloxone, recurred every 3-10 min and lasted up to 2-3 h following the intraamygdaloid injection of the drug. After the intraamygdaloid injection of 500-1000 nmol of naloxone the electrographic background activity in the amygdala was replaced with high voltage fast activity which progressed in high voltage spiking and bursts of polyspiking (Fig. Ic-e), and developed into electrographic seizures. Ictal periods lasted for 1-3 min, recurred every 3-5 min and were followed by periods of depression of the electrographic activity (Fig. 1g,i). Within 24-48 h the electroencephalographic activity slowly returned to the pre-drug pattern (Fig. 1m). Examination of frontal forebrain sections with light microscopy 2-15 days after microinjection of naloxone, 500 and 1000 nmol, revealed widespread damage in amygdala, hippocampus, thalamus, olfactory cortex, substantia nigra and neocortex. The damage to the amygdala was most severe in the region immediately surrounding the injection cannula tip and entended into adjacent regions i.e. pyriform cortex, entorhinal cortex and claustrum. With short-lasting survival times (48-72 h) the damage consisted of prominent edema of the tissue as well as darkened and shrunken neurons. At longer survival time (10-15 days) the morphological structure of the amygdala undervent severe breakdown with loss of most neurons. The damage in the thalamus was limited to dorsomedial, reuniens, rhomboideus and paraventricular nuclei. Neuronal destruction in the hippocampus affected CA1 subfield of the intermediate and rostral hippocampus. The CA3 and CA4 subfields were affected considerably less. The damage in the substantia nigra extended into most caudal portions of the pars reticulata, whereas pars compacta was affected only very rarely. The most injured parts of the neocortex included areas located dorsally to the rhinal sulcus and cingulate cortex. Pretreatment with diazepam, 10 mg/kg, completely blocked the behavioral, electrographic and morphological alterations produced by intraamygdaloid injection of naloxone, 1000 nmol (n=5).

DISCUSSION

Our results demonstrate potent epileptogenic and brain damaging properties of naloxone when injected into the amygdala of rats. High doses of naloxone have been reported to cause generalized clonic convulsions when given systemically (Chugani et al. 1984, Dingledine et al. 1978) whereas lower doses block epileptogenic properties of opiates (Frenk 1983). Two possible explanations for the convulsant action of naloxone in the amygdala exist. The first one implies that naloxone blocks an endogenous anticonvulsant system which is mediated by endogenous opioids (Frenk 1983). In fact, anticonvulsant action of morphine and enkephalins has been reported in animal models of reflex epilepsy and against pentylenetetrazol- and fluorothyl-induced seizures (Frenk 1983; Frey 1986). The second proposal explaining convulsant action of naloxone implies that the drug behaves as a γ-aminobutyrate (GABA)-antagonist. It is known that the opiate antagonist displaces GABA from its binding sites in rat cerebral membranes and facilitates bicuculline induced seizures (Dingledine et al. 1978, Sagratella and

Massoti 1982). Since antagonism at the GABA-receptor could also be demonstrated for morphine, it seems tempting to speculate that both, the opiate as well as the opiate antagonist might act in the amygdala at the same site to initiate generalised limbic seizures, namely the GABA receptor. Studies with intraamygdaloid injections of the GABA antagonists bicuculline and picrotoxin demonstrate that both drugs induce motor limbic seizures and brain damage closely resembling those seen after morphine or naloxone (Turski et al. 1983a; 1985). Diazepam, a compound which enhances GABA-mediated synaptic inhibition in the brain (Haefely et al.1981) abolished the epileptogenic and brain damaging potential of naloxone. This finding however is not indicative of a specific GABA antagonistic action of naloxone, since the benzodiazepine also protects against convulsions produced by systemic or intraamygdaloid injections of kainic acid and cholinomimetics (Ben-Ari et al. 1980; Olney et al. 1983; Turski et al. 1983b).

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DYNORPHIN A (1-13): IN VIVO OPIOID ANTAGONIST ACTIONS AND NON-OPIOID ANTICONVULSANT EFFECTS IN THE RAT FLUROTHYL TEST

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ABSTRACT

Dynorphin A (1-13) acutely elevated the seizure threshold (ST) to the convulsant flurothyl, and this action was not blocked by naloxone. Increases in ST were also observed following i.c.v. injections of the non-opioid fragment dynorphin A (3-13). Pretreatment with dynorphin A (1-13), but not dynorphin A (3-13), non-competitively blocked the anticonvulsant effect of the μ selective opioid DAGO. Furthermore, pretreatment with dynorphin A (1-13) antagonized the δ antagonist properties of naloxone or ICI 154,129 in this seizure model. Thus, in addition to its non-opioid anticonvulsant effects, dynorphin A (1-13) exhibits unique antagonist actions which appear to be specific for the active opioid fragment.

INTRODUCTION

Dynorphin A (1-13). the endogenous ligand for κ opioid binding sites, exhibits κ activity in receptor binding assays, in vitro bioassays, and in vivo analgesia systems. It has also been shown that dynorphin A (1-13) demonstrates opioid antagonist effects in opiate naive and tolerant rats (Lee and Smith 1984). Hence, under the appropriate conditions, dynorphin A (1-13) appears to possess mixed agonist/antagonist properties.

Recently, using the flurothyl seizure test as an in vivo model to differentiate multiple opioid receptor systems (Cowan et al. 1979; Tortella et al. 1985), we demonstrated that β-FNA, another κ agonist with μ antagonist properties (Ward et al. 1982), attenuates the δ antagonist properties of naloxone or ICI 154,129. Therefore, considering the possible similarities between β-FNA and dynorphin A as opioids possessing mixed agonist and antagonist properties, the present studies were aimed at establishing the pharmacological profile of dynorphin A (1-13) in this in vivo model. For these studies, we used the μ selective agonist DAGO (Handa et al. 1981) and the mixed μ-δ receptor agonist DADL (D-Ala^2-D-Leu^5-enkephalin) (Tortella et al. 1985), peptide ligands possessing potent μ (DAGO; Robles et al. 1986) and δ (DADL; Tortella et al. 1985) receptor-mediated anticonvusant profiles in the rat flurothyl test.

MATERIALS AND METHODS

Male Sprague-Dawley rats (225-300 g) obtained from Zivic Miller laboratories (Allison Park, PA) were used. Three to five days prior to testing, rats were anesthetized with ketamine HCL (100 mg/kg, i.p.) and stereotaxically prepared with i.c.v. cannulae aimed at the right lateral ventricle. Three to five days following surgery, the rats (n=8-10 per group) were randomly assigned to treatment groups for testing. The effects of dynorphin A (1-13) (3.9-15.6 nmol, i.c.v.) or dynorphin A (3-13) (15.6 nmol, i.c.v) on flurothyl ST were determined at various times postinjection. Additional rats were

pretreated with naloxone (1 or 10 mg/kg, s.c.) 10 min prior to an i.c.v. challenge of dynorphin A (1-13) (15.6 nmol) and exposed to flurothyl 15 min later. In separate experiments, the μ antagonist actions of dynorphin A (1-13 or 3-13) (15.6 nmol; -2hr pretreatment) were determined in DAGO (0.275-4.4 nmol, i.c.v.) treated animals. Additionally, antagonist-interaction studies were conducted in naive rats pretreated at -2 hr with dynorphin A (1-13) (15.6, nmol), subsequently injected with ICI 154,129 (50 nmol, i.c.v.) or naloxone (290 nmol, i.c.v) 10 min prior to receiving an i.c.v. injection of DADL (35 nmol) and tested 20 min later in the rat flurothyl test.

To determine flurothyl ST's, rats were exposed to the volatile convulsant flurothyl and tested as previously described (Tortella et al. 1985). All i.c.v injections were given in a maximum volume of 7.5 μl (including a 2.5 μl flush). All the control groups received i.c.v. injections of saline at the appropriate treatment times.

RESULTS AND DISCUSSION

Mean ST's for all the control groups ranged from 340 to 370 seconds. The administration of dynorphin A (1-13) (fig. 1A) caused a significant dose- and time-dependent increase in ST. At the highest dose tested (15.6 nmol), ST's were increased 140 + 11 % of control. Peak effect occurred 15-30 min postinjection, with a duration of action of 60 min. The anticonvulsant action of dynorphin A (1-13) was not antagonized by naloxone (fig. 16). Moreover, dynorphin A (3-13) (15.6 nmol) was as effective as dynorphin A (1-13) as an anticonvulsant in this seizure model (data not shown). Collectively, these data clearly indicate that the actions of dynorphin A to increase the ST to flurothyl do not require the active opioid fragment, suggesting that a non-opioid mechanism of action is responsible for dynorphin's anticonvulsant effect in rats.

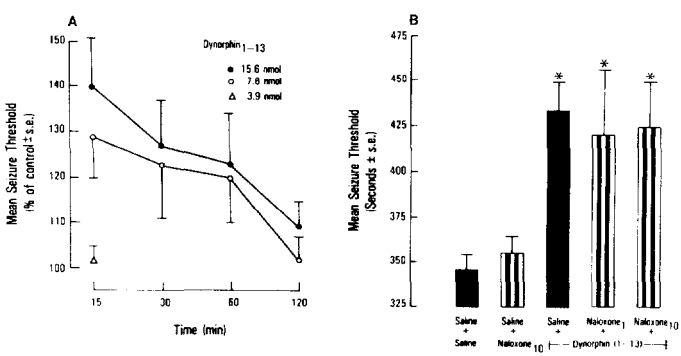

FIGURE 1. A. Dose- and time-dependent anticonvulsant effect of dynorphin A (1-13). B. Failure of naloxone to antagonize dynorphin A (1-13). *p<0.05, compared to saline control (Student's test).

The μ antagonist actions of dynorphin A (1-13) are seen in table 1. In rats

pretreated with dynorphin A (1-73)(15.6 nmol), but not (3-13)(15.6 nmol), the ability of DAGO to increase the threshold to flurothyl seizures was completely antagonized. Although not shown, dynorphin pretreatment shifted the DAGO anticonvulsant dose-response curve to the right and decreased its maximal effect, indicating a non-competitive antagonism. The results of these experiments demonstrate a μ selective antagonist action of dynorphin A which, unlike the non-opioid actions of dynorphin A, is dependent upon the intact opioid fragment.

TABLE 1. Dynorphin A (1-13) antagonism of the anticonvulsant effects of DAGO.

Antagonist	Dose Range of DAGO (nmol, i.c.v.)	Maximal increase in ST (% of Control)
Saline	0.275-2.2	164 ± 11
Dyn A (1-13)	0.275-4.4	115 ± 5
Dyn A (3-13)	0.275-2.2	158 ±12

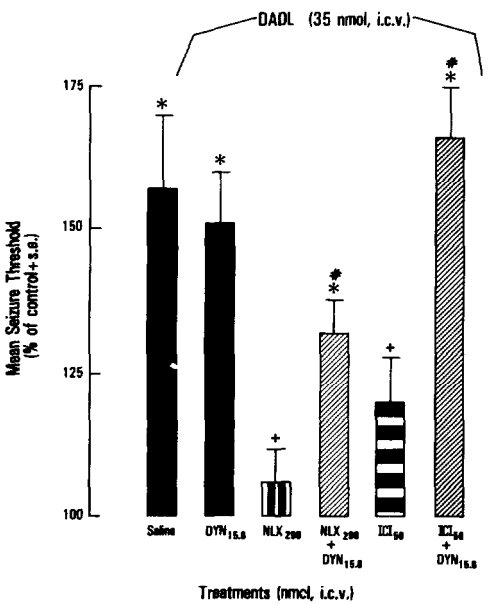

FIGURE 2. Dynorphin A (1-13) blockade of the 6 antagonist actions of naloxone or ICI 154,129 on DADL-induced increases in seizure threshold. All rats were injected with DADL in combination with Qther substances as the indicated dosed (substcripts). *p<0.05, compared to control, $^{+}$p<0.05, compared to the 35 nmol DADL group (Dunnett's test). $^{\#}$p<0.05, compared to ICI_{50} + DADL and NLX_{290} + DADL groups (Student's test).

The effect of dynorphin A (1-13) to diminish the ability of either naloxone or ICI 154,129 to antagonize the anticonvulsant actions of DADL (Tortella et al. 1985) is shown in figure 2. In the absence of dynorphin A (1-13), pretreatment with i.c.v. naloxone (290 nmol) or ICI 154,129 (50 nmol) antagonized the anticonvulsant effect of DADL (157±13 %) to 106±6 % and 120±8 % of control, respectively. However, in the presence of dynorphin A (1-13), which alone had no effect on DADL anticonvulsant activity (151±9 %), the DADL-induced increase in ST was only

partially antagonized by naloxone (132±6 %) and no longer antagonized by ICI 154,129 (166±9 %). Since the doses of naloxone and ICI 154,129 used here have been previously shown to selectively antagonize δ opioid receptor activity in this seizure model (Tortella et al. 1984; Tortella et al. 1985), we conclude that under the appropriate conditions of pretreatment time and dose, dynorphin A (1-13) is capable of antagonizing the selective δ antagonist actions of naloxone and ICI 154,129 in the rat flurothyl test.

In addition to the κ agonist properites of dynorphin A (1-13), the in vivo pattern of results described in the present report are suggestive of three different actions for this opioid ligand: 1) an anticonvulsant action which is clearly non-opioid, 2) μ antagonist activity and 3) the ability to antagonize the actions of δ antagonists (ICI 154,129 or naloxone), but not the δ agonist properties of DADL. Interestingly, a remarkably similar pattern of results has been observed with the κ agonist/irreversible μ antagonist β-FNA (D'Amato and Holaday 1984; Tortella et al. 1985). Given the noncompetitive nature of the μ antagonist properties of dynorphin A reported here, it seems possible that irreversible μ receptor blockade may be a prerequisite for the types of antagonist interactions observed in the present report. In this regard, dynorphin A and β-FNA may share a common mechanism of action at an opioid receptor complex.

Obviously, several mechanisms can be proposed to explain the interactions observed between dynorphin A and β-FNA and the δ antagonists. These include actions upon second messenger systems, altered metabolism, or simple pharmacological interactions of the various ligands at individual μ and δ receptors (Holaday et al. 1986). Alternatively, since several investigators have performed in vivo, in vitro and binding studies indicating that μ and δ receptor sites are isverconvertible (Bowen et al. 1981), and may interact through non-competative molecular mechanisms (see Holaday et al. 1986), we and others have postulated that a functional coupling (possibly via allosterism) of opioid binding sites on a common receptor macromolecule may account for these interactions.

Regardless of the mechanisms involved, this consistent pattern of interactions observed with dynorphin A in the present report, and elsewhere with β-FNA (see above), may have important significance relevant to the concept of multiple opioid receptors. At least in the case of dynorphin A, an endogenous neuropeptide ligand, the results reported in this study strongly support the hypothesis proposed by Lee and Smith (1984) suggesting that dynorphin may function in the CNS primarily as a modulator of opioid receptor systems.

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MU- AND DELTA-OPIOID MODULATION OF ELECTRICALLY-INDUCED EPILEPTIC SEIZURES IN MICE

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ABSTRACT

Graded seizure responses to suprathreshold cerebral electroshock in mice were modified by drugs acting at mu- and at delta-opioid receptors. Morphine exerts a proconvulsant effect at a non-mu opioid receptor and may exert a simultaneous anticonvulsant effect at a mu-opioid receptor. Delta-opioid receptor blockade increases seizure severity, suggesting a predominantly anticonvulsant nature of the delta-opioid system in the seizure model tested here.

INTRODUCTION

Dua et al. (1985) demonstrated that blockade of central delta-opioid receptors can attenuate electroencephalographic seizures initiated by mu-opioid receptor stimulation in rats. In contrast, Tortella and Long (1985) had earlier reported on a putative endogenous delta-opioid agonist which can diminish flurothyl-provoked seizures. We have therefore examined for this study the effects of mu- and delta-opioid receptor agonists and antagonists on the severity of electroshock seizure (ECS) activity in mice, to determine whether modulation of seizure activity might be a general property of such opioid agents.

MATERIALS AND METHODS

Electrical stimulation and seizure response measurement. ECS was produced in Swiss-Webster albino mice by passing 60-Hz sinusoidal constant current (generator output impedance > 50 k ohms) for 200 msecs via electrode jelly-coated pinnae through spring-loaded metal clips. We determined from pilot experiments in untreated mice that a stimulus strength of 5.6 mA root mean square could produce full seizure activity while even slightly higher currents often produced immediate death. With 5.6 mA strength all untreated mice displayed a gradeable continuum of motorobehavioral responses which permitted

us to guantitate seizure response severity in treated and untreated animals on a scale of from 0 to 5, as follows: 0 = no observable seizure activity: 1 = mouse stunned, immobile, may subsequently run, Straub tail; 2 = brief (<2 sec) clonic seizure, stunned, runs and becomes hyperactive; 3 = sustained (>2 sec) clonic seizure, stunned: 4 = brief tonic seizure, stunned, running, all often accompanied by brief clonic activity; 5 = sustained tonic-clonic seizure, stunned, running, followed by postictal depression and, occasionally, death. The seizure severity grade for each mouse was multiplied by total response duration (from initial application of current until cessation of seizure activity) to obtain a Seizure Index (SI) score. Animals which died during seizure were assigned the mean of the two highest SI scores for surviving animals in the same treatment group. Effects of drugs were examined on mean SI scores for each group.

Drugs and administration. Mice were pretreated with morphine sulfate (MOR; BDH, at 0, 8, 32 or 128 mg/kg intraperitoneally (ip) administered 20 min prior to electroshock), naloxone hydrochloride (NIX; Endo, at 0, 0.16, 0.64, 1.0 or 2.56 mg/kg ip administered 10 min prior to electroshock) and ICI 154,129 (I/l; at 0, 6 or 24 mg/kg ip administered 30 min prior to electroshock). All drugs were dissolved in saline and injected in volumes of 10.0 ml/kg ip.

Statistical procedures. Differences between treatment groups were evaluated by analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Linear regression was done by the method of least squares.

RESULTS

Reproducibility and reliability of SI measurement. There were no statistical differences between SI values obtained by two different observers who evaluated SI in different groups of saline-treated (control) mice in separate experiments. Statistical inferences concerning the effects of MOR 8 mg/kg on SI were identical between the two experimenters and identical for tests done at different times in separate experiments.

The effects of pretreatment with opioid drugs on SI. These results are summarized in Table 1. NLX 1.0 mg/kg ip by itself did not alter SI but the combination of NLX with MOR, in a fixed molecular NLX:MOR ratio of approximately 1:50, produced significantly greater SI scores than did MOR alone at 32 and 128 mg/kg. The NLX/MOR combination with MOR at 8 mg/kg produced SI scores numerically greater than those with MOR alone at

8 mg/kg. The chosen NLX:MOR ratio of 1:50 was arrived at mainly from in vitro opioid receptor kinetics data (Kosterlitz and Watt 1968; Lord et al. 1977) and was aimed at maintaining essentially complete blockade of mu-opioid receptors at all doses of MOR used in combination with NLX. Differences between SI scores with MOR alone and with the given combinations of MOR and NLX were plotted against the dose of MOR over the range from 0 to 128 mg/kg. The data resulted in a straight line of best fit (forced through origin) with slope = 2.5, r = 0.65 and $p<0.001$, to indicate the slope as being significantly different from zero. Administration of I/1 6 mg/kg by itself provoked Severity Index scores significantly greater than those from untreated mice. SI in mice treated with a combination of I/1 and MOR was always numerically greater, by at least 117 SI points, than with MOR by itself at corresponding doses. Differences for these comparisons were statistically significant with MOR at 8 and at 128 mg/kg.

TABLE 1

Effects of Pretreatment with Opioid Drugs on Seizure Index

	PRETREATMENT			SEIZURE INDEX
GRP	MOR	NLX	I/1	mean ± S.E.
A	0	0	0	99 ± 26
B	0	1.0	0	129 ± 30
C	0	0	6	338 ± 8
D	8	0	0	219 ± 11
E	8	0.16	0	305 ± 57
F	8	0	6	405 ± 49
G	32	0	0	128 ± 16
H	32	0.64	0	508 ± 30
J	32	0	6	245 ± 49
K	128	0	0	85 ± 19
L	128	2.56	0	330 ± 55
M	128	0	24	312 ± 83

For all groups n=6, except A and D where n=12. Mean SI for Grp A is significantly different ($p<0.05$; ANOVA) from C, D, E,F, H, J, L. M; B from C, E, F, H, L, M; C from A, B, D, G, H; D from A, C, F, H, K; E from A, B, H, K; F from A, B, D, K; G from C, E, F, H, L, M; H from all except F; J from A, F, H, K; K from C, D, E, F, H, J, L, M; L from A, B, G, H, K; M from A, B, G, H, K.

DISCUSSION

The approximated linear relationship between dose of morphine and seizure severity in the presence of a fixed mu-opioid receptor-blocking NLX:MOR ratio supports the possibility that morphine exerts a proconvulsant action at a non-mu opioid receptor. The significant proconvulsant effect of morphine, which with doses greater than 8 mg/kg was unmasked only in the presence of naloxone, suggests the existence of a mu-receptor related anticonvulsant mechanism. This mechanism becomes evident only after blockade of mu-opioid receptors and not with morphine administration alone. Our results suggest also the existence of an anticonvulsant delta-opioid receptor which is tonically activated by endogenous delta-opioid ligand(s), a concept supported here by the significantly proconvulsant effect of I/1, a relatively specific delta-opioid antagonist (Gormley et al. 1982), by itself at 6 mg/kg. This is consistent with the work of Tortella et al. (1984) and Tortella and Long (1985) who maintain that delta-opioid agonism can be anticonvulsant. Thus, depending upon the particular seizure model being tested (Dua et al. 1985), the central delta-opioid receptor system may support both anticonvulsant and proconvulsant mechanisms.

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THE THRESHOLD FOR LIMBIC SEIZURES IN RATS IS DECREASED BY INTRANIGRAL MORPHINE

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ABSTRACT

Microinjection of morphine hydrochloride into the substantia nigra pars reticulata, bilaterally, converts non-convulsant dose of pilocarpine hydrochloride, 100 mg/kg, into a convulsant one. The ED_{50} of morphine for the generation of seizures after pilocarpine, 100 mg/kg, is 3.8 nmol (2.5-5.8). Electrographic and behavioral monitoring both show a pattern of convulsant activity similar to those produced by pilocarpine in doses exceeding 350 mg/kg. Morphological analysis of frontal forebrain sections reveals epilepsy-related damage to the hippocampus, thalamus, olfactory cortex, substantia nigra, neocortex and amygdala. The proconvulsant action of morphine in the substantia nigra is reversed by co-administration of naloxone hydrochloride. The results show that the threshold for limbic seizures may be modulated by opiates in the substantia nigra.

INTRODUCTION

The basal ganglia are concerned with the propagation of motor seizures and in the generation of seizure activity in the limbic system. Clinical observations in man and lesion studies in primates established that substantia nigra governs the spread of seizures elicited from the motor cortex (Hayashi 1952). The substantia nigra undergoes irreversible degeneration in rodents subjected to status epilepticus (Turski et al. 1986). γ-Aminobutyrate (GABA), substance P and dynorphin are tentatively suggested to subserve neurotransmitter role in the striatonigral pathway (Fallon and Loughlin 1985). An excitatory amino acid, L-glutamate and/or L-aspartate, are the most likely transmitter candidates of corticonigral pathway (Fallon and Loughlin 1985). Manipulating GABAergic functions in the substantia nigra (SN) modulates the threshold for limbic seizures (Turski et al. 1986). Blockade of excitatory amino acid- or substance P-mediated excitation in the SN raises the threshold for seizures (Gale 1985). It remains uncertain whether opiates in the SN modify the threshold for limbic seizures. Morphine (MF) potentiates the convulsant action of systemically administered kainic acid and pilocarpine and facilitates the rate of development of amygdala kindling (Fuller and Olney 1979; Turski et al. 1985; Stone et al. 1982). The morphological substrate of this action is largely unknown, although preliminary observations indicate that the concentration of opioid peptides changes in the SN in the course of amygdala kindling (Iadarola et al. 1986). The SN is essential for the motor regulatory function of opiates. There is electromyographic evidence that MF elicits increase in the muscle tone when microinjected into the SN pars reticulata in rats (Turski et al. 1983). Application of opiate receptor antagonist naloxone into the SN blocks the muscle rigidity produced by systemic morphine (Turski et al. 1982). These experiments furnish corroborative evidence for the role of nigral opiates in the

control of spinal motor functions. The purpose of this study is to investigate the function of opiates in the SN in the motor expression of pilocarpine-induced seizures. Seizures produced by pilocarpine in rats provide a useful animal model of temporal lobe epilepsy which permits studying mechanisms of initiation of and pathways operative in the propagation of convulsive activity in the brain (Turski et al. 1986).

MATERIALS AND METHODS

Male Wistar rats, 230-250 g in weight, were subject to stereotaxic surgery for the implantation of stainless-steel guide cannulae directed towards the SN (AP 1.61; L $\pm$ 1.9; V -2.6)(Turski et al. 1986). After a 5-day recovery period microinjections into the SN were performed bilaterally in unanesthetised rats. The drugs were delivered in a volume of 0.5 µl (0.1 µl/min). Morphine hydrochloride (MF)(Polfa) was administered into the SN in doses of 1, 2, 5 and 10 nmol, while naloxone hydrochloride (NAL)(Endo) was microinjected into the SN in the dose of 10 nmol, 15 min prior to i.p. injection of pilocarpine hydrochloride (Sigma), 100 mg/kg. Methylscopolamine nitrate (Sigma), 1 mg/kg, was administered s.c. 30 min prior to the injection of pilocarpine. MF, NAL, pilocarpine and methylscopolamine were dissolved in saline. In all experiments the animals were used once only. For depth recordings, bipolar twisted electrodes were positioned in the dorsal hippocampus. Surface recordings were led from screws positioned bilaterally over the occipital cortex. The brains were processed for morphological examination by light microscopy 1-3 and 5-15 days after the administration of pilocarpine. The methods for histological processing of the tissue were similar to those described elsewhere (Turski et al. 1986).

RESULTS

Microinjection of MF into the SN pars reticulata, bilaterally, increased the severity of behavioral and EEG alterations produced by pilocarpine, 100 mg/kg, in a dose-dependent manner. None of four rats injected with MF, 1 nmol, presented motor limbic seizures or status epilepticus after pilocarpine, 100 mg/kg. Two of seven rats injected with 2 nmol of MF developed motor limbic seizures, while microinjection of 5 nmol resulted in seizures and status epilepticus in six out of eight rats. Seven out of eight animals receiving MF, 10 nmol, dis-

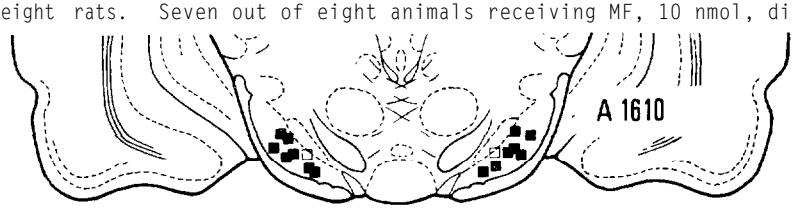

FIGURE 1. Schematic reconstruction of the injection sites of morphine (MF) in the substantia nigra of the rat. MF, 10 nmol was microinjected bilaterally into the substantia nigra 15 min prior to the i.p. administration of pilocarpine, 100 mg/kg. Dark squares represent sites from which microinjections of MF convert non-convulsant dose of pilocarpine, 100 mg/kg, into a convulsant one. Open squares represent sites from which microinjections of MF do not affect the action of pilocarpine, 100 mg/kg.

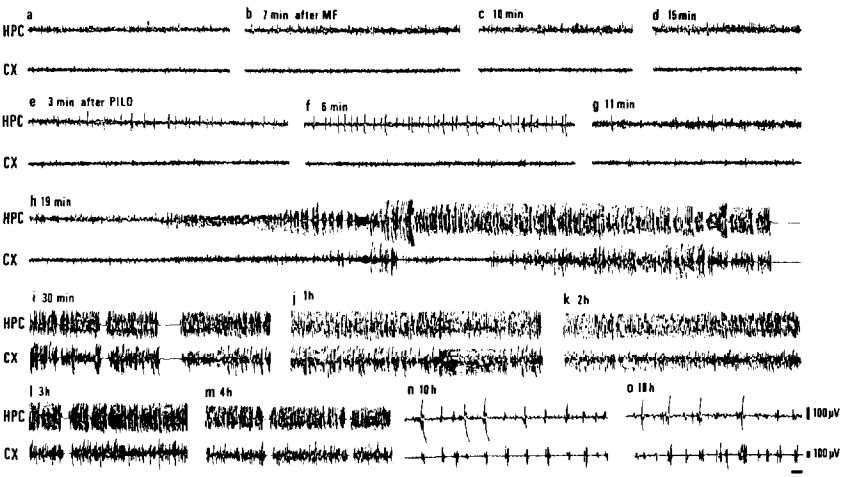

FIGURE 2. Electrographic recordings demonstrating the effect of morphine hydrochloride (MF) on the convulsant action of pilocarpine (PILO) in rats. MF, 10 nmol, was microinjected bilaterally into the SN pars reticulata 15 min prior to systemic administration of PILO, 100 mg/kg. (a) Pre-drug control recordings. (b-d) Unchanged records 7-15 min after microinjection of MF into the SN. (e-o) Electrographic recordings to illustrate the alterations registered during 3 min - 18 h after the administration of PILO in rats given MF into the SN. HPC, hippocampus; CX, cortex.

played severe status epilepticus (Fig. 1). The ED_{50} (effective dose) for MF, as a factor triggering seizures in rats treated with pilocarpine, 100 mg/kg (n=27), was 3.8 nmol (2.5-5.8). The proconvulsant effect of MF, 10 nmol, was blocked by NAL, 10 nmol, when co-injected into the SN (n=4) prior to pilocarpine, 100 mg/kg. The range of EEG changes produced by pilocarpine, 100 mg/kg, in rats pretreated with microinjection of saline into the SN is described elsewhere (Turski et al. 1986). The nature of EEG changes induced by pilocarpine, 100 mg/kg, in MF, 10 nmol, pretreated rats is best illustrated in Fig. 2. The electrographic activity produced by pilocarpine, 100 mg/kg, in rats pretreated intranigrally with MF, 10 nmol, resembles the build-up and EEG components of seizures produced by pilocarpine, 380 mg/kg (Turski et al. 1986). The pattern and topography of the damage to the forebrain produced by pilocarpine, 100 mg/kg, in rats pretreated with MF, 10 nmol, in the SN resemble those found in the brains of animals treated with pilocarpine, 380 mg/kg (Turski et al. 1986). Extensive morphological alterations were found throughout the hippocampus (subfields CA 1 and CA 4), thalamus (mediadorsal lateral, reuniens, rhomboideus, paratenial and paraventricular nuclei), olfactory cortex, amygdala, neocortex and substantia nigra pars reticulata.

DISCUSSION

A deep controversy surrounds the function of endogenous opioids in the pathogenesis of seizure disorders. The present report on proconvulsant action of MF in the SN pars reticulata in pilocarpine-model of

limbic seizures in rats provides further proof for differential actions of opiates in the complex neuronal networks regulating the seizure threshold of the forebrain. Evidence is presented that the action of MF in the SN is opiate specific since naloxone reverses anticonvulsant action of the drug. This finding is in apparent contrast however with anticonvulsant action of intranigral morphine against maximal electroshock (MES)-induced seizures in rats. Garant and Gale (1985) showed that very high doses of MF sulphate when injected into the SN protect rats from tonic hindlimb extension in the MES-test. This action of MF was blocked by systemic naloxone. Surprisingly, anticonvulsant action of MF in the SN in the MES fits well protective effect of the opiate against MES when administered systemically (Czuczwar and Frey 1986). Similarly, proconvulsant effect of intranigral MF in pilocarpine-induced seizures reflects perfectly the proconvulsant action of systemic MF in this model of convulsions (Turski et al. 1985). Several mechanisms have been proposed as explanation of the pro- and anticonvulsant action of MF in different experimental situations (Frenk 1983). It seems likely that SN may be proposed as a site at which opiates are operative in the propagation and generation of seizures. The apparent disparity between proconvulsant action of MF in the SN in an animal model of limbic seizures and potent anticonvulsant action of the drug in MES-induced convulsions probably reflects basic differences between pathways and neurotransmitters determining the spread of seizures in these two models of epileptogenesis. Alternatively, selective interactions with precise neuronal networks differentially involved in regulation of the seizure threshold may well provide a basis for differential response to intranigral MF in various epilepsy models. The emerging picture of the function of opiates in the SN in the modulating of the seizure threshold is further complicated by multiplicity of opiate receptors in this brain region. Further studies are therefore urgently required to dissect out the contribution these receptors make to the epileptogenesis.

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OPIOIDS WITH DIFFERENT AFFINITY FOR SUBRECEPTORS INDUCE DIFFERENT EFFECTS ON EARLY AND LATE SENSORY EVOKED POTENTIALS (SEP) IN MAN

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ABSTRACT

Amplitude changes of event-related sensory potentials (SEP's) present objective measurements for the evaluation of pain perception in man.
Alfentanil, an opioid with dominant binding to the mu-receptor was given in graded doses (5-, 10- µg/kg) to volunteers. Nalbuphine, an opioid with reported kappa-agonist activity, was given in graded doses (100-, 500-, 1000- µg/kg) to another group i.v. Both groups were also exposed to progressively increased electrical stimulations (mA) in order to determine tolerance threshold before and after drug administration. In the alfentanil group (n=5) there was a dose- related decrease of the amplitude of the early N_{20}-peak. The late N_{100}-peak was not, however, affected. This correlated closely (r=0.87) with a naloxone-reversible increase of tolerance to electrical current. In the nalbuphine group (n=15), there was a dose-related, naloxone non-reversible reduction of amplitude of the late N_{100}-peak which showed a good correlation (r=0.9) with an increase in current threshold. As the early evoked potentials are primarily generated in the pontine-thalamic region, it is suggested that the mode of action of alfentanil resembles that of a blockade of sensory impulses. The latter takes place before impulses are transmitted to more rostrally located CNS structures which are responsible for pain identification. The late evoked potential affected by nalbuphine indicates an activity on thalamo-cortical projection sites which are involved in subjects' cognitive activities. Kappa-ligands, therefore, seem to exert a different mode of action than mu-opioids. which results in a modulation of the negative emotional response associated with pain, rather than an attenuation of pain impulses.

INTRODUCTION

Monitoring of somatosenory-evoked potentials (SEP's) has gained accep- tance as a suitable tool for the objective measurement of efficacy of analgesics in man (Buchsbaum et al.1981; Chapman et al.1982). As central analgesics differ in regard to their interaction with opioid receptor sites, the study was undertaken in order to evaluate the possible mode of action of alfentanil, a predominant mu-opioid,

and that of nalbuphine, an agonist-antagonist which mediates analgesia through kappa-receptor interaction (Schmidt et al.1985).

MATERIALS AND METHODS

After institutional approval and informed consent, twenty adult male volunteers, aged 38-45 years, were studied. Unilateral right meadian nerve SEP's were obtained from Ag/AgCl surface electrodes fixed with collodion to the contralateral sensory cortex site (FpZ-C_3', ground right arm strap). Stimulation was of square wave nature with a duration of 0.2 ms, at a rate of 5/s, a stimuli repetition of 1024, and a current twice above motor threshold (supramaximal). At a sampling rate of 256 KHz, and a filter setting between 5-800 Hz, potentials were averaged over a period of 150 ms (Neurotrec, Interspec Medical, Wisconsin, USA). The cortical wave forms were assayed in regard of early (less than 50 ms) and late (more than 70 ms) major deflection changes in terms of base to peak amplitude (µV), compared to control, and identified as either N(=negative) or P(=positive) polarity. Alfentanil was administered as a slow bolus of 5 µg/kg, followed by 10 µg/kg 20 min later. 5 min thereafter, naloxone was given (5 µg/kg) i.v. SEP's were derived at peak drug activity 1 min after injection. Nalbuphine was given in graded doses (100-, 500-, 1000- µg/kg) every 15 min. Prior to the next dose, SEP's were derived. After the last injection, naloxone (5 µg/kg) was given. In addition, subjective pain was evaluated by determination of tolerance threshold to progressively increased stimulations from an electrical current (mA), applied to the median nerve before and after each dose.

RESULTS AND DISCUSSION

Compared to control, alfentanil induced a dose-related decrease in the amplitude of the early N_{20}-, however, not of the late N_{100}-wave. This effect correlated closely (r=0.87) with a concomitant increase in tolerance to electrical current threshold (table 1). The opioid antagonist naloxone reversed the effects of early amplitude suppression and the concomitant increase of tolerance to current threshold.

TABLE 1.
The effect of increasing doses of alfentanil on early and late SEP changes and the corresponding increase of tolerance to electrical stimulation (mean +/-SD).

dose µg/kg	N_{20}(µV)	N_{100}(µV)	tolerance (mA)
control	1.5 +/-0.6	1.9 +/-0.9	13.2
5	1.2 +/-0.6	1.9 +/-1.4	18.5
10	0.6 +/-0.5	1.9 +/-1.4	24.8
naloxone	0.9 +/-0.8	2.4 +/-2.2	11.9

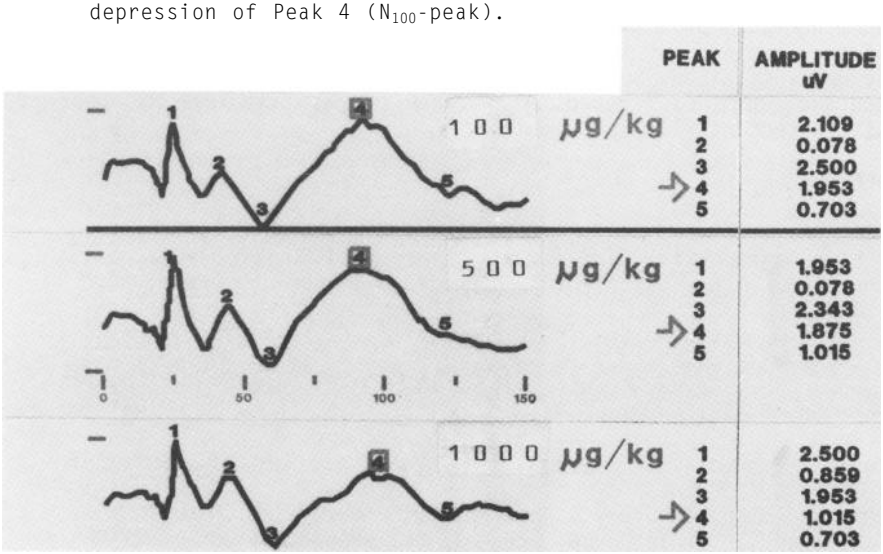
Nalbuphine induced a dose-related suppression of the amplitude of the late N_{100}-peak which showed a good correlation (r=0.90) to an increase of threshold to painful stimuli. This effect could not be reversed by naloxone (table 2).

TABLE 2.
The effect of increasing doses of nalbuphine on early and late amplitude changes of median nerve SEP's and the corresponding increase of tolerance to electrical current (mean +/-SD).

dose(µg/kg)	N_{20}(µV)	N_{100}(µV)	tolerance
control	1.5 +/-1.8	1.4 +/-1.1	13.8
100	1.6 +/-1.4	1.4 +/-0.9	18.2
500	1.3 +/-1.1	1.2 +/-0.8	21.7
1000	1.7 +/-2.3	1.1 +/-0.6	23.5
naloxone	1.3 +/-1.1	1.0 +/-0.8	23.5

The peaks of the evoked potentials are the result of stimuli, which, along their way through the CNS, generate an excitatory response in different anatomical structures (Buchsbaum et al.1981). Thus, a possible funtional deficit as induced by an analgesic is reflected in the generated SEP. By means of identifying the peaks which present drug-induced changes, the possible site(s) of action may be localized (fig.1).

FIG.1
Representative example of increasing doses of nalbuphine on late evoked potentials. Note, there is a dose-related depression of Peak 4 (N_{100}-peak).



In addition, by the use of an antagonist, an identification of the type of receptor mediating the observed neurophysiological changes is possible.

Early evoked potentials appearing less than 50 ms post stimuli are generated in the pontine-thalamic region (Allison et al.1980). This suggests that any change in the early peak (N_{20}) is initiated in these structures. Since the mu-specific opioid alentanil depresses early evoked potentials in a dose-related fashion, the main mechanism seems to be that of a blockade of sensory impulses in subcortical structures. As a result, less activity will reach the more rostrally located CNS structures which are responsible for the identification of incoming impulses. The net effect is that of a loss of pain sensations. One possible subcortical structure involved in the identification of pain is the pallidum, a part of the limbic system and the alleged psychomotoric center for pain sensations and associated conflict feelings (Hassler 1970). As a result of mu-opioid activity, limbic structures are deprived from incoming impulses and so the pain is not felt as an aggrevation. Mu-receptor sites seem to play an important part in mediating this effect.

The suppression of amplitude of the late evoked potential (N_{100}) by nalbuphine, which was not naloxone-reversible, suggests a receptor site which is not of mu, but rather of kappa nature (Schmidt et al.1985). Late-evoked potentials are generated in more rostrally located thalamo-cortical projection sites, where the subjects' cognitive capabilities are situated (Chapman et al.1982). The agonist-antagonist, therefore, seems to exert its analgesic activity mainly through a modulation of impulses coming from the extralemniscal sensory pathway. These neurophysiological findings are in line with displacement and autoradiographic studies demonstrating a predominance of kappa-binding sites in the deep layers of the cortical projection sites (Maurer et al.1963; Goodman and Snyder 1984). Nalbuphine, contrary to the mu-opoid alfentanil, although inducing a centrally mediated effect, is functionally different than the pure agonist. This difference is reflected mainly in the supression of the late and not the early SEP, an effect which is not reversible by naloxone. Thus, an attenuation of the endless discomfort associated with pain is induced. A blockade of pain impulses as observed with alfentanil is not present.

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CENTRAL AND PERIPHERAL B-ENDORPHIN RESPONSE TO TRANSCUTANEOUS ELECTRICAL NERVE STIMULATION

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ABSTRACT

The effects of transcutaneous electrical nerve stimulation on plasma and cerebrospinal fluid (CSF) levels of B-Endorphin (B-EP) were studied in 6 groups of pain-free patients. Different modes of TENS were applied for 20 minutes. Regardless of mode and/or frequency of the stimulation plasma and/or CSF B-EP levels were significantly increased. Plasma and CSF responses were not correlated suggesting that TENS activates different opiatergic pathways at central and peripheral level.

INTRODUCTION

The efficacy of antalgic stimulations are known from thousands. However, one of their mechanism of action has been elucidated only after the discovery of the endogenous opioid systems in our species. Chinese Acupuncture (Mayer et al. 1977; Facchinetti et al. 1981). electrical acupuncture (Clement-Jones et al. 1980; Nappi et al. 1982), transcutaneous electrical nerve stimulation (TENS) (Facchinetti et al. 1984) all are believed to induce analgesia through an activation of peptides related to Proopiomelanocortin (POMC) such as B-endorphin (B-EP). Substantial differences seems to exist between the various kind of stimulation.

A concomitant release of B-EP and ACTH in peripheral plasma is detectable after electroacupunture (Abbate et al. 1980; Nappi et al.1982) and stress situations (Guillemin et al.1977), while only B-EP is increased upon traditional Chinese acupuncture (Facchinetti et al.1981). As far as TENS is concerned, both opioid and non-opioid mechanisms have been hypothesized depending on frequency of stimulation (Sjolund and Eriksson 1979; Cheng and Pomeranz 1979). Anyway, either low frequency (LoF) TENS or high frequency (HiF) TENS are accompanied by a significant increase of pain threshold (Facchinetti et al.1984). In this study we compared the effect of TENS application on the changes of plasma and CSF B-EP in pain free patients.

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MATERIALS AND METHODS

Subjects

Six groups of eight patients entered the study.They were pain-free patients (aged 46-83 ys ; 65.4 on average) undergoing transurethral resection of benign prostatic hyperplasia,performed in local peridural anesthesia. No premeditation was given and any drug treatment was stopped for at least two weeks. TENS electrodes were applied paravertebrally, at Th 12-L 2 level (2.29 cm of surface). The different kinds of stimulation (lasting 20 min) were reported in the table. TENS units were EPIX model kindly supplied by EMPI Inc (Minneapolis/MN). Ten ml heparinized blood and 5 ml CSF samples were collected either before or at the end of treatment.Plasma and CSF samples were added of Trasylol and then stored at -20 C until assays.

MODE	FREQUENCY (Hertz)	INTENSITY (mA)	WAVE	CSF BEP (% M ± SE)
Multimod	80-100	50-99	Changing	40.7 ± 18.8
Continous	2-10	10-30	square,250µsec	71.6 ± 16.2
Continous	80-100	50-99	" "	52.8 ± 22.5
Continous	2-10	50-99	" "	54.0 ± 27.7
Burst (2Hz)	85	50-99	" "	109.2 ± 29.2
Burst (2Hz)	2-4	10-30	" "	13.4 ± 10

Table 1: Details on the different TENS used and effects on CSF B-EP as % on basal value.

Hormone Assays

Silicic acid extracts of plasma samples and 2 ml freeze-dried CSF samples were reconstituted to 0.5 ml with 0.1 M Acetic Acid, 0.01% BSA and applied on Sephadex G-75 column (1.5 x 45 cm) eluted through the same solution. According to the retention coefficient of cold B-EP (0.81), a 18 ml fraction was collected, freeze-dried, redissolved in 0.12 M phosphate buffer, 0.1% BSA, pH 7.4 and submitted to Radioimmunoassay (RIA) as previously described in detail (Facchinetti et al.1984). Cortisol was measured in plasma samples through a commercially available RIA Kit (Radim-Italy).

RESULTS AND DISCUSSION

Both plasma and CSF B-EP levels were increased by TENS, with the exception of burst LoF-LoI stimulation which should be considered like a placebo. Regardless of mode of stimulation (Bursted, Continous, Multimod) there is no difference in the B-EP changes in either CSF or plasma (table 1). Similarly, regardless of the frequency of stimulation, plasma and CSF B-EP levels were increased by at least 40% in

respect to basal values (fig.1). Interestingly, similar data were obtained in plasma by Hughes et al.(1984). Thus these data are opposite to the hypothesis that LoF-HiI TENS would induce analgesia through an opiate mediation, while HiF-LoI analgesia is supported by a

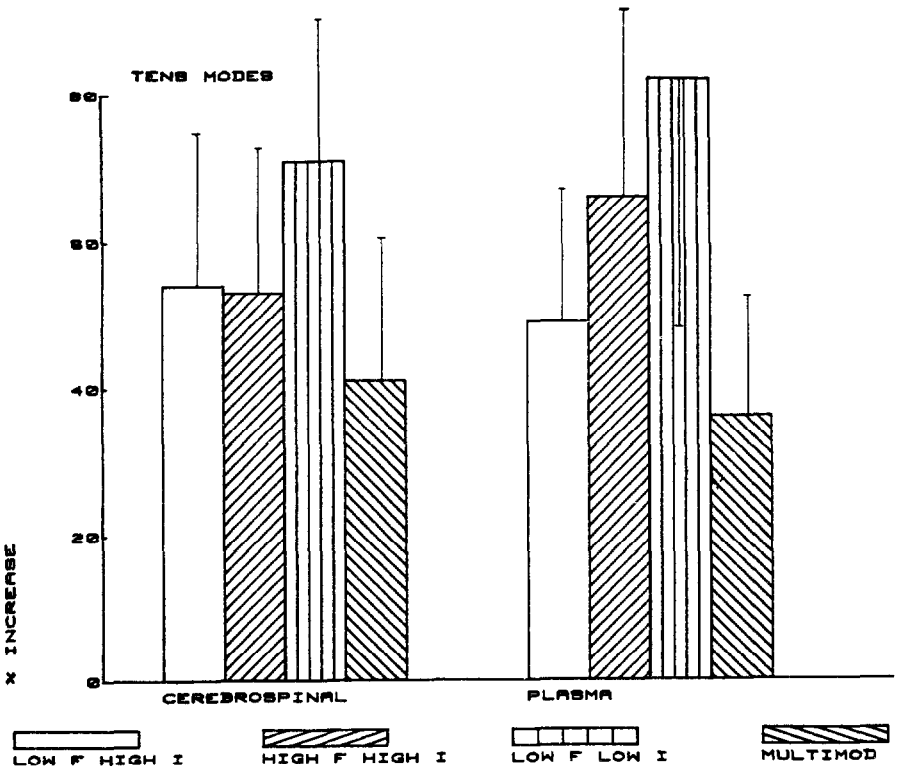

Figure 1: CSF and plasma B-EP changes after different kinds of TENS (M±SE).

non-opiate mechanism (Sjolund and Eriksson 1979; Cheng and Pomeranz 1979).

It should be pointed out that few studies reported the direct measurement of endogenous opioids and the hypotesis was build only on indirect evidences, i.e. the antagonism of analgesia through naloxone. However, as reviewed by Sawynok et al. (1979), naloxone reversal is not a sufficient criterion since different subclasses of opiate receptors exist and it is still unknown if just one of them mediate analgesia (Panerai et al.1984). Moreover, many studies were not performed in healthy controls, but in chronic pain patients (Abraham et al.1981) where an impairment of endogenous opioid system has been demonstrated (Genazzani et al.1984). Accordingly, we previously reported that patients affected by malignant diseases were unable to release B-EP into CSF upon bursted HiF TENS (Facchinetti et al. 1985). Like electroacupuncture (Nappi et al. 1982), the TENS

induced plasma B-EP increase is accompained by a concomitant release of cortisol levels in many cases, indicating the anterior pituitary cleavage of POMC in this condition.If this is depending on corticotrophin-releasing hormone release from the hypothalamus or to a direct effect on the pituitary remain to be clarified. However, despite TENS increases both plasma and CSF B-EP levels, there is no correlation between the two phenomena.Indeed, there are cases with a poor response in CSF (less than 20%) and an important release in plasma(more than 100%) and viceversa. In conclusion regardless of mode and/or frequency of the stimulation plasma and/or CSF B-EP levels were significantly increased. Plasma and CSF responses were not correlated suggesting that TENS activates different opiatergic pathways at central and peripheral level.

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LIMBIC REGIONS OF THE BRAIN OF ALZHEIMER'S DISEASE PATIENTS SHOW SELECTIVE CHANGES IN MU, DELTA AND KAPPA OPIOID RECEPTOR BINDING

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SUMMARY

Total opioid binding and levels of the three major types of opioid binding sites were measured in homogenates of various limbic structures from post-mortem brains of Alzheimer's Disease (AD) patients and age-matched control individuais. AD brains showed an increase in kappa binding in ail six areas of the limbic system examined, with the putamen and caudate regions showing significant increases of 114% and 53%, respectively. In addition, the AD putamen showed a significantly higher level of total binding (85% increase). The amygdala of AD patients exhibited significantly lower levels of mu and delta binding (41% and 55% decrease, respectively).

INTRODUCTION

Deterioration of cognitive function in AD has been imputed in part to neurochemical deficiencies and pathological changes in limbic regions of the brain, an area involved in the complex processes of memory storage and recali. We have previously reported (Hiller et al. 1973) that in the human brain, most areas containing high leveis of opioid receptors lie within or are closely associated with the limblc system. The possible involvement of the endogenous opioid system in memory storage and physiological amnesia has been indicated in several studies. The similarity between the anatomical distribution of opioid receptors and pathoiogical markers of AD and the putative role of the opioid system in cognitive functions prompted us to undertake a study comparing opioid receptor populations in various limbic structures obtained at autopsy from AD patients and age matched individuais unaffected by this disease.

MATERIALS AND METHODS

Brain tissue samples, obtained at autopsy less than 24 hours after death, from control individuals and AD patients were stored at -70°C until processed into crude membrane preparations (Lin and Simon 1978). The regions dissected for this study, using standard anatomical landmarks, were : caudate, frontal cortex, amygdala, temporal cortex, putamen and hippocampus. Brain samples were examined for the presence of pathology by Drs. D.J. Selkoe (Harvard Medical School. Boston, MA) J. Pearson (New York University Medical Center, New York, N.Y.) and J. Uirich (Basel University, Basel, Switzer-

land), from whom these post mortem brain samples were obtained.

Binding assays were carried out essentially as previously described (Hiller et al. 1973) at 25° for one hour in the presence and absence of a 1000-fold excess of naloxone. Mu site binding was measured with ^{3}H-D-Ala^{2} MePhe^{4}-Gly-ol^{5}-enkephalin (^{3}H-DAGO, 2 nM, 49 Ci/mmol, Amersham, Des Plaines, IL). To assess delta site binding, ^{3}H-D-Ala^{2}-D-Leu^{5} enkephalin (^{3}H-DADLE, 2 nM, 43 Ci/mmol, New England Nuclear, Boston, MA) was used. ^{3}H-bremazocine (2 nM 32 Ci/nmol, New England Nuclear, Boston, MA) possessing almost equal affinity for mu, delta and kappa binding sites, was used to assess total binding levels. Selective binding to kappa sites was determined using 2 nM ^{3}H-bremazoclne in the presence of 100 nM each of unlabeled DAGO (Reckitt and Coleman, Hull, England) and DADLE (Peninsula Lab., Inc., San Carlos, CA). AnalySiS Of data was carried out using the Mann-Whitney U test.

RESULTS AND DISCUSSION

Binding studies were carried out on samples from twelve autopsied brains, seven from AD patients (ages 58-68 years) and five from control individuals (ages 54-60 years). The presence of the characteristic neuropathological changes, consisting of neurofibrillary tangles and neurotic plaques in AD samples were found as well as the absence of anomalies in control samples. In those cases where the number of individual determinations of binding site levels differs from the number Of autopsied cases. the amount of material recerved was insufficient to carry out the assay. Results from equilibrium binding studies indicated that, in some regions of the brain of AD patients, statzistically significant differences from control samples were observable. A summary of results from AD and control samples is presented in tabie 1.

Total opioid receptor binding, as determined by the binding of ^{3}H-bremazocine, was increased in the AD putamen over control samples by 85% ($p<0.02$). Assessment of mu opioid binding sites with ^{3}-DAGO demonstrated a 41% decrease in AD amygdaia ($p<0.06$). Delta opioid binding, as determined by ^{3}H-DADLE binding was decreased in the AD amygdaia by 55% ($P<0.03$). In addition, delta opioid binding was reduced in AD temporal cortex by 41%. This change from control level, however, was not significant ($p<0.09$). Kappa opioid binding was assessed by the binding of ^{3}H-bremazocine in the presence of saturating concentrations of DAGO and DADLE. In all six areas of the AD limbic system kappa binding was increased (12% to 114%) over levels seen in control samples. Of these six areas the caudate and the putamen showed statistically significant increases of 53% ($p<0.03$) and 114%.

TABLE 1

Opioid receptor binding in limbic areas of control (C) and Alzheimer's Disease (AD) brains

	fMoles/as protein			
	^{3}H-DAGO	^{3}H-DADLE	^{3}H-BREM	^{3}H-BREM BLOCKED
Caudate(C)	15.45	20.02	49.36	124.40
±S.E.	1.17	1.66	6.70	7.84
(N)	(4)	(5)	(5)	(5)
Caudate(AD)	14.40	25.29	75.59	155.20
±S.E.	1.95	6.00	8.71	17.28
(N)	(4)	(7)	(7)	(7)
P	N.S.	N.S.	<0.03	N.S.
Frontal Cortex(C)	17.50	24.66	60.94	152.80
±S.E.	2.10	3.74	11.08	17.59
(N)	(5)	(5)	(5)	(5)
Frontal Cortex(AD)	24.93	27.16	94.64	208.37
±S.E.	5.61	4.84	15.08	21.17
(N)	(7)	(7)	(7)	(7)
P	N.S.	N.S.	N.S.	N.S.
Amygdala(C)	25.74	30.48	117.50	239.20
±S.E.	3.45	6.90	27.83	18.29
(N)	(5)	(5)	(5)	(5)
Amygdala(AD)	15.14	13.69	131.53	191.41
±S.E.	4.77	4.31	23.60	31.58
(N)	(7)	(7)	(7)	(7)
P	<0.06	<0.03	N.S.	N.S.
Temporal Cortex(C)	26.40	37.90	92.47	188.65
±S.E.	5.51	8.18	26.74	36.02
(N)	(4)	(4)	(3)	(4)
Temporal Cortex(AD)	20.41	22.33	113.45	171.44
±S.E.	4.21	3.60	27.29	23.41
(N)	(7)	(7)	(4)	(4)
P	N.S.	<0.09	N.S.	N.S.
P u t a m e n	9.63	13.60	27.97	66.23
±S.E.	3.37	2.69	5.49	8.16
(N)	(3)	(3)	(3)	(3)
Putamen(AD)	14.77	16.04	59.84	122.66
±S.E.	2.63	3.04	7.12	8.84
(N)	(7)	(7)	(7)	(7)
P	N.S.	N.S.	<0.05	<0.02
Hippocampus(C)	4.20	6.83	39.53	77.83
±S.E.	0.02	0.54	2.97	7.26
(N)	(3)	(3)	(3)	(3)
Hippocampus(AD)	4.18	8.72	64.18	93.52
±S.E.	1.04	2.31	11.52	26.89
(N)	(6)	(6)	(4)	(6)
P	N.S.	N.S.	N.S.	N.S.

(p<0.02), respectrveiy. if total kappa binding in all control and AD brain areas is compared, an increase in total kappa binding of 35% is noted in the AD group with a highly significant p value of <0.01.

The simplest explanation for our observation of lowered levels of mu and delta binding is that the manifestation of AD involves cellular patterns of pathology that affect those cells bearing mu and delta type opioid receptors. However, other possible explanations do exist. One that seems quite plausible is "homospecific up- or down-reguiation" in which certain ligands can modulate the number or affinity of their own receptors. For example, chronic exposure of HG108-15 cells to opiate agonists leads to a down-regulation of opioid binding (Chang et al. 1982). In contrast, chronic exposure of rats to opiate antagonists caused a 140% increase in ^{3}H-etorphine binding in limbic regions (Zukin et al. 1982). This last phenomenon may be explained by a mechanism which up-regulates the receptors by chronic displacement of endogenous opioid ligands. It is possible that our observed increase in kappa receptor binding may be due to an up-regulation caused by a decrease of dynorphin content in the AD limbic system. Support for this theory may be derived from the report (Pongdhana et al. 1985) showing that intraperitoneal injection of ketamine increased the dynorphin content of rat brain cortex while decreasing it in the septal area. Simultaneously, the binding affinity of the kappa ligand, ethylketocyciarocine, was decreased in the cortex but increased in the septal area. Dynorphin content of the human caudate and putamen is among the highest in human brain (Gramsh et al. 1982).

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DIFFERENTIAL EFFECTS OF MU AND KAPPA OPIOID SYSTEMS ON MOTIVATIONAL PROCESSES

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ABSTRACT

The role of multiple opioid receptor types in mediating the motivational properties of opioid agonists and antagonists was investigated by means of a place-preference conditioning procedure. Mu-agonists produced positive reinforcing effects in drug-naive animals whereas kappa agonists and the opioid antagonist, naloxone, were aversive. Chronic infusion of naloxone during conditioning, at a dose sufficient to block mu- but not kappa-receptors, antagonized the effects of both morphine and naloxone. This treatment did not alter the aversion produced by the kappa agonist, U69593. These data demonstrate that opioid reinforcement results from an activation of mu-receptors whereas aversive effects are produced by either an antagonist action at this receptor or an activation of kappa receptors. Such results indicate that mu and kappa opioid systems differentially modulate motivational processes.

INTRODUCTION

There is now substantial evidence to indicate the existence of multiple opioid receptor types within the mammalian CNS (Lord et al. 1977; Martin et al. 1976). However, despite extensive study, the role of these various receptor types in the regulation of motivational processes remains ill-defined. In the present study, a place preference conditioning procedure was utilized to characterize the motivational properties of opioids and examine the role of mu (μ)- and kappa (κ)-receptors in mediating their reinforcing and/or aversive effects. Conditioning produced by the μ-agonist, morphine; the K-agonist, U69593; and the opioid antagonist, naloxone (NLX) were evaluated in drug-naive animals and those chronically treated with NLX at a dose which selectively blocks mu-receptors. Evidence is presented to indicate that μ- and κ- opioid systems exert opposite effects upon motivational processes.

MATERIAL AND METHODS

Subjects were male Sprague-Dawley rats weighing 120-160g. One day prior to the commencement of

experiments, rats were implanted, under light ether anethesia, with osmotic minipumps (Alzet Model 2001) containing either the opioid antagonist, NLX, at a dose (0.55 mg/kg/hr) sufficient to block μ- but not K-receptors (Millan et al. submitted) or distilled water. Using a place-preference conditioning procedure (Mucha et al. 1982), separate groups of implanted and drug-naive (control) rats were administered (s.c.) various doses of morphine, U69593, or NLX. Each rat received alternate day injections of drug or vehicle for 6 days. Following drug injections, rats were immediately confined to a visually and texturally distinct environment i.e. black box with smooth black floor for either 60 min (morphine and U69593) or 30 min (NLX). Following injections of vehicle, rats were confined to a different environment, i.e. white box with rough white floor. The order of injections and drug-paired environments were counterbalanced within groups. On day 7, preference for a particular place was determined by allowing uninjected rats free access to both environments and measuring the time spent in each. Analyses of variance and the Student- Newman Keuls test were used to determine the statistical significance of results (Zarr 1974).

RESULTS AND DISCUSSION

Figure la demonstrates that, in control rats, morphine was positively reinforcing as indicated by a significant preference for the drug-paired place. However, in rats chronically treated with NLX, at a dose which selectively blocks B-receptors, the reinforcinq effect of morphine (3.0 mg/kg) was abolished. These results suggest that the reinforcing properties of opioid agonists results specifically from an activation of the μ-opioid receptor.

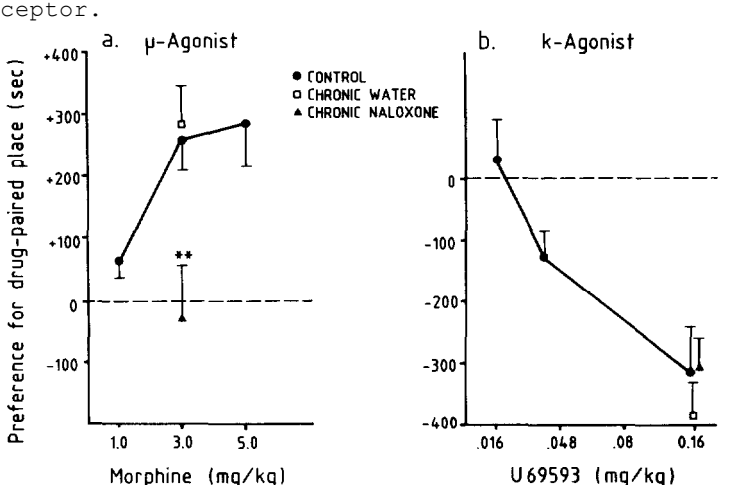

FIGURE 1: Motivational properties of μ- and κ-receptor agonists in control and chronic NLX-treated rats. Each point represents the conditioning score (mean ± S.E.M.) of 8-10 rats. **p < 0.001: significant difference between control and NLX-treated rats.

In contrast to morphine, U69593 produced dose-related aversions for the drug-paired place (figure 1b). If the aversion resulted from an agonist action at k-receptors, selective p-receptor blockade should be ineffective in altering its motivational properties. As shown in figure 1b, 0.16 mg/kg U69593 was aversive in both control and NLX-treated rats. Taken together, these data indicate that μ-and κ-opioid systems have opposing motivational effects: activation of the former is reinforcing while activation of the latter is aversive.

Previous studies using the place conditioning procedure (Bechera and Van der Kooy 1985, Mucha et al. 1982) have demonstrated that NLX is aversive in drug-naive animals. As the reinforcing effect of morphine is mediated by an activation of μ- receptors, we hypothesized that the aversive property of NLX results specifically from an antagonism of this receptor. Therefore, chronic p-receptor blockade during training should mask the b-antagonist activity of acute NLX, resulting in an attenuation of its aversive effects. The results shown in figure 2 confirm this prediction. Administration of NLX (5.0 mg/kg) was highly aversive in control animals. Mu-receptor blockade abolished this effect. These data indicate the aversive properties of NLX result from antagonist effects at the μ-opioid receptor.

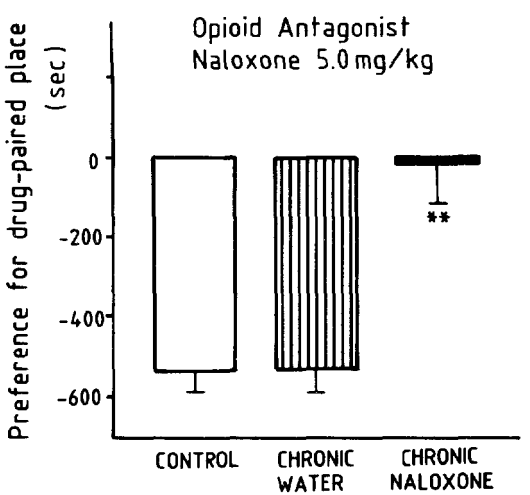

FIGURE 2: Effect of b-receptor blockade on the aversive properties of NLX. Each point represents the mean ± S.E.M. of 10 rats (**P < 0.001).

In conclusion, these studies demonstrate that opioids with agonist actions at the μ-receptor function as positive reinforcers whereas κ-agonists are aversive. Such results suggest the existence of opposing opioid systems which regulate motivation. Moreover, these data reveal that contrasting mechanisms underlie the aversive effects of NLX as compared to κ-agonists.

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AVERSIVE PROPERTIES OF OPIATE WITHDRAWAL STUDIED IN RATS

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ABSTRACT

Preference conditioning models were applied to the study of the motivational properties of opiate withdrawal. Conditioned place and taste aversions were produced by a single injection of an opiate antagonist in rats implanted with a morphine but not a placebo pellet. The hypothesis that withdrawal-produced jumping reflects the aversive properties of withdrawal was not confirmed. The use of central and peripheral administration of antagonists suggest that the aversive effects reflect antagonist activity in the brain.

INTRODUCTION

Although threats of drug intake reduction *or* of withdrawal are powerful stimuli for activating drug seeking behaviors in humans, the motivational properties of withdrawal have received little attention in the experimental literature on opiate physical dependence. The best examined models are contractures in the isolated opiate-treated guinea pig ileum (Schulz and Herz 1976), and "wet dog shakes", body weight loss and jumping or escape attempts in opiate-treated rodents (Blasig et al. 1973, Wei et al. 1973). However, only in the case of jumping has there been any attempt to relate a withdrawal sign to the discomfort described by withdrawing addicts; withdrawal-produced jumping is said to be similar to behavior elicited by electric shock and can be modified by variables believed to be motivational (Siegel et al. 1975).

Previous work suggested that taste (Pilcher and Stolerman 1976) and place preference conditioning procedures (Mucha et al. 1982) could be used to directly assess motivational properties of opiate withdrawal, but no attempt has been made to systematically extend these findings. Accordingly, we describe here progress on studies designed 1) to establish efficient models of withdrawal aversiveness in rodents, 2) to test the hypothesis that withdrawal jumping reflects withdrawal aversiveness and 3) to determine the general location of the receptors involved in the aversions.

MATERIALS AND METHODS

Male Sprague-Dawley rats obtained from St. Constant, Quebec, were used. Conditions of housing, testing, and general experimental design were outlined previously (Mucha and Herz 1986, Mucha and Iversen 1984), except that the composition of the flavors for the taste conditioning here comprised a) 92.7 mM NaCl, and 9.36 mM MSG and b) 1.13 mM citric acid and 0.71 mM saccharine. All subjects were implanted using halothane with a morphine (containing 75 mg morphine base) or placebo pellet. Where necessary, a cannula was implanted stereotaxically in the lateral ventricle. Drug administration was SC (approximately 1 ml/kg) or ICV through the cannula (4 µl). Peripheral doses of antagonists were expressed as free base.

Withdrawal aversiveness was assessed by measuring the preference of the subject for a cue paired with a single administration of an opiate antagonist as opposed to a different cue paired with the corresponding vehicle administration. With place training, animals were injected first with antagonist or vehicle and then placed for 1 h into one of the two place-training boxes and 7-24 h later injected with vehicle or antagonist and placed into the other box. For the taste training, rats were given 3 mls of the appropriate flavor to drink after water deprivation overnight and then given the necessary injection. Unless otherwise stated, training was carried out on the 5th and 6th day after pellet implantation; testing was always one day after training. Place testing involved giving individual rats access for 15 min to a rectangular box with the two sets of training boxes (cues) separated by a neutral grid platform. Taste testing involved one day ad lib access to the two flavors. Other assessment of withdrawal signs was carried out after the antagonist administration during the place training sessions. For the first 0.5 h after injection rats were observed for diarrhea, jumping, writhing and head or body shakes.

The place conditioning score was the time on the side of the test box where withdrawal was experienced (administration of antagonist) minus time on the control side. The taste score comprised the amount consumed of antagonist-paired flavor minus the amount consumed that paired with vehicle, expressed as a percent of total intake. An effect was considered significant with a $p<.05$.

RESULTS AND DISCUSSION

As assessed with taste conditioning using a low training dose (0.015 mg/kg) in animals trained at

different times after implantation with morphine pellets, naloxone was most potent when the training was 4-8 days after pellet implantation. Mean serum morphine levels measured (Kim and Kats 1984) in tail vein blood of 6 rats run in parallel were 126.0 ± 15.2 ng/kg on the 4th and 70.9 ± 19.0 ng/ml on the 9th day after implantation but fell sharply thereafter (Day 11: 18.0 ± 10.1 ng/ml). On Day 14 naloxone produced almost no effects (X = +17.3 ± 24%, n = 8) in contrast to significant conditioning on Day 7 (X = -55.3 ± 16,4%, n = 11).

Dose response curves were measured during the above-mentioned period of peak naloxone sensitivity (fig. 1) and revealed maximal aversions with 0.05 mg/kg in pellet animals; however, even 1.5 mg/kg failed to produce significant aversions in placebo rats. As seen in fig. 2, potent aversive effects in pellet animals were not limited to the use of naloxone nor the SC route; corresponding significant aversive effects were not seen in placebo controls (not shown). Given the different conditions and the dose ranges where the drugs were effective, it was concluded that the potent aversive effects of opiate antagonists in morphine pellet-implanted animals are probably due to their antagonistic action and therefore reflect the aversive properties of opiate withdrawal.

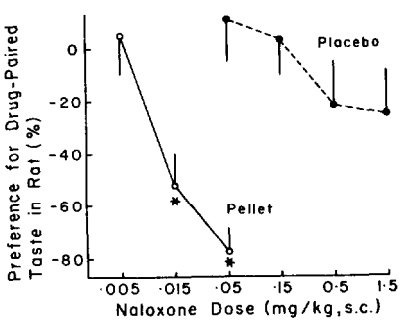

FIGURE 1 Taste conditioning scores in rats (ns = 8-10) implanted for 5 days with a morphine or placebo pellet.

In addition, the jumping response and the aversive state produced by withdrawal were contrasted over different conditions of antagonist administration. With SC naltrexone, the aversion paralleled jumping, but in rats trained with ICV quaternary-naltrexone it did not (fig. 2). Accordingly, we could not support the hypothesis that presence of aversive effects was reflected in jumping. Also, none of the other signs of withdrawal showed a good correlation with the place aversions (not shown).

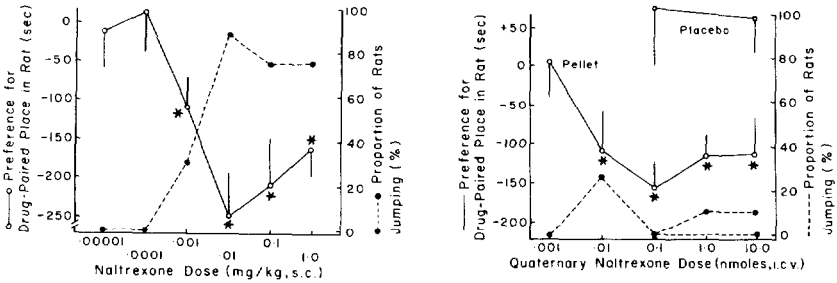

FIGURE 2 Place conditioning scores and jumping measured in the same animals after naltrexone given SC (left) or quaternary naltrexone ICV (right). Implantation: circles, pellet; squares, placebo.

It should be noted that quaternary naltrexone does not readily cross the blood brain barrier but produced clear place aversions when given centrally (fig. 2). Therefore, it is likely that the receptors responsible for the withdrawal-related aversive effects are somewhere in the brain.

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MORPHINE WITHDRAWAL IN GUINEA-PIG ILEUM IS CONFINED TO ONE ELECTROPHYSIOLOGICALLY DEFINED CLASS OF MYENTERIC NEURONE.

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ABSTRACT

Intracellular microelectrodes were used to record changes in myenteric neurones underlying morphine dependence in the guinea-pig ileum. Of the two electrophysiologically defined classes of neurones (S and AH), only a proportion of S-neurones were involved in the naloxone-induced withdrawal responses. Withdrawal caused direct depolarization and discharge of action potentials in S-neurones and also resulted in synaptic activation of neurones of this class. Both fast and slow synaptically mediated depolarizations were evoked by naloxone. The experiments suggest that morphine withdrawal is expressed in quite specific neuronal circuits in the myenteric plexus.

INTRODUCTION

The analysis of opiate dependence in the guinea-pig myenteric plexus has been based largely on studies of the nerve-mediated contraction of the longitudinal muscle evoked in morphine-pretreated tissues by naloxone. The naloxone-induced contraction is due predominantly to activation of cholinergic motor neurones since it is depressed by tetrodotoxin and atropine (Collier et al. 1981). The residual non-cholinergic component of the contraction may be due in part to the release of substance P (Gintzler 1980; Tsou et al. 1985). The present study aimed to analyse the changes underlying morphine dependence more directly by using intracellular microelectrodes to record from myenteric neurones.

MATERIALS AND METHODS

Guinea-pigs were pretreated either by injections three times daily with increasing doses of morphine (10 - 100 mg/kg s.c.) for 3-5 days, or by subcutaneous implantation of a pellet containing a mixture of morphine base (120 mg) and morphine hydrochloride (30 mg) for 7 days. Longitudinal smooth muscle-myenteric plexus (LSM-MP) preparations from the treated animals were set up in physiological saline solution (PSS) containing 1 μM morphine in order to maintain continuous exposure to morphine <u>in vitro</u> (Schulz and Herz 1976). These preparations responded to naloxone (1 μM) with a powerful nerve-mediated contraction of the longitudinal muscle, the characteristic index of morphine dependence in the guinea-pig ileum (Schulz and Herz 1976; Collier et al. 1981). Control preparations, from untreated guinea-pigs, were unaffected

by naloxone.

Similar LSM-MP preparations were mounted in a tissue chamber for intracellular recording from myenteric neurones. In addition to morphine (1 μm), the PSS perfusing the preparations contained hyoscine (1 μm) and nicardipine (3 μm) to prevent contractions of the longitudinal muscle during intracellular recording from myenteric neurones.

Intracellular recordings were made from both S and AH neurones (Hirst et al. 1974). Neurones in which electrical stimulation of fibre tracts evoked fast cholinergic excitatory postsynaptic potentials (e.p.s.p.'s) were classified as S-neurones. AH neurones received no fast synaptic input and were defined by the long lasting (2 - 30 sec) after-hyperpolarization accompanying an action potential evoked in the soma. After a stable impalement of a neurone had been obtained, naloxone (1 m) was added to the perfusate to displace morphine from its receptors so that electrophysiological changes associated with withdrawal could be recorded.

RESULTS AND DISCUSSION

1) S-neurones

Intracellular recordings were obtained from 53 S-neurones and 40 AH-neurones in LSM-MP preparations from 51 morphine-pretreated guinea-pigs.

50% of S-neurones in morphine-dependent LSM-MP preparations showed changes in their properties during naloxone-induced withdrawal. Naloxone did not alter the electrophysiological properties of S-neurones in control tissues. The most frequent withdrawal response was a membrane depolarization (12.1 ± 1.2 mV; mean ± S.E.M.) observed in 40% of neurones. The change in membrane potential, which was sustained throughout the 5-10 minute perfusion with naloxone, was associated with a slight but not statistically significant increase in input resistance (120 ± 30 M Ω cf. 101 ± 22 M Ω ; means ± S.E.M.). Twenty per cent of S-neurones responded with a depolarization which was accompanied by discharge of action potentials (0.5 - 10 Hz).

In 12% of S-neurones, naloxone evoked fast depolarizations which were similar in amplitude (5 - 20 mV) and time course (20 - 50 msec) to the fast cholinergic e.p.s.p. These fast depolarizations dissapeared after synaptic transmission was blocked by raising the concentration of Mg^{++} in the PSS from 1.7 mM to 10 or 20 mM, or by lowering the concentration of Ca^{++} from 2.5 mM to 0.5 mM, or by a combination of reduced Ca^{++} and raised Mg^{++}. This observation suggested that the withdrawal response was initiated by excitation of a cholinergic interneurone synapsing with the impaled S-neurone. The activation of interneurones during withdrawal was further suggested by the observation that the naloxone-induced slow depolarization was also reduced in some cells after synaptic

transmission blockade. Naloxone-induced withdrawal may therefore involve activation of not only the final motor neurones (leading to contraction of the longitudinal muscle) but also of cholinergic interneurones and interneurones producing slow synaptic depolarizations in S-neurones (Johnson et al. 1980).

In a small proportion (6%) of neurones naloxone evoked action potentials which were not associated with any change in the underlying membrane potential or with fast e.p.s.p.'s. The discharge of action potentials persisted after synaptic transmission blockade. These neurones therefore represent those in which withdrawal responses are initiated.

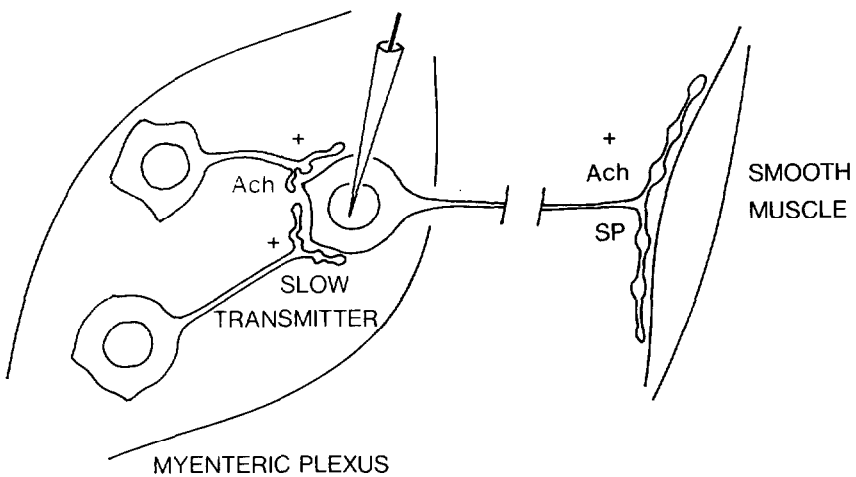

FIGURE 1. Possible arrangement of myenteric S-neurones involved in naloxone-induced withdrawal responses. During withdrawal the impaled neurone receives synaptic input from cholinergic interneurones (Ach) and from interneurones which release transmitter(s) producing slow depolarizations. Excitation of final motor neurones, either directly or by synaptic activation, leads to contraction of the muscle by release of Ach and substance P (SP) (not necessarily from the same motor neurone). The axon of the impaled neurone has been interrupted in the diagram to denote that it is not identified as a motor neurone by the intracellular recording technique.

2) AH-neurones

In contrast to its effects on S-neurones, naloxone did not alter the membrane potential or evoke action potential discharge in AH-neurones of morphine-dependent LSM-MP preparations. Furthermore, naloxone did not alter the input resistance, soma action potential configuration or the slow hyperpolarization following a soma spike.

The specificity of the withdrawal responses for S-neurones and their restriction to only a proportion of those neurones suggests that morphine withdrawal may occur in quite specific circuits in the myenteric plexus. Fig. 1 shows the possible arrangement of neurones participating in the withdrawal responses. The further characterization of these neurones will provide a better understanding of the cellular basis of morphine dependence.

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ENKEPHALINASE INHIBITORS ATTENUATE NALOXONE-PRECIPITATED WITHDRAWAL SYNDROME

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ABSTRACT

Enkephalinase inhibitors phosphoramidon, thiorphan or phelorphan suppressed naloxone-precipitated withdrawal symptoms in acute and chronic morphine dependent mice and rats. It is suggested that decreased biotransformation of the endogenous opioid peptides might compensate the relative shortage of exogenous opiates during withdrawal in opiate dependent subjects and could be a new and promising way to attenuate the severity of abstinential syndrome.

INTRODUCTION

Exogenous administration of opioid peptides suppress the opiate withdrawal in animals (Blasig and Herz 1976) and man (Wen and Ho 1982). Similarly, blockade of biodegradation of endogenous opioid peptides by the unspecific peptidase inhibitors aprotonin and bacitracin or by the relatively specific enkephalinase inhibitor phosphoramidon, suppressed the severity of the opiate withdrawal syndrome in rats and nitrous oxide convulsant withdrawal in mice, respectively (Pinsky et al. 1982, Dzoljic et al. 1984). These data indicate that enhancing of endogenous opioid peptides, by decreasing a biotransformation of these substances, might have an attenuating effect on the narcotic withdrawal syndrome, similar to the exogenously administered opioid peptides or opiates.
In order to study the role of endogenous opioid peptides in morphine addicted animals we examined the effect of relatively specific enkephalinase inhibitors, phosphoramidon, thiorphan or phelorphan, on the withdrawal syndrome in acute or chronic morphine dependent in mice and rats.

METHODS AND MATERIALS

Rats. Male wistar rats (200-225 g) were used in experiments. A steel cannula for intracerebroventricular (i.c.v.) administration of artificial cerebrospinal fluid (CSF) or enkephalinase inhibitors was implanted stereotaxically into the left lateral ventricle. Chronic morphine dependence was induced by subcutaneously (s.c.) implanted pellets (75 mg morphine), according to the method described by Blasig et al. (1973).
Mice. Adult male mice of B10A strain were used. Administration of CSF or enkephalinase inhibitors was carried out by means of a stainless guide cannula stereotaxically implanted in the left lateral ventricle. Acute morphine dependence was induced by a single s.c. injection of morphine sulphate (32 mg/kg). In order to observe the withdrawal syndrome, two hours later mice were injected intraperitoneally (i.p.) with naloxone hydrochloride (16 mg/kg). In general, the experimental

model of Kosersky et al. (1974) for acute withdrawal jumping in mice was followed.

Chronic morphine dependence was induced by two daily s.c. injections of morphine sulphate with a starting dose of 50 mg/kg and increasing daily by 25 mg/kg to reach a final dose of 200 mg/kg on the fifth day. On the 8th day a last morphine injection of 225 mg/kg was given, followed by naloxone 16 mg/kg i.p. (Takemori and Sprague 1978).

Drugs. Phosphoramidon (Cambridge Research Biochemical, New York). thiorphan (CRB, Cambridge) and phelorphan (donated by the Division of Pharmaceutical Chemistry, University of Amsterdam) were dissolved in CSF before i.c.v. administration. Naloxone hydrochloride (Endo Lab.) and morphine sulphate (Chemiefarma NV, Maarsen) were dissolved in saline.

Statistical analysis. The significance of the differences in the different group means are evaluated by the Mann-Whitney U test. Significance was accepted at $P<0.05$.

RESULTS

Phosphoramidon (50-200 µg i.c.v.) suppressed, in a dose-dependent manner, the naloxone precipitated withdrawal jumping but potentiated forelimb shakes in both acute and chronic dependent mice. Both thiorphan and equimolar doses of phelorphan decreased the severity of the naloxone-precipitated withdrawal syndrome in rats (table 1). However, some symptoms remained unchanged or even potentiated (table 1).

DISCUSSION

The essential observation in this study is that enkephalinase inhibitors, phosphoramidon, thiorphan or phelorphan attenuate the severity of morphine withdrawal syndrome in mice and rat. However, some of the withdrawal symptoms were either not altered or even potentiated. For example, phosphoramidon suppressed withdrawal jumping in the mice, while forelimb shakes were potentiated. The failure of phosphoramidon to inhibit the forelimb tremor is not clear. The withdrawal syndrome is a complex behavioural pattern, mediated by a balance of many transmitter systems in various brain regions. Unequal distribution of i.c.v. administered phosphoramidon and other enkephalinase inhibitors might be a reason that various opiate withdrawal symptoms were unequally affected. Furthermore, a comparison of the effects of equimolar concentrations of thiorphan and phelorphan shows that phelorphan has a less prominent antiwithdrawal effect than thiorphan. These differences might be due to the unequal penetration into the various brain regions or to the unequal capacity to inhibit the biodegradation of the enkephalins. It is of interest to note that some sexual manifestation during naloxone-precipitated withdrawal were potentiated by administration of thiorphan or phelorphan (penile licking, erection, ejaculation). It supports the idea that sexual response might be potentiated by any condition activating the endogenous opioid system (Henry 1982), including the enkephalinase inhibitors (this study). The decrease or abolition of the abstinential diarrhea by i.c.v. administered enkephalinase inhibitors is in accordance with suggestion that opioid peptides might regulate the intestinal transit by a central mechanism (Galligan and Burks 1982).

Incidence of the withdrawal signs (counted signs)

WITHDRAWAL SIGNS	CSF 2 ul (n=9)	THIORPHAN 40 ug/2 ul i.c.v. (n=9)	PHELORPHAN 62 ug/2 ul i.c.v. (n=8)
teeth chattering	5.6 ± 2.4	1.9 ± 0.6*	6.7 ± 1.8
wet dog shakes	10.5 ± 1.7	10.0 ± 1.7	12.5 ± 1.5
grooming	4.0 ± 0.7	3.2 ± 0.9	5.3 ± 1.2
scratching	0.2 ± 0.1	0.4 ± 0.2	0.9 ± 0.5*
penile licking	5.5 ± 0.4	8.7 ± 1.0*	9.2 ± 1.5*
writhing	2.3 ± 0.5	-	0.6 ± 0.3*
rearing	9.5 ± 2.1	6.8 ± 1.4	8.7 ± 2.0
digging	31. ± 5.3	4.0 ± 0.9"	11.1 ± 4.0*
head hiding	27.1 ± 5.7	1.2 ± 0.8"	5.6 ± 2.4
chewing	69.1 ± 11.0	32.9 ± 8.0*	33.8 ± 10.0*
paw tremor	1.8 ± 0.5	2.2 ± 0.8	5.0 ± 0.5*
head shakes	1.7 ± 0.3	3.0 ± 1.8	3.9 ± 1.1*
stretching	13.2 ± 3.1	8.9 ± 2.4	3.0 ± 1.0*
Presence of the withdrawal signs (checked signs)			
erection	3/9	3/9	8/8*
ejaculation	-		7/8*
diarrhea	9/9	2/9*	- *
Straub tail	8/9	3/9*	- *

Table 1. The effects (mean ± SEM) of equimolar dose of the enkephalinase inhibitors thiorphan or phelorphan on naloxone-precipitated (4 mg/kg, i.p.) withdrawal symptoms in chronic morphine dependent rats. The control group received artificial cerebrospinal fluid (CSF) instead of an enkephalinase inhibitor. Observation period: 30 min after naloxone administration. *Means significant difference compared to control ($P<0.05$). Note that enkephalinase inhibitors attenuated some of the withdrawal signs.

Concluding, the saturation of the opiate receptors by enkephalins, following administration of enkephalinase inhibitors, alleviates the several symptoms of morphine withdrawal syndrome in mice and rat. Although not all withdrawal symptoms were alleviated by enkephalinase inhibitors, it seems that the potentiation of the functional activity of endogenous opioid peptides could be a promising tool in future for treatment of opiate dependence.

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PRESYNAPTIC ALPHA$_2$ ADRENORECEPTOR FUNCTION IN DEPENDENT RATS BEFORE AND AFTER MORPHINE WITHDRAWAL

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ABSTRACT

Changes in the number of alpha$_2$ adrenoreceptors in the hippocampus, as measured by the specific binding of ^{3}H-clonidine, were compared to changes in presynaptic alpha$_2$ adrenoreceptor function in electrically stimulated hippocampal slices. In dependent rats the specific binding of ^{3}H-clonidine was reduced significantly. At 32 h after withdrawal binding began to return toward normal values, and at 72 h after withdrawal was significantly greater than that seen in saline-treated rats. In hippocampal slice experiments sensitivity to clonidine was significantly decreased in morphine-dependent animals and returned toward normal values after withdrawal. At 72 h after withdrawal, the sensitivity to clonidine of the presynaptic alpha$_2$ adrenoreceptor remained depressed although receptor density as measured in the binding studies was increased. This study suggests that the changes in the function of presynaptic alpha$_2$ adrenoreceptors might be important in the development of dependence upon morphine.

INTRODUCTION

Acute administration of morphine stimulates markedly the turnover of catecholamines in the brains of rats (Garcia-Sevilla et al. 1978) and mice (Smith et al. 1972). Tolerance develops to this effect of morphine, and after withdrawal, norepinephrine (NE) turnover is decreased in the brains of dependent mice (Rosenman and Smith, 1972). Neuronal release of NE is regulated in part by the presynaptic alpha$_2$ adrenoreceptor. Several investigators have reported that chronic treatment with morphine decreases the number of alpha$_2$ adrenoreceptors in specific areas of the rat brain as measured by the binding of ^{3}H-clonidine, an agonist at alpha$_2$ adrenoreceptors (Smith et al. 1983, Vincentini et al. 1983). The purpose of the present study was to determine whether changes in ^{3}H-clonidine binding to hippocampal membranes that occur after chronic morphine treatment and during withdrawal result in corresponding alterations in presynaptic alpha$_2$ adrenoreceptor mediated inhibition of stimulation-evoked NE release from hippocampal slices.

METHODS

Male Sprague-Dawley rats (220-240 g) were injected i.p. every 8 h with saline or with morphine sulfate as follows (day, mg/kg/injection): 1-3, 10; 4-6, 20; 7-9, 40; 10-12, 70; and 13-14, 100. Hippocampi were isolated from 6 rats (220-240 g). Specific binding of ^{3}H-clonidine to hippocampal membranes was measured as

described previously (Smith et al. 1983). The non-linear regression analysis program, LIGAND (Menson and Rodbard 1980) was used to develop the final values for K_D and B_{max} For the electrically stimulated brain slice experiments two sections, 0.5 mm thick, were made perpendicular to the longitudinal axis of each hippocampus. The slices were incubated with ^{3}H-NE, 300 nM, for 15 min, washed twice with fresh Krebs buffer and transferred to superfusion chambers. Each slice was superfused at a rate of 0.5 ml per minute for a 30 min equilibration period prior to the onset of field stimulation. Field stimulation consisted of trains of square-wave pulses (100 mA, 2 msec duration) at 4 Hz for 2 min periods. There were nine stimulation periods at 16 min intervals in each experiment. Two of each set of four slices were stimulated but did not receive any drug. For the other 2 slices drugs were introduced into the superfusion buffer immediately after the second (the baseline response period) and each subsequent period of stimulation. Drug concentrations were increased by approximately 3-fold increments in order to construct complete concentration-effect curves. Each slice was equilibrated in the presence of each drug concentration for a period of 13.5 min after which the slice was stimulated for an additional 2 min. Aliquots of the superfusate (2 ml) were collected at 4 min intervals. Immediately after the last aliquot of superfusate was collected, the tissue slice was removed from the superfusion chamber, blotted and weighed. Release of ^{3}H-NE was expressed as the amount of labelled amine in each aliquot given as a percentage of the amount of labelled amine in the tissue immediately before collection of that aliquot. The stimulation-evoked release was calculated as the difference between the total release during the period of stimulation less the estimated basal release. EC 50's were calculated by probit analysis.

RESULTS

After treatment of rats with morphine for 14 days, the specific binding of ^{3}H-clonidine was reduced significantly. Eight h after the last injection of morphine, the B_{max} for ^{3}H-clonidine was decreased by 26.3 % (TABLE 1) . At this time the rats were dependent but did not show signs of withdrawal. Thirty-two h after the last morphine injection the B_{max} had returned only slightly toward control values. At this time the rats were at the peak of the withdrawal syndrome. Seventy-two h after the last morphine injection, no signs of withdrawal were noted. At this time there was a marked (45.8%) increase above normal control values in the specific binding of ^{3}H-clonidine.

Prolonged treatment of rats with morphine caused a decrease in the sensitivity to clonidine of presynaptic alpha$_2$ adrenoreceptors in the electrically stimulated hippocampal slice preparation. Eight h after the last injection of morphine the clonidine concentration-effect curve was shifted to the right (FIGURE 1) and the EC50 for clonidine was increased from a control value of 3.4 ± 0.8 nM to a value of 26.3 ± 9.8 nM in the dependent rats. Thirty-two h after the last morphine injection the clonidine concentration-

TABLE 1. Specific binding of ^{3}H-clonidine to hippocampal membranes isolated from rats treated with morphine or after withdrawal from morphine.

Treatment[a] Duration	B_{max} [b]	% Change in B_{max}	K_D [c]	n
Control	96.5 ± 2.6	0	3.71 ± 0.54	12
1 Day	83.5 ± 4.9[d]	-13.5	3.31 ± 0.35	4
14 Days				
8 hr	71.1± 3.2[f]	-26.3	3.36 ± 0.69	7
32 hr	78.3 ± 4.5[e]	-18.9	2.64 ± 0.41	5
72 hr	140.7 ± 7.5[g]	+45.8	3.22 ± 0.25	6

[a] Chronically treated animals were sacrificed at 8, 32 or 72 h after the last injection of morphine. [b] fmoles/mg protein. [c] nM. Values represent the mean of n observations ± the standard error of the mean. [d] P < .05, [e] P< .0005, [f] P < .0001, [g] P < .00001.

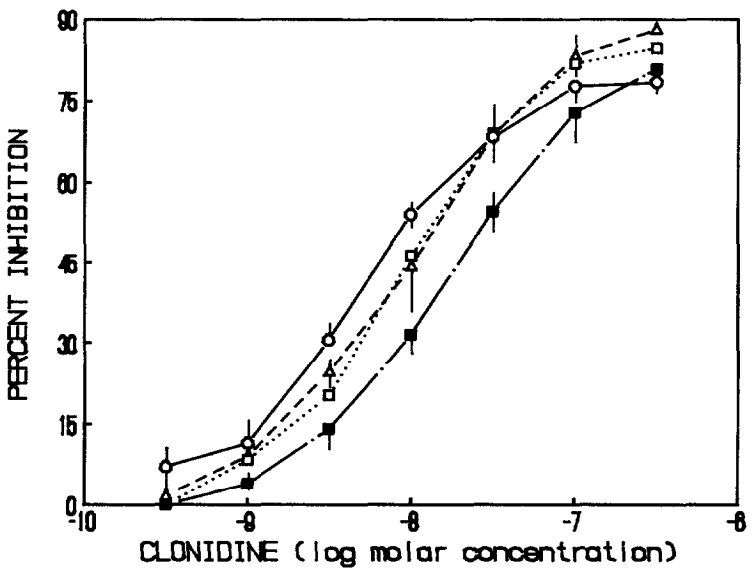

FIGURE 1. Effects of clonidine upon electrically evoked release of ^{3}H-NE from hippocampal slices from dependent rats before and after morphine withdrawal. Abscissa: con entration of clonidine. Ordinate: percent inhibition of ^{3}H-NE release. Open circles: controls; solid squares, 8 h after the last injection; open squares, 32 h after

withdrawal; open triangles, 72 h after withdrawal. Each point represents the mean of 5-9 values. Vertical bars, standard error of the mean.

effect curve was shifted back toward the left. At that time the EC50 for clonidine was 8.1 ± 2.2 nM. Seventy-two h after the last morphine injection there was no further change in the clonidine concentration-effect curve, and the EC50 for clonidine was 12.9 ± 3.4 nM.

DISCUSSION

Both the density and function of $alpha_2$ adrenoreceptors in the brain change when rats are made dependent upon morphine and after withdrawal of dependent rats from morphine. Although Hamburg and Tallman (1981) reported an increase in the density of $alpha_2$ adrenoreceptors in cerebral cortex from rats implanted with morphine pellets for 4 days, the present study and previous studies (Smith et al. 1983, Vincentini et al. 1983) indicate that the maximum number of specific binding sites for ^{3}H-clonidine is decreased in morphine-dependent rats. This decrease in binding is well-correlated with a decrease in the sensitivity to clonidine of presynaptic $alpha_2$ adrenoreceptors in electrically stimulated hippocampal slices. Seventy-two h after withdrawal from morphine the sensitivity to clonidine of presynaptic $alpha_2$ adrenoreceptors in the hippocampus remains depressed although there is a marked increase in the number of specific binding sites for ^{3}H-clonidine. This observation suggests that during withdrawal an increase in the number of postsynaptic $alpha_2$ adrenoreceptors occurs in the hippocampls. The finding of changes in $alpha_2$ adrenoreceptor number and sensitivity in the hippocampus of morphine-dependent rats suggests that alterations in central presynaptic $alpha_2$ adrenoreceptor function might be have an important role in opioid dependence.

ACKNOWLEDGEMENT

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THE EFFECT OF CHRONIC MET-ENK ON VARIOUS DOPAMINE SYSTEMS IN THE RAT

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ABSTRACT

Morphine and enkephalins act preferentially via different receptors, mu and delta, respectively. Chronic administration of morphine or methionine enkephalin results in development of physical dependence. Data from our laboratory suggests that there are some differences in the effect of these two opioids with regards to certain neurochemical systems.

INTRODUCTION

Several types of opioid receptors have been demonstrated. Among these are the mu (μ) and delta (6) receptors which interact with morphine and enkephalins, respectively. Distinctions between these 2 classes of receptors have been clearly defined on the basis of pharmacological differences, localization studies, and peripheral bioassays (for review see Paterson et al. 1983). There is a dense population of μ as well as 6 receptors in the striatum and hypothalamus. Here neuroregulatory interactions occur with various neurotransmitter systems. Both μ and δ receptor agonists can elicit alterations in dopaminergic transmission in these areas. The relationship between catecholamines and responses induced by opioids have been investigated extensively to determine whether amines play an important role in the adaptive mechanism(s) of physical dependence. Chronic administration of either morphine or enkephalin peptides results in development of physical dependence. Continuous exposure to morphine has been demonstrated to result in an increased sensitivity of striatal and hypothalamic dopamine (DA) systems as measured by apomorphine (APO)-induced stereotypy and hypothermia, respectively (Ritzmann et al. 1983). Changes in the high affinity component of D2-DA receptor binding for agonists in these brain regions were found to correlate with these APO-induced behavioral/physiological parameters in morphine-dependent rats (Ritzmann et al. 1983). It remains to be determined whether various opioid receptors are regulated by or linked to different molecular processes. Therefore, the many differences in characteristics of μ and δ receptors leads one to question whether the mechamism(s) of adaptation during chronic exposure to morphine and enkephalins are similar. Thus, using a method which produces physical dependence, the effect of chronic methionine enkephalin administration on striatal and hypothalamic dopamine systems was investigated.

MATERIALS AND METHODS

Male Wistar rats, (Fredricks Cancer Center) weighing between 200-300 grams were housed in a controlled environment (temperature 23 ± 2°C; light 0800 to 1800 hours). Animals were anesthetized with Equithesin (2.7 cc/kg; Jen Sail) and a stainless steel cannula was implanted into the lateral ventricle of the brain. Methionine^5-enkephalin (met-enk) was administered into the ventricle at a rate of 10 µg/µl/hr for 5 days via an osmotic mini-pump (Tm, Alzet; model 2001). Control animals received 0.9% sterile saline in place of a met-enk solution and underwent the same procedures as the met-enk treated animals. A mini-pump was connected to a cannula via polyethylene tubing. Withdrawal was initiated by disconnecting the tubing and sealing off the cannula. Rectal measurements of body temperature were monitored every 2 hours for the first 8 hours and at the 24 hour time point. Twenty four hours after initiation of withdrawal, animals were either tested for behavioral/physiological responses to the dopamine agonist apomorphine (0.5 mg/kg, i.p.) or sacrificed for binding studies. In animals receiving an APO injection, behavior was monitored at 5 minute intervals for 30 minutes post-injection and rated according to the following scale: a score of 0 - normal behavior; 1 - increased locomotor behavior; 2 - rearing and head bobbing (non-continuously); 3 - rearing and head bobbing (continuously); 4 - stereotyped sniffing continously; 5 - licking and gnawing behavior. An animal was considered stereotypic if a score of at least 4 was obtained. Rectal temperatures were monitored in these animals prior to and 30 minutes after APO injection. Animals for DA binding assays were sacrificed by decapitation at the 24 hour time point. Hypothalamus and striata were dissected out for determination of specific D2-DA receptor binding. Binding assays were performed as previously described (Ritzmann et al. 1983). Parametric data was analyzed by t-tests. Values are expressed as means ± SEM. Non-parametric data was analyzed using Fisher's Exact Probability Test. Binding data was analyzed by the computer program LIGAND (Munson and Rodbard 1980).

RESULTS AND DISCUSSION

Chronic 5 day exposure to met-enk resulted in a marked degree of physical dependence as evidenced by a significant ($p<0.01$) degree of withdrawal hypothermia up to 8 hours after initiation of withdrawal. The maximum temperature change occured in the peptide treated animals at the 4 hour time point (-0.69 ± 0.14°C met-enk group; 0.09 ± 0.07°C control group). Temperatures returned to control levels in the met-enk group 24 hours post-withdrawal. Animals dependent on met-enk were more sensitive than controls to the hypothermic response to APO 24 hours after initiation of withdrawal. This was evident by a temperature change of -0.67 ± 0.22°C in the met-enk treated animals compared to a change of -0.08 ± 0.18°C in control animals (Fig. 1). This increased sensitivity to APO-induced hypothermia is similar to that observed in animals physically dependent on morphine (Ritzmann et al. 1983). In contrast, met-enk treatment groups

exhibited a response to APO-induced stereotypy that was not statistically different from their controls (0% stereotypic, met-enk group; 12% stereotypic, control group; Fig. 1). This is unlike the effect produced by chronic morphine treatment which results in an increased sensitivity to APO-induced stereotypy (Ritzmann et al. 1983). Chronic morphine exposure produces an increase in percentage of receptors and/or increase in affinity of D2-DA receptors in the high affinity state in both hypothalamus and striatum for DA (Ritzmann et al. 1983). Similarly, 5 day met-enk treatment increased the affinity in the hypothalamus of the high affinity component for DA compared to controls <K_D - 10.0nM control group; K_D - 0.008nM, met-enk group; Table 1). This increased affinity of hypothalamic DA receptors correlates positively with the increased sensitivity to APO-induced hypothermia observed in these animals. In contrast to morphine treatment (Ritzmann et al. 1983), the high affinity striatal D2-DA receptor binding was not different in control versus met-enk treated animals (K_D - 53nM, control group; K_D - 46 nM, met-enk; Table 1). Again this corresponds with the lack of change in response to APO-induced stereotypy seen in these animals compared to controls. There was no change in receptor binding capacity (B_{max}) in either striatal or hypothalamic D2-DA agonist binding with chronic met-enk similar to that observed previously with chronic morphine (data not shown). Although met-enk preferentially interacts with δ receptors it also interacts with μ receptors at higher concentrations. Recently Pasternak and Wood (1986) have postulated the existence of 3 receptor types that bind morphine and enkephalins selectively: μ1, which has very high affinity (<1 nM) for both morphine and enkephalins; μ2, a site which preferentially binds morphine far more potently than enkephalins; and δ, which selectively binds enkephalins. The hypothalamus has been demonstrated to contain a high concentration of μ1 receptors and these have been linked to effects on DA turnover in this region (Pasternak and Wood 1986). On the other hand, the striatum contains high concentrations of μ2 and δ receptors which have been demonstrated to effect DA turnover in this area (Pasternak and Wood 1986). Therefore, met-enk may be affecting the DA system in the hypothalamus via μ1 receptors and via δ receptors initially in the striatum. However, desensitization or down-regulation of δ but not μ receptors can occur during chronic exposure to opioid peptides in various cell cultures and brain slice preparations (for review see Zukin and Temple 1986). Therefore the possibility exists that the effect on DA turnover by 6 receptors during chronic opioid exposure is different than that produced by either μ1 or μ2 receptors. Recent data from our laboratory suggests that this phenomena of opioid receptor down-regulation does indeed occur in vivo in the striatum during 5 day met-enk exposure (Steece et al. 1986). Hence, such an adaptive mechanism of 6 receptors in the striatum could conceivably account for the overall lack of effect of chronic met-enk on the DA system in the striatum.

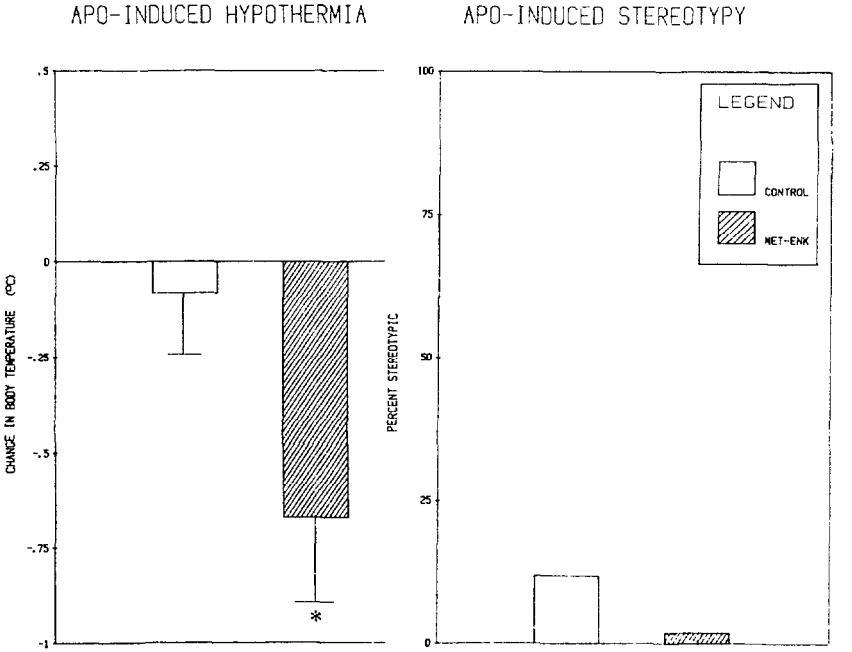

FIGURE 1. The effect of 5 day met-enk treatment on APO-induced hypothermia and stereotypy 24 hours after initiation of withdrawal. (n $\geq$ 12; *p<0.05)

EFFECT OF MET-ENK ON THE AFFINITY (K_D) OF D2-DA RECEPTORS

Treatment	Striatum	Hypothalamus
Control	46.0	10.0
Met-Enk	53.0	0.008

TABLE 1. The effect of 5 day met-enk treatment on the high affinity component of D2-DA receptors in the striatum and hypothalamus. Each group represents at least four determinations. Data is KD expressed in nM.

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EFFECTS OF PRIOR EXPOSURE TO MORPHINE ON THE OPIOID INHIBITION OF THE STIMULATED RELEASE OF [^{3}H]NOREPINEPHRINE FROM GUINEA PIG CORTEX SLICES

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ABSTRACT

The potassium stimulated release of [^{3}H]norepinephrine ([^{3}H]NE) from terminal fields of locus coeruleus projections can be inhibited in a dose-dependent manner by mu and kappa selective opioids. Chronic exposure to morphine for six days decreases the maximum achievable depression by the mu selective agonist Tyr-D-Ala^2-Gly-Me(Phe)-Gly-ol (DAGO), but has no effect on the degree of inhibition produced by the kappa selective opioid U50,488H.

INTRODUCTION

Opioids inhibit release of several neurotransmitters in the central nervous system, presumably by interactions with receptors located presynaptically on the nerve terminals. There are reports of the opioid inhibition of the release of dopamine and acetylcholine (Mulder et al. 1984), and of norepinephrine (Montel et al. 1974; Hagan and Hughes, 1984). In the rodent, cell bodies of the locus coeruleus project to several areas of the brain, including the cortex (Ungerstedt 1971). Locus coeruleus neurons are hyperpolarized by the application of opioids in electrophysiological studies using slices of brain tissue from the rat (Williams and North 1984) and the guinea pig (Pepper and Henderson 1980). The terminal fields of these noradrenergic fibers contain opioid receptors, as shown autoradiographically (Lewis et al. 1983) and in binding studies (Werling et al. 1985). We therefore investigated the possible opioid regulation of the release of [^{3}H]NE by the mu selective agonist DAGO and the kappa selective agonist U50,488H in naive guinea pigs compared to those implanted with osmotic pumps which delivered chronic morphine to the animals for six days.

METHODS

Male Hartley guinea pigs (300 - 600 g) were subcutaneously implanted with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) containing either 0.9% saline or morphine-HCl at the appropriate concentration to deliver 100 µg/hr. After 6 days, animals were sacrificed, their brains removed to ice, and the cortices dissected. The cortices were chopped in two planes into 250 µm slices on a Sorvall TC2 tissue slicer (DuPont Instruments, Newtown, CT), then suspended in oxygenated modified Krebs Ringers (MKR) solution (127 mM NaCl, 5 mM KCl, 1.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 15 mM HEPES, 10 mM Glucose). Subsequently, all steps of the preparation and experiment were performed in a warm room which was maintained at a constant temperature of 37°C. The tissues were washed 3 times in MKR (20 ml/half cortex), resuspended in MKR and incubated for 15 min with 15 nM

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[^{3}H]NE (New England Nuclear, Boston, MA). The tissues were then washed twice for 5 min each in MKR, and once for 5 min in MKR containing 1 μM each desiprimine and yohimbine. All subsequent steps were carried out in buffer containing these drugs to prevent feedback inhibition of release through presynaptic alpha receptors and reuptake of released [^{3}H]NE. The tissue slices were resuspended in MKR and distributed onto a series of nylon mesh baskets attached to Costar (Cambridge, MA) multi-well plastic culture plate lids. The baskets rested in the wells which contain 2 ml each MKR. To change treatment, the lids were lifted from one plate of wells to the next, which contained buffer with or without drug as appropriate. In all cases, MKR had been pregassed with 95 % O_2/5% CO_2 and pipetted into the wells immediately before tranferring the baskets. In this way, 24 different conditions could be examined simultaneously in quadruplicate by employing 4 plates of 24 baskets each. Preliminary experiments established that a stable baseline release was achieved by 10 min incubation time in wells. After this initial 10 min incubation, baskets were transferred to another set of wells containing MKR, this time containing drug as appropriate, for 10 min. The tissues were then stimulated to release [^{3}H]NE by transferring the baskets to wells containing 20 mM potassium plus or minus drug as appropriate for 10 min. Finally, tissue was transferred to wells containing 0.2 N HCl for 45 min to extract the remainder of the loaded [^{3}H]NE. All releasates were sampled and counted, and fractional release calculated according to the total amount of radioactivity in the tissue samples during the release interval.

RESULTS

Both mu and kappa selective opioids produced a dose-related depression of stimulated [^{3}H]NE release. The maximum inhibition produced by DAGO was 60% in animals which received saline containing pumps, and that produced by U50,488H was about 85% (Fig 1). The inhibition Of release produced by the mu agonist DAGO was more readily reversible by naloxone than the inhibition produced by the kappa agonist U50,488H, consistent with their action through mu and kappa receptors, respectively. However, at doses of agonist greater than 500 nM, the inhibition produced by either agonist could not be completely reversed. In the absence of any added agonist, naloxone did not produce an increase in control resting or stimulated release at doses between 1 and 1000 nM.

In the cortices of animals chronically treated with morphine, the maximum achievable depression by DAGO was about 40%, but that by U50,488H was unchanged at 85% (Fig 1). The shape of the DAGO dose response curve was changed in the morphine tolerant tissues relative to that in controls. Whereas in cortex from naive animals, DAGO produced a broad displacement over the concentration range 0.05 - 500 nM in chronic morphine treated animals, lower concentrations showed similar potency in inhibiting the release of [^{3}H]NE while higher doses produced relatively little additional depression. At concentrations of 5.0 nM and above, means of values representing per cent control release from tissues of saline treated (n = 6) animals were significantly different from those from chronic morphine treated (n = 3) animals as determined by a two-tailed T test ($p < 0.05$). The T statistic employed a pooled variance estimate derived from a two way analysis of variance. As in the control animals, in the absence of added agonist, naloxone failed to increase resting or stimulated

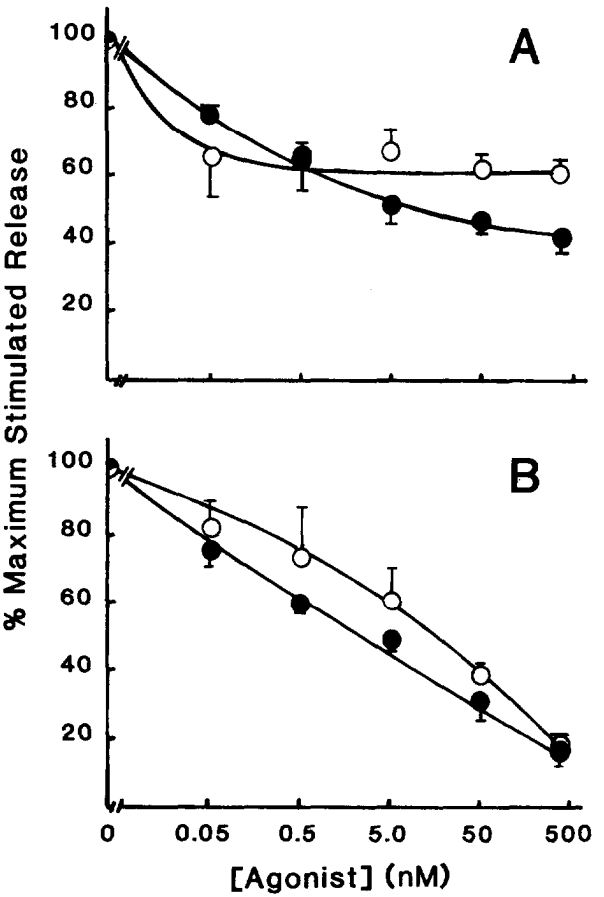

Fig. 1 Inhibition of the stimulated release of [^{3}H]NE by DAGO (A) and U50,488H (B) in chronic morphine treated (○) and saline treated (●) guinea pigs. Data are expressed as percent of release of [^{3}H]NE elicited by 20 mM K^+ in the absence of any added agonist. Control release from the cortices of morphine tolerant animals was approximately 70% that of naive animals. Data are means from three morphine treated and three naive animals.

[^{3}H]NE release in tissue from chronic morphine treated animals. Thus the overall effect of the chronic treatment was a reduction in the maximum effect of the mu agonist, while the inhibition of [^{3}H]NE release by the kappa agonist was not changed significantly.

DISCUSSION

The data presented suggest that both mu and kappa opioid receptors may be involved in the regulation of release of norepinephrine in the cortex of the guinea pig. These observations are consistent with electrophysiological studies on neurons of the locus coeruleus and receptor binding studies. Presumably opioid peptides within the brain are available to interact with the receptors which modulate this release. However, naloxone was not able to increase either resting or stimulated release, suggesting that the release of norepinephrine from the cortex is not under tonic suppression by these opioids. In the morphine dependent state, the regulation of release of this neurotransmitter by opioids which work through interaction with mu type receptors is apparently altered. DAGO produces a lower maximum inhibition of release in the morphine tolerant animal. However, the ability of kappa opioids to inhibit release remains relatively unaffected. This result is not unexpected since morphine exerts its effects primarily through mu opioid receptors. Further characterization of the effects of opioids on release of catacholamines in the dependent state may help to elucidate adaptive mechanisms of tolerance and dependence.

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TOLERANCE TO OPIOIOS IN SINGLE LOCUS COERULES NEURONS OF THE RAT

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ABSTRACT

Chronic treatment of rats with morphine caused tolerance to the membrane hyperpolarizing action of morphine in single locus coeruleus neurons in vitro. Tolerance was less pronounced for Try-D-Ala-Gly-MePhe-Gly-ol (DAGO) than for normorphine. No changes were found in affinity of naloxone for μ-receptors on these neurons, nor in the properties of the potassium conductance increase caused by μ-receptor activation. These results suggest that the mechanisms underlying tolerance involve either a reduction of the number of μ-receptors on each cell, or a reduced coupling of μ-receptors to potassium channels.

INTRODUCTION

Opioid inhibition of firing of locus coeruleus (LC) neurons is due to membrane hyperpolarization (Williams et al. 1982). This hyperpolarization results from increase in conductance to potassium ions and is brought about by activation of p-receptors (Williams et al. 1982; Williams and North 1984). α_2-Adrenoceptor agonists also hyperpolarize LC neurons by increasing the same potassium conductance as that which is activated by μ-receptor agonists (North and Williams 1985). The mechanisms underlying the development of opioid tolerance could involve adaptations of μ-receptor binding sites, the coupling of binding sites to potassium channels, potassium channels themselves, or subsequent processes. Some of these possibilities were investigated in the present study by measuring the potassium conductance increased by μ-receptor and α_2-adrenoceptor agonists in LC neurons from rats chronically treated with morphine.

METHODS

Rats were treated with morphine by subcutaneous implantation of morphine-containing pellets (75 mg base) on alternate days for 7 days (1,3,5 and then 7 pellets/day). Intracellular recordings were made as previously described (North and Williams 1985) from the LC of rats killed 2 to 4 days after the last implantation. Slices of pons containing the LC were submerged in a continuously flowing artificial cerebrospinal fluid at 37°C to which drugs could be added. Membrane current was measured by single electrode voltage clamp technique (Axoclamp II)(North and Williams 1985). All data are presented as mean ± SEM. The unpaired Student t-test was used.

RESULTS

Membrane properties of LC neurons from animals chronically treated with morphine were not distinguishable from controls. Both fired action potentials spontaneously with amplitudes close to 80 mV, which arose from a threshold potential of -55 mV and had durations of about 1.5 ms. Steady-state conductances were plotted directly on an X/Y recorder by applying a ramp potential change (-130 to -45 mV, at 1 mV/s). Cells from both control and treated animals had similar steady-state current/voltage (I/V) relationships. Between -90 and -60 mV the I/V plot was linear (resting conductance denoted G_{rest}, table 1). At potentials negative to -90 mV the slope of the I/V relation was increased (inward rectification).

	Control	Morphine-treated
Grest (nS)	8.34 ± 0.51 (38)	7.59 ± 0.54 (27)
G_{op} (nS)	3.35 ± 0.28 (38)	2.79 ± 0.24 (27)
I_{op} (pA)	286 ± 19.3 (38)	242 ± 20.0 (27)
$I_{op}/I_{\alpha 2}$	1.16 ± 0.05 (14)	1.10 ± 0.05 (11)

TABLE 1. Membrane properties of LC neurons following chronic treatment with morphine. G_{rest} is resting conductance between -60 and -90 mV. G_{op} is maximal additional opioid conductance at same potential range. I_{op} is maximal opioid current at -60 mV. $I_{\alpha 2}$ is maximal current induced by UK14304 or noradrenaline at -60 mV. Number of cells in parentheses. No significant differences were found.

The increase in conductance induced by maximal concentrations of DAGO (3 μM) or Met-enkephalin (ME, 30 μM) was determined both by measuring the outward current induced by superfusion of the agonist while holding the membrane potential at -60 mV, and by constructing steady-state I/V plots in the absence and presence of DAGO (3 μM) or ME (30 μM) and then subtracting the resting conductance (table 1) from that found in the presence of agonist over the same potential range (-60 to -90 mV). The increase in conductance and outward current induced by DAGO (3 μM) or ME (30 μM) was not different from that induced by maximal concentrations of α_2 agonists (noradrenaline in the presence of cocaine (10 μM) or 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK14304, 1 μM)). $I_{\alpha 2}$ (table 1) was the same in cells from control and morphine-treated rats.

The EC_{50} for DAGO was shifted to the right two-fold in the morphine-treated group although the maximal outward current induced by DAGO was not significantly affected (figure 1). In control tissues, the maximum outward current induced by normorphine (30 μM) was similar to that produced by DAGO. In contrast, the maximum response evoked by

normorphine in cells from morphine-treated rats was only 55 ± 2% (figure 1) of that produced by DAGO, and the EC_{50} was shifted to the right two-fold (figure 1). The degree of tolerance to normorphine ranged from about six-fold at the foot of the concentration-response curve to infinite at about 55% of the maximum response (figure 1). Such reductions in the maximal response to normorphine were observed for up to 6 h after removal of tissue from the animal.

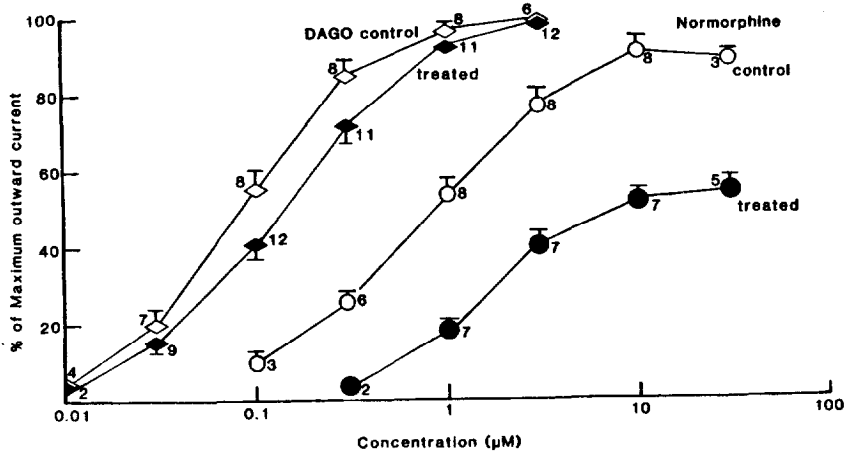

FIGURE 1. Maximum outward currents induced by superfusion of DAGO and normorphine following chronic morphine treatment are expressed as percentage of maximum possible current for the same cell. EC_{50} for DAGO were: control 91 ± 17 nM (8); treated 167 ± 26 nM (11)($p \leq 0.05$). EC_{50}'s for normorphine were: control 0.91 ± 0.23 μM (8); treated 1.9 ± 0.17 μM (7) ($p \leq 0.005$).

Naloxone was without effect on the membrane current of neurons for either control or morphine-treated animals. Concentration-respone relations for DAGO or ME were constructed in the presence of increasing concentrations of naloxone in both control and treated tissues. Chronic morphine treatment had no effect on the naloxone pA_2 (control 8.8 ± 0.1 (10): treated 8.7 ± 0.1 (8); Schild analysis or method of Kosterlitz and Watt 1968).

DISCUSSION

The present results demonstrate that chronic treatment of rats with morphine induces opioid tolerance in single LC neurones when their properties are later measured in vitro with an intracellular electrode. These results are consistent with extracellular recordings of LC neurons both in vivo and in vitro (Aghajanian 1978; Andrade et al. 1983).

Because no significant effects of treatment were observed on the properties of the potassium conductance, nor on the ability of maximal concentrations of full μ-or α_2-agonists to increase potassium conductance, it can be concluded that the mechanisms underlying tolerance do not directly involve the potassium channel which is activated by μ- and α_2-receptors. More likely, the mechanisms underlying tolerance development occur between p-receptor and potassium channel.

Two conclusions can be drawn from the effects of naloxone on LC neurones following chronic morphine treatment. Firstly, consistent with extracellular recordings from LC slice preparations (Andrade et al. 1983), there was no change in membrane conductance or potential following application of naloxone to tissue from treated animals. With extracellular recording from LC neurones in vivo, a meagre increase in firing rate was observed (Aghajanian 1978). This discrepancy could result from the presence of circulating morphine, or from increased activity of excitatory afferents to the LC during in vivo experiments. Secondly, no change was observed in the affinity of naloxone for μ-receptors.

The observation that tolerance to normorphine is more pronounced than that to DAGO following chronic treatment is consistent with the interpretation that the site of adaptation resides between k-receptor occupancy and potassium channel activation. This interpretation is based on two findings. First, full activation of potassium channels in normal LC neurons occurs with low fractional occupancy of μ-receptors by full agonists (Williams and North 1984), i.e. the tissue posesses considerable receptor reserve. Second, normorphine must occupy a greater fraction of μ-receptors than DAGO to elicit an equivalent response; ie. it posesses lower intrinsic efficacy (Miller et al, 1986). Thus, in control tissue both normorphine and DAGO are capable of eliciting the same maximal response since there is considerable receptor reserve. However, in treated tissue normorphine is no longer capable of eliciting the same maximal response as DAGO because fewer receptor sites are present, or because coupling of receptor to channel has become less efficacious. In either case the net result is a decrease in the μ-receptor reserve of LC neurons following chronic morphine treatment.

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EFFECT OF MATERNALLY ADMINISTERED OPIATES ON THE DEVELOPMENT OF THE β-ENDORPHIN SYSTEM IN THE OFFSPRING

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ABSTRACT

Pregnant rats received subcutaneous implantation of either morphine or placebo pellets at various stages of gestation. Untreated animals served as basic controls. The effect of morphine treatment on the growth of the offspring as well as on the ontogeny of β-endorphin in the pituitary gland and the hypothalamus was investigated. Results obtained indicated that pregnant rats treated with morphine from day 1 of pregnancy to day 21 presented complete resorption. Subcutaneous implantation of a morphine pellet on days 11, 13 and 19 of gestation allowed pregnancy to term, however the post-natal mortality rate was very high. Implantation of a morphine pellet on days 9 and 13 of gestation allowed pregnancy to term and after birth the withdrawal reaction of the offspring was mild and the mortality rate was low. At early stages of development there were differences in the pituitary and hypothalamic β-endorphin content between the offspring of the morphine treated animals and the offspring of the control animals, but by day 22 no significant differences among the various groups were noticed.

INTRODUCTION

Prenatal exposure to morphine has been shown to induce a number of morphological and behavioural changes (Kirby and Holtzman 1982; Lichtblau et al. 1984). Since prenatal exposure to opiates alters the responsivity of mature rats to noxious stimuli e.g. hot plate, inducing a decreased analgesic response (Lichtblau et al. 1984), opiates may alter the development and function of the endogenous opiate system (opiate peptides and opiate receptors) in the pituitary gland and specific areas of the brain. Thus the objective of the present studies was to investigate the effect of exposure to morphine in utero on the ontogeny of the β-endorphin related peptides in the pituitary gland and hypothalamus.

MATERIALS AND METHODS

Female Sprague Dawley rats at the stage of late proestrous were placed individually into breeding cages with experienced male breeders (Sprague Dawley). The following morning the presence of sperms in the vagina was tested. If sperms were detected in the vagina this was considered as day 1 of pregnancy. The pregnant animals were divided into three major groups. Animals of group I received subcutaneous (s.c.) implantation of morphine pellets (75 mg morphine base) at the following stages of gestation: (a) on days 1, 4, 7, 10, 13, 16 and 19; (b) on days 11, 13 and 19; and

(c) on days 9 and 13 of gestation. Animals of group II received s.c. implantation of placebo pellets while animals of group III received no treatment and served as basic controls. All animals were given water and food ad lib. Following birth the offspring were counted and weighed. On day 2 of post-natal life the number of offspring per litter were limited to eight. The offspring were weighed and sacrificed on days 1, 4, 8, 14 and 22 post-natally. The pituitary and hypothalamus were dissected free and extracted by sonication in 0.2 N HCL with protease inhibitors (Gianoulakis and Gupta 1986). The extracts were stored at -75°C for estimation of the total content of immunoreactive β- endorphin. Some animals receiving a morphine or a placebo pellet on days 9 and 13 as well as some untreated control animals, were sacrificed on days 19 and 20 of gestation. The pituitary and hypothalamus were dissected and extracted as described previously. For the estimation of the total β- endorphin like inununoreactivity β-EPLIR) in the peripheral circulation trunk blood was collected at the time of sacrifice, centrifuged and the serum was stored at -75°C till the time of radioimmunoassay. The content of β-EPLIR in the tissue extracts and the unextracted serum was estimated by radioimmunoassay using a specific antiserum to β- endorphin at 1:30,000 final dilution (Gianoulakis et al. 1981). The specificity of the antiserum was directed to the C-terminal portion of β- endorphin (Gianoulakis et al. 1981).

RESULTS

In general, prenatal exposure to morphine increased the rate of mortality of the offspring. Animals receiving morphine treatment from day 1 of pregnancy and throughout the whole gestation period presented complete resorption. In fact, the process of resorption took place at the early stages of pregnancy as was shown by the changes in body weight. Subcutaneous implantation of a morphine pellet on days 11, 13 and 19 allowed pregnancy to term, however, the mortality rate was very high (≈66%). Implantation of a morphine pellet on days 9 and 13 of pregnancy allowed pregnancy to term and after birth the withdrawal reaction of the offspring was mild, and the mortality rate was low (≈10%). Offspring of morphine treated animals had lower body weight at the time of birth, however at later stages of development no significant difference was observed among the various groups. On days 19 and 20 of gestation as well as on day 4 post-natally, the content of β- EPLIR in the pituitary gland was lower in the offspring exposed to morphine in utero. No significant difference was noticed in the other stages of development (figure 1). The pattern of ontogeny of β- endorphin was similar in the pituitary of the three treatment groups, showing a sharp increase between days 1 to 4, a smaller increase between days 4 to 8 and a sharp increase between days 14 to 22. On days 19 and 20 pre-natally and day 1 post-natally, a lower content of β- EPLIR was found in the hypothalami of the animals exposed to morphine in utero, while on day 4 post-natally a higher content of β- EPLIR was noticed in the hypothalami of the offspring of both the morphine and the placebo treated animals (figure 1). No significant difference in the content of

β-EPLIR in the hypothalamus was noticed at the other stages of development investigated. Likewise to the pituitary gland, a similar pattern of the ontogeny of β-endorphin in the hypothalamus was presented by the offspring of all treatment groups, showing a small rate of increase between days 4 to 8 followed by a sharp increase between days 8 to 14 and 14 to 22. On day 1 post-natally the content of β-EPLIR in the serum of the offspring exposed to morphine in utero was significantly higher than in the offspring of the control groups. No significant difference was noticed in the serum β-EPLIR content at the other stages of development among the various groups.

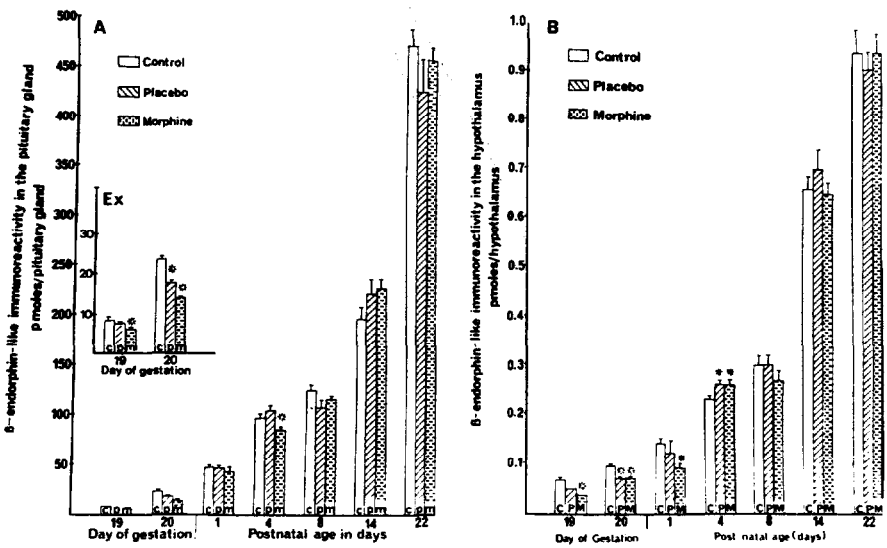

FIGURE 1. Content of β-endorphin-like immunoreactivity in the pituitary gland (A) and hypothalamus (B) at various stages of pre-natal and post-natal development. Ex = Expanded scale. *Significantly different from controls $p \leq 0.05$

DISCUSSION

The present results clearly indicate that pre-natal exposure to morphine may induce pre-natal death and increase the post-natal

mortality. The high incidence of post-natal mortality may be attributed to the withdrawal reaction of the offspring following birth, however, the cause of the pre-natal mortality is not clear. Since offspring of animals receiving s.c. morphine pellets on days 9 and 13 showed mild withdrawal reaction and high survival rate, they were used for estimation of tissue and serum β-EPLIR content. Though a lower content of β-EPLIR was observed on pre-natal days 19 and 20 in the morphine exposed offspring at later stages of post-natal development no significant differences were noticed among the offspring of the three treatment groups. These observations indicate that the effect of pre-natal exposure to morphine on the β-EPLIR content in both the pituitary gland and the hypothalamus is transient and complete recovery to control levels occurs at early post-natal life (day 8). Similar changes in the hypothalamic β-endorphin content were noticed in the offspring of both the placebo and the morphine treated animals, suggesting that the changes observed in the content of β-EPLIR may be partially due to the stress of ether exposure and surgery. Furthermore, both the pituitary and the hypothalamus showed a slower rate of increase in the total content of β-EPLIR between days 4 and 8, the period of development known as the stress non responsive period (Childs et al. 1982). The higher content of serum β-EPLIR on day 1 of development observed in the offspring exposed to morphine in utero, may be attributed to the withdrawal reaction following birth.

In summary, prenatal exposure to morphine alters the content of β-EPLIR in the pituitary and hypothalamus pre-natally and at early stages of post-natal life with complete recovery to control levels by day 8 post-natally. However, though no significant difference is present in the total pituitary and hypothalamic β-EPLIR content, the ability of the β-endorphin system to respond to stress may have been modified by prenatal exposure to morphine.

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SUPPRESSION OF IMMUNOLOGICAL FUNCTIONS IN MORPHINE ADDICTED MICE

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ABSTRACT

Lymphocytes isolated from morphine addictedmice show a number of impaired immunological functions. They include Con A stimulated mitogensis, NK cell activity, plaque forming cell activity and delayed-type of hypersensitivity. In addition, the met-enkepalin and β-endorphin level in the lymph nodes of addicted mice were also depressed.

INTRODUCTION

The role of the opioid peptides in modulating immune response has received much attention since Wybran et al. (1979) first demonstrated the existence of opiate receptors in human blood lymphocytes. Since then, the action of the opioid peptides, particularly the enkephalins and β-endorphin, in stimulating a variety of immune response has been documented (Fischer and Falke 1984). Chronic opiate addicts are known to have higher rates of infection (Brown et al. 1974). In human as well as in animal studies, it has been shown that lymphocytes isolated from addicted subjects have depressedmitogenic activity (Brownetal. 1974, Ho and Leung 1979). As chronic addiction can also depressed the level of enkephalin and β-endorphin (Clement-Jones et al. 1979, Ho et al. 1980), it is notunreasonableto expect that there may be a causal relationship between the lower level of circulating opioid peptides and the depressed immune status of the addict. The present study aims to 1) establish if other immunological parameters, besides mitogensis, can be depressed by addiction and 2) to determine whether a depressed immune response is correlated with a lower level of opioid peptides in the lymphoid tissues.

MATERIALS AND METHODS

Addiction of animals: Inbred female Balb/c mice weighing between 25 to 30 gm were used in this study. The mice were randomly divided into three groups; untreated, saline-treated and morphine addicted. Addiction tomorphine was inducedby two daily injections of 1 ml saline containing morphine-HCl for three weeks. The dose used ranged from 10 to 50 mg/kg/day. As 30 mg/kg/day turned out to give the Optimal result, this dose was used for most of the experiments. The development of addiction was confirmed by behavioral criteria. The body weight of the animals remained constant throughout the entire experimental period and there was no sign of malnutrition.

Assay of immune functions: Con A stimulated mitogensis was performed according to Ng et al. (1978). Endogenous and activated natural killer (NK) cell activity wasmeasured by the release of Cr-51 0n

prelabeled YAC-1 cells. Plaque forming cell (PFC) activity was determined by the formation of plaques on lyzed sheep red blood cells after the addition of complement. Delayed-type of hypersensitivity was measured by the swelling of the foot pad when animals previously sensitized to sheep red blood cells were challenged with a dose of the antigen. Unless specified otherwise, lymphocytes isolated from the spleen were used in these studies.

Radioinmunoassay and enzyme assay: The level of met-enkephalin and β-endorphin in the lymph nodes were determined by RIA according to Clement-Jones et al. (1980) and Guillemin et al. (1977). Met-enkephalin degrading activity of lymph node membranes was determined by the rate of disappearance of met-enkephalin in the assay mixture. HPLC was used to separate met-enkephalin from contaminants and by-products.

RESULTS AND DISCUSSION

In this study we have used four imnune function tests to determine the effect of addiction on immunity. The results are presented in table 1 and 2 and fig. 1. In the Con A stimulation test, the mitogenic activity of lymphocytes isolated from addicted mice was significantly depressed. In order to prove that this effect was mediated by morphine, the antagonist, naloxone, was coadministered. As indicated in table 1, mitogenic activity was suppressed to a significantly lower level when animals were treated in this manner.

TABLE 1 Effect of Addiction on Con A Stimulated Mitogensis

Treatment	H-3 Thymidine Uptake ($\times 10^{-3}$ cpm/10^5 cells)	% of Untreated Control
Untreated	140 ± 2.8	100
Saline treated	140 ± 5.0	100
Morphine addicted	73 ± 7.0	52
Naloxone	131 ± 9.5	93
Morphine/nax	114 ± 4.5	81

The dose of morphine and naloxone used was 50 mg/kg/day. In the naloxone experiment, it was given immediately before morphine. Spleen cells were used in this study.

The suppressive effect of addiction on mitogensis was determined to be dependent on the dose of morphine used (data not shown). At a daily dose of 10 mg/kg, there was no significant suppression of activity. Only when the dose was increased to 30 mg/kg/day did the effect on mitogensis become noticeable. Other than dose, the organ from which the lymphocytes were isolated may also influence the results. Thus, cells isolated from the spleen were in general less suppressed than those isolated from the lymph nodes. This may be due to a difference in the response of the lymphoid tissue to neuroendocrine control (to be discussed later).

As in the case of Con A stimulated mitogensis, addiction also suppressed both NK and PFC activities (table 2). In the delayed-type

of hypersensitivity assay, addicted animals had a slower and less active response in general. Maximum swelling of the footpads was delayed by one day on the average and the degree of inflammation was never as severe as the controls (fig. 1).

Table 2. Effect of Addiction on Natural Killer Cell and Plague Forming Cell Activities

Treatment	NK Activity (% Cr-51 released) Endogenous	Activated	PFC per 10^6 Cells
Untreated	19	45	354
Saline-treated	20 (105)	41 (91)	364(102)
Addicted	14 (73)	25 (55)	77(22)

NK activity of animals was preactivated by injection of C. parvum (350 ug/mouse) 4 days prior to measurement. Similar results were obtained from at least 3 independent trails. Values in parethesis are % of untreated control.

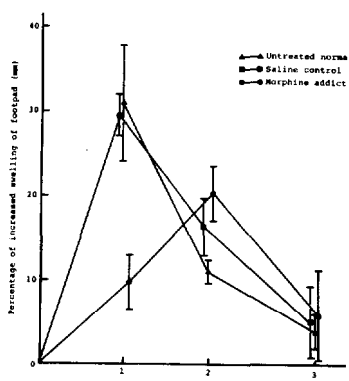

Fig. 1. Effect of Addiction on Delayed-Type of Hypersensitivity.

The mechanism of how opiate addiction can suppress immune response may be related to the inhibition of endogenous opioid peptide level. In both animal and human studies, it is known that chronic addiction can decrease the level of enkephalin and β-endorphin (Clement-Jones et al. 1979, Ho et al. 1980). Since these peptides can activate lymphocyte response (Wybran 1985), it is not unreasonable to speculate that factors which can reduce the level of opioid peptides would also have a negative influence on the immune system. To assess this, we have measured the level of met-enkephalin and β-endorphin in the lymph nodes of addicted and control animals. As indicated in table 3, the concentration of both peptides were significantly reduced in the addicted group.

Presumably, this would not be due to a higher level of degrading enzyme activity since membranes isolated from lymph nodes of addicted mice hydrolyzed met-enkephalin at the same rate as that of the controls. Further work is in progress to evaluate the role of

the opioid peptides in modulating immune responses.

Table 3 Effect of Addiction on Opioid Peptide Level and Met-Enkepalin Degrading Activity of the Lymph Nodes

Treatment	β-EP	Met-ENK	Met-ENK Degrad.Act.
Untreated	120 ± 6	1.82 ± 0.43	8.92 ± 4.75
Saline-treated	105 ± 22	1.75 ± 0.03	8.82 ± 3.68
Addicted	29 ± 11*	1.33 ± 0.22*	9.48 ± 2.74

β-EP was measured as ng/mg protein, met-ENK as ng/mg wet weight and met-ENK degrading activity as nmol/min/mg protein.
* A high level of significance of difference compared with control ($p<0.01$).

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MORPHINE METABOLISM IN MOUSE BRAIN

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ABSTRACT

Morphine UDP-glucuronyltransferase activity was demonstrated in the brain of mice from recombinant inbred strains of the BXD series. The formation rate of morphine-3-glucuronide was about 4 fold higher in the progenitor DBA as compared to the C57BL strain.

INTRODUCTION

Morphine is still the mainstay in the treatment of chronic severe pain. When used as an oral medication its effects vary between patients. To a large extent this is due to wide interindividual variation in the presystemic elimination of this drug (Säwe et al. 1985). The possibility that also the formation of active morphine metabolites may contribute to the pharmacological effects and its variation has previously been put forward (Joel et al. 1985, Osborne et al. 1986). A genetic difference in the quantitative metabolism as well as a difference in metabolic pattern would be compatible with a variation in effect. Whereas the major metabolite morphine-3-glucuronide (M3G) is inactive, the morphine-6-glucuronide (M6G) (Shimomura et al. 1971), and morphine-6-sulphate (M6S) (Mori et al. 1972) have been demonstrated to possess analgesic activity in mice. However, these metabolites represent minor metabolic pathways of morphine. The present study was designed to investigate if there is a measurable morphine UDP-glucuronyltransferase activity (UDP-GT) in the mouse brain and if the enzyme activity i affected by one or several genes.

MATERIALS AND METHODS

Microsomes from the whole male mouse brain were isolated by standard procedures. The glucuronidation assay was performed according to Pacifici et al. (1982). The morphine metabolites were analyzed by high performance liquid chromatography according to Svensson et al. (1982). The reproducibility of the whole assay was 11% determined as coefficient of variation from 5 repeated assays of the same sample. The detection limit of M3G in the incubate was 2 ng. The genetical model used was the BXD series of recombinant inbred (RI) strains and the two progenitor strains C57BL/6J and DBA/2J.

RESULTS

The morphine UDP-GT activity was measurable in the brain microsomes from the progenitor strains as well as in the brain of all the RI-strains. The reaction was linear with protein up to a concentration of 3 mg per ml incubate (fig 1). The reaction proceeded linearly with time from 1 to at least 4 hours (fig 1). The rate of glucuronidation was 4 times higher in the DBA than in the C57BL mice ($p<0.001$).

The variation between individuals was also larger in the DBA strain; 6.8 ± 4.0[1] pmol/mgxmin (n=10) versus 1.8 ± 0.68[1] pmol/mgxmin (n=9) in the C57BL strain. The morphine UDP-GT activity in the different RI-strains is shown in fig 2. Each bar represents one strain and the mean of four animals. As is seen in the figure, the activity profile from one experiment to another was not fully reproducible. The values of the RI-strains were within the range of the two progenitor strain values.

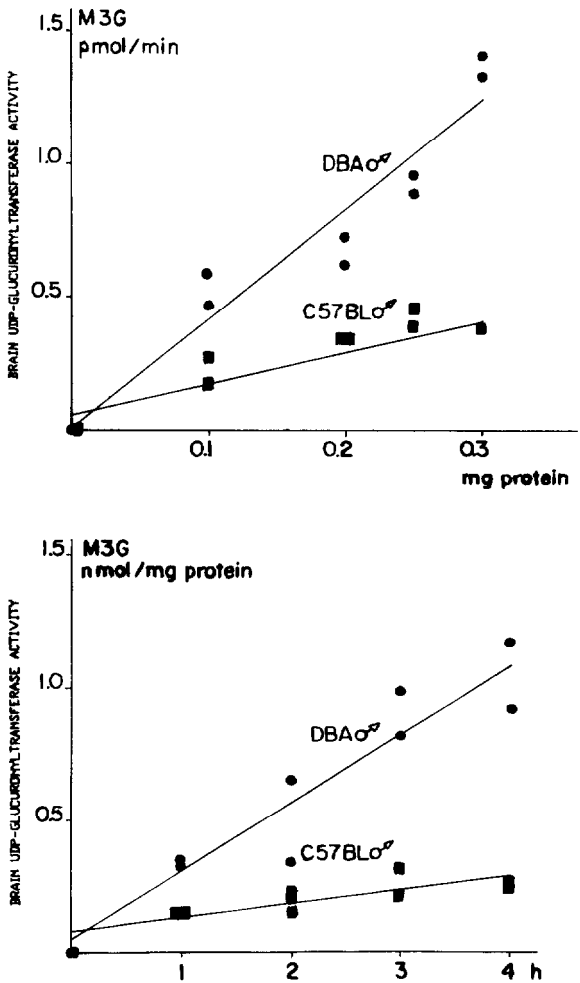

FIGURE 1.

Morphine UDP-GT activity in brain of DBA and C57BL mouse strains, at different protein concentrations (upper panel) and the time course of morphine-3-glucuronide (M3G) formation (lower panel).

Standard incubations (100 ul final volume) were carried out at 37°C for 2 hours, 0,2 mg protein in TRIS-buffer pH = 7.4 with 8.3mM Mg Cl_2, 3mM morphine and 15mM uridine diphosphoglucuronic acid (UDPGA).

[1] Mean ± standard deviation

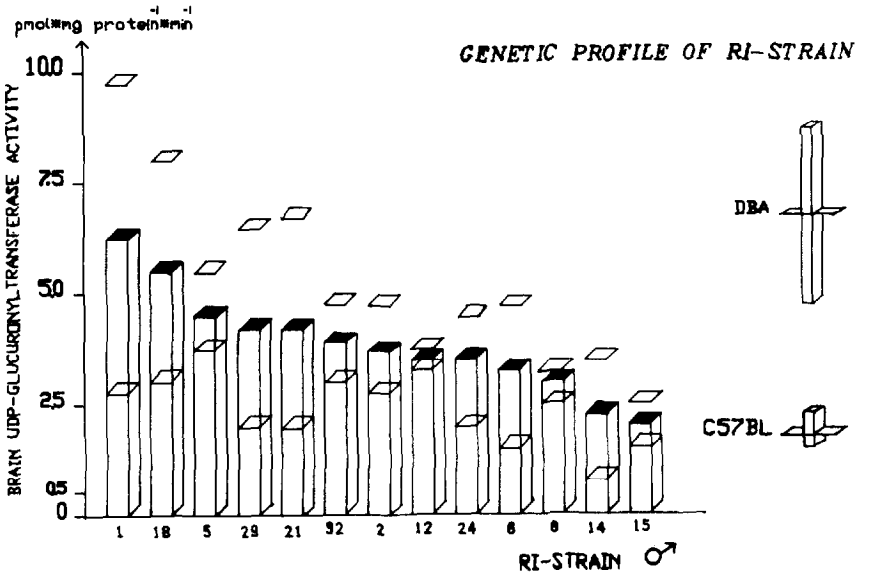

FIGURE 2. Morphine UDP-glucuronyltransferase activity in mouse brain. The profile of 13 RI-strains is shown. The averages of two experiments each including two animals are shown as black cuts in the histogram. The activities of the progenitor strains were determined in 10 (DBA) or 9 (C57BL) male individuals. Mean values and standard error of the mean for these are indicated to the right. (Details in fig 1.)

DISCUSSION

Morphine UDP-GT activity was measurable in brain microsomes from all strains tested. The activity differed by a factor of 4 in the two progenitor strains indicated a strong genetic influence. However, the distribution of morphine UDP-GT activity among the RI-strains does not allow us to easily interpret the mode of inheritance. One explanation might be that there is a family of morphine UDP-GT enzyme forms (Mackenzie et al. 1985), of which more than one contributes to the variation in our results. Environmental factors may also influence the activity. We did not attempt to monitor the crowding conditions in the cages which may bear importance for the brain metabolism. Such factors may effect certain pain mechanisms as suggested by Rodgers and Hendrie (1983). They showed that social conflict in male mice is a potent biologically relevant stimulus in the activation of endogenous naloxone-sensitive pain mechanisms. These authors also found that social status is an important determinant of nociceptive response to such experience. Indeed the DBA strains that had a higher and more variable activity "seemed" more aggressive than the other progenitor strain.

The hypothesis that different metabolic routes of morphine could be responsible for the analgesic effect and side effects has not been

fully investigated. The other pathways of morphine metabolism, as the formation of morphine-ethereal sulphate and normorphine are currently investigated in our laboratory. Although these metabolites are formed in minor amounts they may contribute quantitatively to the side effects by virtue of their high potency. In the light of the recent evidence of biosynthesis of morphine in the mammalian body (Donnerer et al. 1986) more information is neededd about the metabolism of this drug and its relation to the pharmacological effects.

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OPIOID REGULATION OF PHOSPHOINOSITIDE TURNOVER. M.E. Abood, N.M. Lee. H.H. Loh, Depts. of Pharmacology and Psychiatry, University of California, San Francisco, CA 94143, USA.

Since a number of studies indicate the involvement of Ca^{++} in the action of opioids, as well as a role for G proteins, the regulation of phosphoinositide (PI) turnover may be a possible second messenger system for opioids in the brain. This possibility was addressed by measuring PI turnover in rat brain cerebral cortical slices. In the presence of 10 µM etorphine, a small (23 - 83%) but significant ($p < .01$, $n = 9$) increase in all three products of PI hydrolysis was observed. It is worth noting that with smaller sample numbers ($n = 5$), it was difficult to demonstrate a significant increase in PI turnover in response to etorphine. However, with smaple numbers of $n = 9$ or more the increase was always statistically significant. Etorphine at 1 µM produced a 10% increase in phosphoinositides. The etorphine effect was a specific opioid effect, as it was reversible with 20 µM naloxone. This data indicate another second messenger system in the rat brain through which opioids may act.

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OPIOID REGULATION OF PHOSPHOINOSITIDE TURNOVER. M.E. Abood, N.M. Lee, H.H. Loh, Dept of Pharmacology, UCSF, San Francisco, CA 94143, USA

Since a number of studies indicate the involvement of calcium in the action of opioids, as well as a role for G proteins, the regulation of phosphoinositide (PI) turnover may be a possible second messenger system for opioids in the brain. This possibility was addressed by measuring PI turnover in rat brain cerebral cortical slices. In the presence of 10 µM etorphine, no significant increase in the three products of PI hydrolysis was observed (n = 15). It is worth nothing that with smaller sample numbers (n = 5), it was difficult to demonstrate reproducible effects on PI turnover in response to etorphine. The effect of etorphine on PI turnover in the midbrain was also examined.

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SYNAPTOSOMAL MEMBRANES CONTAIN A MEMBRANCE-BOUND FORM OF ENDOPEPTIDASE 24.15 THAT GENERATES LEU- AND MET-ENKEPHALIN FROM LARGER PEPTIDES. G.R. Acker, C.J. Molineaus and M. Orlowski. Dept. of Pharmacol., Mount Sinai Sch. of Med. New York, NY 10029.

There is evidence that enkephalins are derived not only from proenkephalin, but also from prodynorphin. A soluble metalloendopeptidase (SMEP) (EC 3.4.24.15) from rat brain was previously shown to cleave dynormphin A-(1-8) (D8); α- and β-neo-Enaorpnin (α- and β-nE) to Leu enkephalin (LE) (Chu and orlowski, Endocrinol., 116: 1418-1425, 1985). We have found that a membrane-bound form of this enzyme accounts for 20-25% of the total activity. The specific activity of this membrane-bound form was higher in synaptosomal membranes than that of endopeptioase-24.11 ("enkephalinase"). Purified synaptosomal membranes were incubated with the prodynorphin-derived peptides D8,α- and β-nE, and the proenkephalin-derived Met-enkephalin-Arg-Gly-Leu (ME-RGL), in the presence of bestatin, captopril and N-01-(R,S)-carboxy-2-phenylethyl]-Phe-p-AB, specific inhibitors of aminopeptioase, engiotensin-converting enzyme (EC 3.4.-15.1) and memberane-bound MEP (EC 3.4.24.11), respectively. After 3 h, the rate of enkephalin generation was greater than 70% from ME-RGL, D8, β-nE. A slower rate of conversion was observed for α-nE, consistent with the turnover rate constants found for the purified SMEP (above ref.). Addition of N-[1-(R,S)-carboxy-2-phenyletny1]-Ala-Ala-Phe-p-AB, an active site-directed inhibitor of endopeptidase-24.15 innibited LE and ME formation by 80% indicating the involvement of this enzyme in the observed conversions. These results demonstrate that synaptosomes contain a membrane-bound form of enaopeptidase-24.15 that efficiently generates enkephalin from proenkephailn and prodynorphin sources.

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EFFECTS OF MONOSODIUM GLUTAMATE ON THE LEVELS OF BETA-ENDORPHIN IMMUNOREACTIVE MATERIAL IN RAT DORSAL AND VENTRAL CAUDAL MEDULLA.

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Neuronal perikarya containing beta-endorphin immunoreactivity (BE-IR) are found in two regions of the brain, the hyopthalamic arcuate nucleus and nucleus tractus solitarius (NTS) in the dorsal caudal medulla (Khachaturian et al., Trends Neurosci. 8:111, 1985). BE-IR is also found in fibers in both the dorsal caudal medulla (DCM) and ventral caudal medulla (VCM). The exact origin of these fibers, i.e. arcuate versus NTS is currently not known. Biochemical studies which have characterized BE-IR in medulla have used either the entire caudal medulla or the DCM (Alessi and Quinlan, Peptides. 6 Suppl 2:137, 1985; Dores et al., Brain Res. In press). In order to clarify the contributions of the arcuate versus NTS to total BE-IR in caudal medulla, we have used monosodium glutamate (MSG) lesions which selectively damage the arcuate neurons. Neonatal male Sprague-Sawley rats were injected with MSG (4 mg/gm body weight) on postnatal days P2, P4, P6, and P8. Control animals were injected with 0.9% saline. At 100 days of age, the animals were decapitated and the brain stem rapidly frozen in powdered dry ice. The caudal medulla was cut at the central canal into DCM and VCM. Levels of BE-IR was determined with a BE RIA using antiserum Brenda (Drs. Watson and Akil). In the saline injection group BE-IR in the DCM and VCM was 7.53 ± 0.99 (mean ± SEM) and 4.23 ± 0.44, respectively, with BE-IR being significantly higher in the DCM (paired t-test. $p < 0.025$). MSG treatment resulted in a decrease in BE-IR in DCM (3.51 ± 0.53). but not in VCM (3.38 ± 0.67) (n.s.). A comparison of the DCM in the saline versus MSG groups demonstrates a significant drop in the BE-IR levels with MSG ($p<0.005$). A similar comparison of the VCM demonstrates no significant change ($p< 0.375$). The extent of cell damage in the arcuate nucleus and NTS was also determined immunohistochemically using a 16K antiserum. MSG and control rats were treated with colchicine prior to sacrifice. The preliminary results indicate a significant cell loss in the arcuate nucleus (approximately half of control), whereas no perikaryal loss was apparent in the NTS. The results of this study suggest that a significant portion of the BE-IR identified in the DCM oriainates from cells in the arcuate nucleus which might be affected by MSG. Furthermore: since no significant drop in BE-IR was noted in the VCM, BE fibers in this region of the medulla may originate either from NTS or portions of the arcuate nucleus not affected by MSG.

P152

HIGH EXTRACELLULAR K^+ ION AND THE DOPAMINE (DA) AGONIST BROMOCRIPTINE INHIBIT β-ENDORPHINS SECRETION FROM PERIFUSED RAT NEUROINTERMEDIATE (NIL) PITUITARY CELLS IN A VERY DIFFERENT MANNER. Richard G. Allen and Julianne Stack. Departments of Medicine and Physiology, Oregon Health Sciences University, Portland, Oregon 97201, U.S.A.

The secretion of β-endorphins derived from the pro-hormone pro-opiomelanocortin (POMC) is tonically inhibited by the neurotransmitter dopamine in vivo. It has been demonstrated that ion fluxes, voltage dependent ion channels and membrane potentials contribute to the mechanism(s) of release of neuropeptides from secretory vesicles. We measured the effects of potassium, sodium and bromocriptine on β-endorphins release from the NIL-cells to investigate the relationship between ion fluxes, membrane potential and neurotransmitter actions. Male rat NILs were enzymatically dispersed and perifused with a continuous flow for 5 hrs. Twenty min pulses of control medium were interrupted with 10 min pulses of experimental medium. Control medium was a complete medium with 5.4 mM KCl and 155mM NaCll. Experimental megiums were composed of control medium plus additives: high K^+=60.4mM KCl, high Na^+=209mM NaCl, bromocriptine(10^{-6}M). Two min fractions were collected and analyzed for β-endorphins immunoactivity. The high K^+ caused an immediate inhibition of basal release and in 10 min had reduced secretion to 40% of the expected basal release. When control medium was reintroduced, the secretion rate returned to 100% of the pre-inhibited rate. The high Na^+ had no significant effect on basal release, but when control medium was reintroduced there was an immediate, transient (8 min) increase of secretion, 407% above the predicted basal rate. Bromocriptine had an immediate 20-fold inhibitory effect on secretion of β-endorphins that was never reversed with control medium. The inhibitory effects of high K^+ were specific for this ion and reversible. Inhibition of secretion by bromocriptine was much greater than high K^+ and not reversible, implying that other intracellular mechanisms may be involved in the inhibitory effects of bromocriptine. (Supported by NIH grant NS 19397-02).

05

THE PURIFIED μ OPIOID RECEPTOR AND ITS RELATIONSHIP TO OTHER OPIOID-RELATED BINDING SITES. E.A. Barnard, C.D. Demoliou-Mason and Y. Wong, MRC Molecular Neurobiology Unit, MRC Centre, Cambridge CB2 2QH, U.K.

Soluble highly active opioid receptors from rat brain membranes have been purified by affinity chromatography on 14-β-(C-glycyl)-amido-(N-cyclopropyl-methyl)-norcodeinone (GANC) immobilized on Affi-Gel-10, or on D-ala^{2}-leu^{5}-enkephalin chloromethylketone (DALECK) immobilized on thiol-Sepharose 4B. After elution by opiates and a lectin affinity chromatography step, the completely purified receptors bind opiate agonists and antagonists with high affinity and also opioid peptides, the latter with lower affinity. The subtype specificity depends upon the conditions used in the chromatography. The purified receptor from either column shows a single protein band (apparent mol. wt. 65,000) after SDS-gel electrophoresis and silver staining, when the affinity chromatography was in the presence of Mg^{2+} and Na^{+}. [^{3}H]DALECK itself acts as a u-specific affinity reagent and labels a single polypeptide. Comparison will be made between the receptor subunits as identified thus in the membrane and after purification under various conditions. Separation from other opioid subtypes in solution, and from the initially-associated guanine-nucleotide binding protein, can be demonstrated. Approaches directed towards the cloning of the cDNAs encoding these receptor subunits will also be discussed.

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EXPRESSION OF OPIOID RECEPTORS IN CULTURED EMBRYONIC NEURONS IS REGULATED BY COMPONENTS IN THE CULTURE MEDIUM. J. Barg, R. Levy and R. Simantov, Department of Genetics, Weizmann Institute of Science, Rehovot 76100, Israel.

Cultured aggregating rat brain neurons express mu, delta and kappa opioid receptors (Lenoir, Barg and Simantov, Brain Res., 304, 285-290, 1984). A more recent study shows that chemical depolarization alters the number and possibly the type of receptors expressed (Simantov and Levy, Develop. Brain Res., 1986, in press). It appears, therefore, that the profile of receptors expressed by a given tissue, at least in culture, depends on its neurochemical activity. This study was undertaken to further analyze the factors that regulate the expression of these receptors. Hindbrain, forebrain or whole brain aggregating cell cultures were prepared from dissociated E-15 rat brain ceils and grown in serum containing or serum free media. ^{3}H-Diprenorphine was used to determine the total number of opioid receptors, and DAGO and DPDPE, the number of mu and delta sites, respectively. Cultures grown for only 7 days have different receptor density and subtype profile than cultures grown for 19 days. Serum alters the receptor number, and aggregates cultured in serum free medium have different response to depolarizing concentration of potassium ions than serum containing cultures. Moreover, opiate antagonists have a selective effect on the number of different receptor subtypes. These and additional data using cultures treated with conditioned media from several cell sources indicate that the phenotypic expression of opioid receptors in culture may be regulated by multiple factors.

P110

POSSIBLE IMPLICATIONS OF PITUITARY THYROTROPIN RELEASING HORMONE RECEPTORS IN MORPHINE TOLERANCE IN THE RAT. Hemendra N. Bhargava, Sumantra Das, M. Bansinath and R. Prasad, Departments of Pharmacodynamics and Pathology, University of Illinois at Chicago, Health Sciences Center, Chicago, Il 60612, U.S.A.

The effect of chronic treatment of rats with morphine and subsequent withdrawal on brain and pituitary receptors for thyrotropin releasing hormone (TRH) was investigated. Rate were implanted with four morphine pellets (75 mg each) during a three day period. Placebo pellet implanted rate served as controls. Both tolerance to and dependence on morphine developed as a result of this procedure. The binding of ^{3}H-MeTRH to membranes prepared from whole brain minus cerebellum was unaffected in morphine tolerant-dependent rata as demonstrated from the unaltered K_d and B_{max} values when compared to placebo pellet implanted animals The binding of ^{3}H- MeTRH to five different regions of the brain am well as pituitary were also investigated. Binding of ^{3}H-MeTRH to pituitary was increased during development of morphine tolerance and naloxone preceipitated withdrawal, whereas, binding to the different regions of brain was unaffected under these conditions. There was a decrease in circulating levels of thyroid hormones (T_8 and T_4). However, serum TSH level remained unaltered. In contrast, 24 hour withdrawal of morphine renulted in an increase in ^{3}H-MeTRH binding in pons + medulla whereas binding in other regions of the brain as well as in pituitary was unaffected. Serum T_3, T_4 and TSH levels were also unaffected during 24 hour withdrawal of morphine. The results suggest that the development of tolerance to morphine may be associated with changes in the pituitary- thyroid axis (Supported by a grant DA-02698 from the National Institute on Drug Abuse).

013

SELECTIVE INHIBITION OF OPIOID BINDING BY FAB FRAGMENTS FROM A MONOCLONAL ANTIBODY DIRECTED AGAINST THE OPIOID RECEPTOR. J.M. Bidlack and W.E. O'Malley, Center for Brain Research, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

Fab fragments from a monoclonal antibody, OR-689.2.4, directed against the opioid receptor selectively inhibited opioid binding to rat and guinea pig neural membranes and to the NG108-15 neuroblastoma x glioma cell line. In a titrable manner, the Fab fragments noncompetitively inhibited the binding of the μ selective peptide, [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, and the δ selective peptide, [^{3}H][D-Pen^2,-Pen^5]enkephalin, to neural membranes. The Fab fragments were competitive inhibitors of [^{125}I-Tyr27]β-endorphin binding to neural membranes and to membranes from the NG108-15 cell line. In contrast, κ opioid binding, as measured by the binding of [^{3}H]bremazocine to guinea pig cerebellum in the presence of μ and δ blockers, was not significantly altered by the Fab fragments. The OR-689.2.4 Fab fragments blocked the binding of [^{3}H]bremazocine to μ and δ binding sites. In addition to blocking the binding of μ and δ ligands, the Fab fragments displaced bound opioids from the membranes. When μ sites were blocked with [D-Ala^2,MePhe^4,Gly-ol^5] enkephalin, the Fab fragments suppressed the binding of [^{3}H][D-Pen^2,D-Pen^5]enkephalln to the same degree as when the μ binding site was not blocked. The Fab fragments also inhibited binding to the μ site regardless of whether or not the δ site was blocked with [D-Pen^2,D-Pen^5]enkephalln . This monoclonal antibody is directed against a 35,000 dalton protein. Since the antibody is able to inhibit μ and δ binding but not κ opioid binding, it appears that this 35,999 dalton protein is an integral component of μ and δ opioid receptors but not κ receptors.

P133

INTERACTIONS OF A MORPHINE (μ) RECEPTOR SPECIFIC LIGAND, [^{3}H]Tyr-Pro-NMePhe-D-Pro-NH_2, WITH RAT BRAIN MEMBRANES. S. G. Blanchard, P. Lee, J. S. Hong and K.-J. Chang, Molecular Biology Department, The Wellcome Research Laboratories and N.I.E.H.S., Research Triangle Park, NC 27709, U.S.A.

The highly u-receptor selective ligand Tyr-Pro-NMePhe-D-Pro-NH, (PL017) was custom tritiated and its binding to rat brain membranes was directly measured. The data were well fit by a model assuming the existence of a single homogenous population of binding sites. Scatchard analysis yielded values for Kd and binding sites of 6 nM and .16 pmole/mg, respectively. As expected for binding to an opiate receptor, addition of sodium, magnesium and guanyl nucleotides to the assays resulted in a modulation of ligand binding. In all cases tested, however, no significant deviations of the Scatchard plots from linearity were observed. Furthermore, displacement of ^{3}H-PL017 by all opioid ligands tested was monophasic, consistent with simple competitive inhibition at a single binding site. The IC_{50} values thus obtained showed good agreement with previously published values determined using less selective radiolabeled ligands. The regional distribution of ^{3}H-PL017 binding to rat brain was examined by in vitro autoradiography. The labeling pattern was similar to that seen for other μ-type ligands. Because of its high selectivity, the binding of ^{3}H-PL017 to the μ-type receptor can be measured directly without the need to suppress binding to other sites. As such, ^{3}H-PL017 should be a useful ligand for dissecting the actions of multiple opiate receptors.

P17

IRREVERSIBLE BINDING OF IODO AZIDO DTLET TO RAT BRAIN DELTA OPIOID RECEPTOR.

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Azido DTLET (Tyr-DThr-Gly-pN_3Phe-Leu-Thr) is a photoactivable derivative of the delta specific ligand DTLET and is itself a potent opioid agonist. The iodination of Azido DTLET by the chloramine T method, followed by separation over reverse phase HPLC, yields a pure monoiodinated species, as verified by mass spectrometry. ^{125}I Azido DTLET displays high affinity, levorphanol displaceable binding to rat brain membranes. The behaviour of the ligand in inhibition experiments is very similar to that of DTLET (Table). Irradiation with u.v. light (254 nm), following incubation with ^{125}I Azido DTLET, results in irreversible binding to several rat brain proteins. SDS polyacrylamide gel electrophoresis shows that one of these proteins (apparent molecular weight 43000) is not labeled when the incubation is carried out in presence of a high concentration of levorphanol. ^{125}IAzido DTLET also binds to rat brain slices (20 um thick) with a distribution similar to the localisation of delta receptors. ^{125}IAzido DTLET has also been used to label NG 108-15 cell membranes. The labelled proteins from both rat brain and this opioid receptor rich cell line do not display the same patern.

Inhibition of binding by DAGO, DTLET and ^{127}IAzido DTLET (Ki nM)

competing ligand	Radioactive ligand		
	^{3}HDAGO	^{3}HDTLET	^{125}I Azido DTLET
DAGO	3.9	700	668
^{127}IAzidoDTLET	25	1.4	2.5
	136	13.8	14.9

P29

KAPPA OPIOID BINDING SITES ON PITUITARY ASTROCYTES.

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Opioid binding sites in the rat pituitary are predominantly located over the neural lobe. We have demonstrated that the majority of these sites are of the kappa subtype, and are located on the pituitary astrocytes, the pituicyte. This unexpected observation raises the interesting possibility that astrocytes elsewhere in the CNS may also possess kappa opioid binding sites. A limited study suggests however that this is not the case. Kainic acid injection of the rat striatum induces a marked gliosis, but a concurrent reduction in the density of opioid binding sites. The rat striatum has only very low levels of kappa opioid binding sites. In contrast, the guinea-pig cerebellum ehibites a high density of these sites, located predominantly over the molecular layer (Robson et al. 84). Such a distribution would be consistent with a location on radial Bergman glia, a type of cerebella astrocyte. Once again, although injections of kainic acid produced a very considerable gliosis, there was an almost total abolition of kappa opioid binding sites.

These and other observations suggest that the pitucicytes may be unique among astrocytes in possessing binding sites for kappa opioid ligands. Pituicytes also exhibit a remarkable morphological plasticity, engaging in a dynamic interaction with the neurosecretory nerve terminals (Hatton et al. 84). It is interesting to speculate as to the role of this interaction in regulating neurohypophyseal secretion and as to the possible influence of opioids upon this relationship. As a first step in addressing these questions, and as part of a wider interest in the actions of opioids on astrocytes, we have attempted to establish pituicytes in tissue culture. A wide variety of culturing procedures have been evaluated and the resulting cells identified using immunohistochemical cell-specific markers. Monolayer cultures of three day old rat pituitary gave rise to a number of cell types, including a population of cells intensely expressing the astrocytic marker glial fibrillary acidic protein. The striking stellate morphology often displayed by these astrocytes made them easily identifible in histologically stained preparations. Autoradiograms produced from these cultures show that kappa directed ^{3}H-EKC binding was selectively localized over these cells.

It is our working hypothesis that these in vitro astrocytes are derived from the in vivo pituicytes, an hypothesis supported by the continuity of GFAP and kappa opioid binding site expression. We suggest that the cultured pituicyte preparation may provide a useful model to examine the action of kappa opioid agonists.

Robson et al 1984. Neurosci. 12, 621.
Hatton et al 1984. Peptides 5, 121.

015

DIRECT EVIDENCE FOR THE COMPLEXATION OF A GTP-BINDING PROTEIN WITH DELTA-OPIOID RECEPTORS. K.-J. Chang. 2. Y. Li and S. G. Blanchard, Molecular Biology Department, The Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.

Opioid-receptors in brain are regulated by GTP and cations. The binding affinity of opioid-agonists to receptors is decreased by GTP and sodium ions and increased by divalent cation, Mg^{2+}. In neuroblastoma cells, delta-receptors are similarly regulated by GTP and cations. Enkephalins inhibit the activity of adenylate cyclase in a GTP- and Na^{+}-dependent manner. Pretreatment of cells with pertussis toxin eliminates the opioid inhibitory effect on adenylate cyclase and decreases the affinity of agonists to delta-receptors. These effects of pertussis toxin are correlated with the ADP-ribosylation of a GTP-binding protein. These data suggest that delta-receptors are associated with a GTP-binding protein. In this study, we have used an opioid-affinity column to obtain direct evidence that suggests the complexation of a GTP-binding protein with delta-receptors. The solubilized receptors were assayed with ^{125}I-labeled [D-Ala^2, -D-Leu^5]enkephalin and the GTP-binding proteins were measured by the binding activity of ^{35}S-labeled GTPγS. Delta-receptors were solubilized from neuroblastoma cell membranes with 10 mM CHAPS and subsequently applied to an opioid-coupled affinity column. Nearly 90% of solubilized delta-receptors and significant amounts of GTP-binding proteins are retained by the affinity column. Significant activity of a GTP-binding activity was specifically eluted by [D-Ala^2,D-Leu^5]enkephalin or naloxone. No significant activity of either protein was retained and subsequently eluted in a control column. These data suggest that a GTP-binding protein can be adsorbed to an opioid-affinity column and subsequently be specifically eluted by opioid-ligands. This result provides direct evidence that a GTP-binding protein is a part of the delta-receptor system.

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017

RELATIONSHIP OF OPIATE RECEPTOR BINDING SITES AND OPIATE-INHIBITED ADENYLATE CYCLASE IN BRAIN MEMBRANES. S.R. Childers, P. Nijssen and J. Harris, Dept. of Pharmacology, Univ. of Florida Coll. of Med., Gainesville, Fl 32610.

Opiate agonists inhibit adenylate cyclase in both brain and neuroblastoma cell membranes. Adenylate cyclase appears to be coupled to delta opiate receptors in NG108-15 cells, but the pharmacological nature of the adenylate cyclase-linked opiate receptor in mammalian brain has yet to be established. To determine the relationship between high affinity multiple opiate receptor binding sites and opiate-inhibited adenylate cyclase in brain, we assayed adenylate cyclase in membrane preparations after treatment with various agents which are known to block high affinity opiate agonist binding. Incubation of membranes with phospholipase A (500 ng/ml) inhibited 93-100% of binding of [^{3}H]-DAGO, [^{3}H]-DSTLE and [^{3}H]-EKC to mu, delta, and kappa sites, respectively. However, adenylate cyclase in treated membranes was still inhibited by D-Ala enk (with inhibition blocked by naloxone); moreover, the D-Ala enk agonist dose response curve was not affected by phospholipase treatment. To obtain more specific receptor blockade, membranes were incubated with irreversible opiates, then washed to remove traces of reversibly-acting drugs; control membranes contained equal concentrations of naloxone to control for washing. Incubation of membranes with several irreversible opiates, including antagonists like naloxone azine and beta-FNA, and agonists like p-nitro-phenyl-oxymorphone, did not affect agonist dose response curves in inhibiting adenylate cyclase, but blocked high affinity receptor binding by 75-100%. However, incubation of membranes with beta-CNA (10 μM), which blocked binding by 95-100%, shifted the adenylate cyclase dose response curve for D-Ala enk by approx. ten-fold. These results indicate an irreversible loss in adenylate cyclase-linked receptors by beta-CNA, but not by other irreversible opiates, and suggest that receptors linked to adenylate cyclase in brain do not correspond to any of the sites labeled with nM concentrations of [^{3}H]-agonists.

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DEVELOPMENT OF A SELECTIVE MU_1 OPIATE RECEPTOR BINDING ASSAY.

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Previous studies have identified a binding site of very high affinity which bound both opiates and enkephalins with very high affinity. In most routine assays this binding site corresponded to approximately 20-30% of total specific binding, precluding detailed studies of mu_1 binding. Therefore, we have developed a simple binding assay in which greater than 75% of total specific binding corresponds to mu_1 sites. There are currently no suitable radiolabeled mu_1-selective ligands. An alternative approach is to obtain tissue enriched in mu_1 sites relative to either mu_2 or delta sites. Detailed computerized digital subtraction autoradiograms reveal relatively high levels of mu_1 and mu_2 sites in the thalamus, but few delta sites. We took advantage of the low density of delta sites in the thalamus by using ^{3}H[D-ala^2,D-leu^5]enkephalin which labels mu_1 and delta sites. The low density of delta sites relative to mu_1 sites increased the proportion of mu_1 to delta binding compared to whole brain homogenates. In this assay system, ^{3}H[D-ala^2,D-leu^5]enkephalin labels the mu_1 sites with an affinity constant (K_D) of 0.4 nM and a B_{max_2} of approximately 3 fmoles/mg tissue. Both morphine and [D-ala^2,D-leu^5]enkephalin compete ^{3}H[D-ala^2,D-leu^5]enkephalin binding with IC_{50} values under 1 nM and saturation studies performed in the presence and absence of morphine reveal competitive inhibition. Other opiates and opioid peptides also compete binding effectively. Mu_1 binding in this assay is very sensitive to sodium chloride and guanyl nucleotides and is irreversibly inhibited by naloxonazine. N-Ethylmaleimide at 25 μM lowers binding by greater than 50% and trypsin (1 μg/ml) virtually abolishes binding. The development of this assay system should provide a means of examining the mu_1 binding sites more extensively.

P35

A UNIT WHICH IS NOT SUBSTRATE OF PERTUSSIS TOXIN MAY MEDIATE SODIUM EFFECTS ON OPIOID RECEPTORS IN NG 108-15 CELLS. T. Costa, S. Ott and A. Herz, Department of Neuropharmacology, Max-Planck-Institut für Psychiatric, D-8033 Martinsried, F.R.G.

Target size analysis of the opioid receptor in NG 108-15 cells reveals that a molecular unit of 56 kDa accounts for the binding activity determined with ^{3}H-diprenorphine (^{3}H-DPR) in the presence of Na and GTP, whereas in the absence of these agents a structure of 98 kDa is measured. This suggests that an additional 42 kDa subunit is required for opioid binding in the absence of ions and nucleotide.

Na^+ and GTP have similar but not identical effects on the kinetics of ^{3}H-DPR binding: they both - and synergistically - appear to increase the proportion of a fast-dissociating form of the receptor, but only Na^+ enhances the absolute rate of association and dissociation. The slowly dissociating form is likely to represent in part a N-protein receptor complex, since in membranes exposed to low concentrations of N-ethylmaleimide (NEM) or obtained from cells pre- treated with pertussis toxin (PT) the receptor exists predominantly in a fast dissociating form and no or little GTP effect can be detected; however, the effect of Na^+ is still present. In these membranes, as well as in those from cells treated with cholera toxin (CT), the target size is identical to that assessed in native membranes, whether or not GTP is present in the binding reaction. Conversely, in the presence of Na^+, a 40% reduction of the apparent target size is observed in+ control, PT, CT, and NEM treated membranes. Thus, Na is the sole agent responsible for the change in apparent target size of the opioid receptor. We conclude that a ~ 42 kDa membrane unit, which is not substrate of bacterial toxins, is also necessary for high affinity binding of the opioid receptor and may mediate the effect of Na^+.

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09

DEMONSTRATION AND CHARACTERIZATION OF 3 SUBTYPES OF KAPPA OPIOID RECEPTORS IN BOVINE ADRENAL MEDULLA MEMBRANES.

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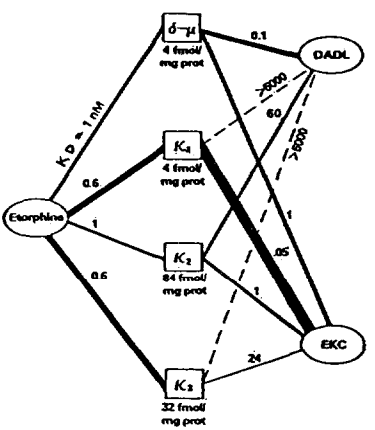
Opiopeptides and their receptors are present in high concentration in adrenal medulla,and may play a role in the regulation of catecholamine release and chromaffin cell function. Previous studies have shown predominance of κ (relative to μ and δ) sites, but there has been controversy about whether these sites represent 1, 2 or 3 classes of sites. We have used frozen bovine adrenal medulla membranes in Tris.Cl pH 7.4, 37°, 30 min with ^{3}H-etorphine (Et), ^{3}H-ethylketocyclazocine (EKC), and [^{3}H]-[D-Ala^{2},D-Leu^{5}]enkephalin (DADL) as tracers, displaced by all 3 unlabeled ligands. Studies were done "unblocked" (μ,δ, κ_1, κ_2, κ_3 sites), and with 100 nM DADL to block μ and δ sites, and with 1, 10 and 100μM DADL to progressively block the κ_2 (or benzomorphan) site. Mathematical modeling with the LIGAND computer program was used to analyze data from more than 100 curves, to compare models with 1 to 5 classes of sites. Results indicate 3 subtypes of κ receptors. κ_1 is selective for EKC relative to Etorphine and has binding capacity of 4 fmoles/mg prot. κ_2 is EKC-Etorphine nonselective (K_d=1 nM), and represents 80% of the sites (84 fmoles/mg protein) and can be blocked by 1 to 100 micromolar DADL. κ_3 is Etorphine selective, with a binding capacity of 32 fmoles/mg protein. DADL at high concentration (100 μM) does not block Etorphine or MC binding to κ_1 or κ_3.



Conclusion: Multiple subtypes of kappa opioid receptors are present in bovine adrenal medulla membranes.

P30

UPREGULATION OF BRAIN KAPPA OPIATE RECEPTORS IN SPONTANEOUSLY HYPERTENSIVE RATS. Sumantra Das and Hemendra N. Bhargava, Department of Pharmacodynamics, University of Illinois at Chicago, Health Sciences Center, IL 60612, U.S.A.

The binding of tritiated ligands for various opiate receptor subtypes to brain membranes prepared from spontaneously hypertensive rats and normotensive Wistar-Kyoto rats was determined. The density (B_{max}) or the apparent dissociation constant (K_d) for the binding of the mu-ligand (naltrexone) and delta-ligand (Tyr-D-Ser-Gly-Phe-Leu-Thr) to brain membranes of hypertensive and normotensive rats did not differ. However, the B_{max} for the binding of kappa-ligand (ethylketocyclazocine) to brain membranes after the suppression of mu- and delta-sites by 100 nM each of unlabeled D-Ala^2-MePhe^4-Gly-ol^5-enkephalin and D-Ala^2-D-Leu^5-enkaphalin, respectively, was significantly greater in hypertensive rats compared to normotensive rats. The K_d values for the binding of ^{3}H-ethylketocyclazocine (EKC) in the two groups did not differ. The binding of ^{3}H-EKC in brain regions was in the order: hypothalamus> midbrain> striatum> cortex> pons + medulla. The increase in the binding of ^{3}H- EKC in the brain of hypertensive rats compared to normotensive rats was due to increased binding in the hypothalamus and cortex. These results provide for the first time evidence for selective proliferation of kappa-opiate receptors in the brain of hypertensive rats, and suggest that brain kappa-opiate receptors may play an important role in the pathophysiology of hypertension. (Supported in parts by grants from the National Institute on Drug Abuse, DA-02598 and Chicago Heart Association A83-30).

P41

PRODYNORPHIN DERIVED PEPTIDES AND mRNA IN RAT LEYDIG CELL LINES.

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The primary structure of the rat precursor for dynorphin peptides has recently been deduced using cDNA hybridization technology. The precursor protein, prodynorphin, contains several opioid peptides that have been isolated as separate products from mammalian neural tissues. In addition, imnunoreactive dynorphins have been found in various reprductive tissues of rat, guinea pig and rabbit. In the rat testis, the immunoreactive dynorphin peptides have been localized to Leydig cells. These observations prompted us to determine the presence of prodynorphin mRNA and dynorphin peptides in two stably transformed rat Leydig cell lines, R2C and LC540. Prcdynorphin transcripts were observed in poly (A+) mRNA isolated from R2C cells but not in LC540 cells. Neither cell lines expressed the proenkephalin gene while both cell lines expressed the proopiomelanocortin gene. Immunoreactive dynorphin A and (Leu) enkephalin were measured in R2C cells by radioimmunoassay. The estimated concentration of 33fmol immunoreactive dynorphin A/mg protein in R2C cells corresponds to a tissue concentration of 14fmol/mg protein in rat testis. Trypsin and carboxypeptidase B treatment of the cell extract did not substantially increase the (Leu)enkephalin immunoreactivity suggesting the absence of any large molecular weight precursor that might have cross reacted poorly with the dynorphin antibody. The observations that the R2C cells contain prodynorphin transcripts and translated products suggest that this Leydig cell line could be used to study the dynorphin gene at the level of transcription, translation and the processing of the propeptide.

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THE ROLE OF ENDOGENOUS OPIOID PEPTIDES IN SUDDEN INFANT DEATH SYNDROME AND OTHER CLINICAL SITUATIONS. William L. Dewey, Edwin C. Myer, Dale Morris and Michael Adams. Departments of Pharmacology and Neurology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298 U.S.A.

Sudden infant death syndrome occurs in approximately two out of every one thousand births. The cause of death is not known but appears to be associated with respiratory malfunction. Since opioid peptides have been shown to cause respiratory depression and because at least one child with documented apneic spells has been treated successfully with a narcotic antagonist, we have investigated whether a correlation exists between β-endorphin levels in CSF and/or plasma and risk for SIDS. The CSF level of β-endorphin in the siblings of SIDS was 78.8 pg/ml, and children with interrupted SIDS had levels averaging 128.9 pg/ml. The CSF level of β-endorphin in children not at a high risk for SIDS (controls) was 32.8 pg/ml. The highest control value was below the lowest value in the high risk group. Thus no overlap existed between the two groups. Two children with high CSF levels of β-endorphin were treated with naltrexone, which resulted in normalization of life-threatening respiratory patterns. These results support the hypothesis that in humans endogenous opioids in excess may result in respiratory events which, under certain circumstances in infants, may result in SIDS. The level of β-endorphin in plasma was not elevated in the high risk group. CSF β-endorphins also were found to be elevated in five children with Rett's Syndrome, and in one child with nonketotic hyperglycemia with ongoing seizures. Two children admitted in status epilepticus had high endorphin levels. CSF β-endorphins would appear to be the result of CNS stress events and may have many etiologic causes. (Supported in part by USPHS Grant No. DA01647).

P42

EXPRESSION OF OPIOID PEPTIDE GENES IN MALE AND FEMALE REPRODUCTIVE TISSUES. J. Douglass, B. Quinn, B. Cox, O. Civelli and E. Herbert, Institute for Advanced Biomedical Research, The Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, U.S.A.

Recent studies suggest a functional role for opioid peptides as modulators of the hypothalamus-pituitary-gonadal axis at a variety of levels in both males and females. We report here the expression of all three opioid peptide genes in rat male and female reproductive tissues. Prodynorphin mRNA is present in the rat ovary, uterus and testis. Proenkephalin mRNA can be detected in the epididymis and vas deferens, in addition to the ovary, uterus and testis. POMC mRNA can be detected in the ovary, testis and epididymis. Immunohistochemical analysis reveals that prodynorphin derived peptides are expressed in Leydig cells in the testis. The distribution of immunoreactive dynorphins in reproductive tissues was also determined by RIA. Dynorphin A and B immnoreactivity was detected in the testis, epididymis and vas deferens of the rat, guinea pig and rabbit. These observations suggest that opioid peptides may exert paracrine and/or autocrine effects in mammalian reproductive tissues.

P108

ACTION AT THE MU RECEPTOR IS SUFFICIENT TO EXPLAIN THE SUPRASPINAL ANALGESIC EFFECT OF OPIATES.

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Mu, delta, and kappa opiate receptors have been implicated in the production of analgesia. In order to study the relative contributions of these receptor types to supraspinally-mediated analgesia, apparent pA_2 values and rank order potencies were determined for intracerebroventricularly-injected highly selective opioid agonists in the mouse using a thermal nociceptive assay. Drugs used included the prototypical mu agonist morphine, put tive mu agonists DAGO [D-Ala^2, N-methyl-Phe^4, Gly^5-ol] enkephalin and BAM 2 P, putative delta agonists DPDPE [D-Pen^2, D-Pen^5] enkephalin, DTLET [D-Thr^2, Thr^6, Leu^5] enkephalin, and DSLET [Tyr-D-Ser-Gly-Phe-Leu-Thr] enkephalin, and the putative kappa agonists U50488H [trans-3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulfonate hydrate] and dynorphin A (1-13).

We were unable to demonstrate significant analgesic potencies for intracerebroventricularly-injected (i.c.v.) dynorphin A (1-13) or BAM 22P in the absence of marked behavioral abnormalities. The rank order potency of the remaining compounds studied was found to be: DAGO > DTLET > DSLET > Morphine > DPDPE > U50488H. Apparent pA_2 values of morphine, DAGO, and DPDPE in naloxone antagonism trials did not differ significantly.

These results indicate that while both mu- and delta-selective ligands produce potent analgesia, a single receptor (mu) is sufficient to mediate the supraspinal effects of opiate drugs.

071

ABSENCE OF HIGH AFFINITY DELTA AND KAPPA BINDING IN RAT PERIAQUEDUCTAL GRAY ENRICHED P_2 MEMBRANE. J. P. Fedynyshyn, G. Kwiat. and N. M. Lee, Department of Pharmacology, University of California, San Francisco, CA 94143, U.S.A.

The periaqueductal gray (PAG) region of the midbrain has been implicated in both stimulation produced and opioid induced analgesia. In the present study the opioid binding characteristics of the PAG were examined with an in vitro radioligand binding technique. 2nM ^{3}H-Ethylketocylazocine (EKG) was used to nonselectively label μ, δ, and κ binding sites in PAG enriched P_2 membrane. The μ selective ligand [D-Ala^2,N-Methyl Phe^4,Glyol^5] enkephalin (DAGO) displaced ^{3}H-EKC from more than one population of binding sites with both high and low affinity. In contrast the δ selective ligand [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and the κ selective ligand U50,488 displaced ^{3}H-EKC from a single population of binding sites with low affinity. DPDPE and U50,488 also displaced 2 nM ^{3}H-DAGO from a single population of binding sites with similar low affinity. DAGO and not DPDPE displaced 2 nM ^{3}H-[D-Ala^2,D-Leu^5] enkephalin (DADLE) with high affinity. This binding data is consistent with the presence of a single population of μ selective high affinity binding sites in PAG enriched P_2 membrane to which δ ligands and κ ligands have low affinity. This data is also consistent with opioid induced supraspinal analgesia being a primarily μ opioid receptor mediated event. This research was supported in part by National Institute of Drug Abuse Grants DA 02643 and 1 F31 DA 05281-01(JPF).

P138

GASTRIC ACID SECRETORY EFFECTS SEPARATE OPIOID RECEPTOR TYPES.

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Morphine and the endogenous opioid peptides produce variable effects on gastric acid secretion. Part of this variability may be due to the activation of different opioid receptor types. The aim of the present investigation was to evaluate the effects of central and peripheral administration of the mu selective agonists DAGO and PL017, the delta selective agonist DPDPE, the kappa selective agonist U50,488H and morphine on basal gastric acid secretion in the rat. Gastric secretion was measured in 24 hour fasted, unanesthetized, pylorus-ligated rats. Drugs were administered immediately after pylorus ligation either intracerebroventricularly (ICV) through an indwelling guide cannula or intravenously (IV) through the tail vein. Eats were sacrificed 2 hours after drug administration and stomach volume and acid were measured. Acidity was determined by titration with 0.01M NaOH to a pH of 7.0. ICV administration of DAGO, PL017 and morphine produced a dose-dependent (0.3-10ug) decrease in both gastric secretion volume and acidity. In contrast, central administration of DPDPE (100ug) or U50,488H (600ug) did not alter gastric acid secretion. IV administration of morphine and PL017 (0.3-3mg/kg) significantly decreased gastric acid secretion. U50,488H administered IV caused a dose-dependent (1-10mg/kg) increase in gastric acid secretion, whereas DPDPE and DAGO did not alter gastric secretion when given IV. The central inhibitory effect of PL017 and the peripheral stimulatory effect of U50,488H on gastric acid secretion were antagonized by naloxone 3mg/kg administered subcutaneously 15 minutes prior to the agonists. These results suggest that stimulation of central and possibly peripheral mu receptors decreases acid secretion, stimulation of peripheral kappa receptors increases gastric secretion and delta opioid receptors are not involved in the regulation of gastric acid secretion. Because of the clear separation of gastric secretory effects observed with these selective agonists, measurement of gastric acid secretion may be useful as an *in vivo* bioassay to determine relative opioid receptor selectivity. (Supported by AM36289 and AM33547.)

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MOLECUIAR BIOLOGY OF ENKEPHALIN CONVERTASE (CARBOXYPEPTIDASE E).

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Enkephalin convertase (carboxypeptidase E) is the carboxypeptidase B-like enzyme involved in the biosynthesis of the opiate peptides, as well as other peptide hormones and neurotransmitters. This enzyme has been purified to homogeneity from bovine pituitary, and a partial amino acid sequence has been determined. Oligonucleotide probes corresponding to the amino acid sequence have been used to isolate a cDNA clone from a bovine pituitary cDNA library. The predicted amino acid sequence of the cDNA clone contains several pairs of basic amino acids, and displays some homology with carboxypeptidases A (CPA) and B (CPB). Although the overall homology is low, all of the amino acids thought to be essential for the catalytic activity of CPA and CPB have been conserved in enkephalin convertase. Major differences between enkephalin convertase and the other carboxypeptidases include an additional 120 amino acids at the C-terminal of enkephalin convertase, and numerous changes in amino acids near the substrate binding sites. These changes may reflect the different specificities of the enzymes: enkephalin convertase is very specific for the enkephalin precursors, and does not further degrade the enkephalin peptides. The homology between the carboxypeptidases suggests a common evolutionary origin, which supports the theory that the peptide processing enzymes existed before the first opiate peptides.

The cDNA encoding enkephalin convertase hybridizes primarily to mRNA of approximately 3.3 kb in bovine brain, pituitary, and adrenal medulla. Restriction fragment analysis of bovine genomic DNA indicates a single gene encodes enkephalin convertase. Results of studies on the regulation of this gene will be presented.

P 90

DYNORPHIN AND ANTERIOR PITUITARY HORMONES: EFFECTS ON PLASMA CONCENTRATIONS IN THE MALE RHESUS MONKEY. P. M. Gilbeau, Y. Hosobuchi and N. M. Lee, Departments of Neurological Surgery and Pharmacology, University of California, San Francisco, CA 94143, U.S.A.

The role of dynorphin-(1-13) and dynorphin-(1-10)-amide in the neuroendocrine control of primate anterior pituitary hormones was studied in nonrestrained, adult male rhesus monkeys. The effects of these opioids on plasma concentrations of prolactin (PRL), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyrotropin (TSH) and growth hormone (GH) were assessed. Dynorphin or its saline vehicle were administered and blood was collected through an indwelling jugular catheter to avoid the stress associated with these procedures. Blood samples were drawn at 20 min intervals 1 hr prior to and 4 hr post administration, additional blood samples were collected at 5 and 10 min post administration. Opioid doses ranged from 0.1 to 120 ug/kg. Hormones were measured by RIA. Intravenous administration of dynorphin-(1-13), 1 to 120 ug/kg, significantly increased plasma PRL levels. Average maximal increases of 100% to 225% occurred within 5 min and levels remained significantly elevated for up to 2 hr. A plateau was reached following the 30 ug/kg dose. No effect was seen with doses lower than 1 ug/kg. Dynorphin-(1-13) had no observable effects on plasma concentrations of LH, FSH, TSH or GH at any dose level studied. Administration of dynorphin-(1-10)-amide significantly increased plasma PRL concentrations. Dose levels of 1 to 120 ug/kg produced average peak increases from 100% to 225%, 5 to 10 min post administration. Dynorphin-(1-10)-amide had no significant effect on plasma concentrations of LH, FSH, TSH or GH. The increases in PRL by dynorphin were naloxone reversible. These results indicate a selective effect of dynorphin on the regulatory mechanisms of PRL secretion over that of other anterior pituitary hormones.

Abstract

DIRECT ANALYSIS OF THE RELEASE OF METHIONINE-ENKEPHALIN FROM GUINEA PIG MYENTERIC PLEXUS. A.R. Gintzler, W.C. Chan and J. Glass. Department of Biochemistry, Downstate Medical Center, Brooklyn, NY 11203, U.S.A.

The in vitro release of methionine-enkephalin (met-enkephalin) from 2 longitudinal muscle myenteric plexus (LMMP) strips from guinea pig ileum has been obtained during continuous superfusion and quantitated directly using a radioimmunoassay specific for this opioid peptide. Electrical stimulation (5-80 Hz) produced a significant increase in the rate of release of met-enkephalin the magnitude of which was not dependent on the frequency of stimulation. Analysis of the release of met-enkephalin per pulse as a function of the frequency of stimulation indicated that the release of this opioid peptide from the myenteric plexus is inversely proportional to the frequency of stimulation. Electrically evoked release (40 Hz) of met-enkephalin was reduced by greater than 80% by substituting $CoCl_2$ for $CaCl_2$ or by pretreatment with tetrodotoxin (TTx; 1 ug/ml for 15 min. Evoked release was also substantially reduced by pretreatment with morphine (1 uM, 1.5 min). Alternatively, pretreatment of naive LMMP strips with the opiate antagonist (-) naloxone caused a significant increase in the rate of met-enkephalin release in the absence of electrical stimulation. In contrast, (+)-naloxone was devoid of any activity. These data, in combination with indirect pharmacological experiments, strongly indicate that met-enkephalin functions as a neurotransmitter in the enteric nervous system. Moreover, the activity of neurons that transmit via met-enkephalin appear to be under opioid regulation.

P120

INTRATHECAL DYNORPHIN (1-13) ATTENUATES WITHDRAWAL IN MORPHINE-DEPENDENT RATS. P.G. Green and N.M. Lee. Department of Pharmacology, University of California, San Francisco, Ca 94143, U.S.A.

Dynorphin is an endogenous opioid peptide and studies have suggested that it may be involved in tolerance and dependence mechanisms (Lee and Smith, TIPS 5 108-110, 1984). Systemically administered dynorphin has been shown to substitute for morphine in morphine-dependent monkeys (Aceto et al., Eur. J. Pharmacol. 83 139-142, 1982). However, it is not known whether dynorphin acts on spinal or supraspinal systems; both these have been implicated in the development of opioid dependence (Yaksh et al., Eur. J. Pharmacol. 42 275-284, 1977).

Male Sprague-Dawley rats (220-240g) were implanted either with chronic catheters to permit injection of dynorphin directly into the spinal subarachnoid space (i.t.) or cannulae for intracerebroventricular (i.c.v) administration. At least 5 days following surgery, rats were made tolerant to morphine by the implantation of morphine pellets. Six hours after removal of the pellets, rats were given either dynorphin or vehicle i.t. or i.c.v. and assessed for withdrawal over a period of time.

The relative importance of spinal and supraspinal sites in the modulation of opioid withdrawal could be determined by the effectiveness of dynorphin administered i.t. and i.c.v. The data from these experiments suggest that dynorphin is able to attenuate morphine withdrawal signs by acting primarily at spinal sites.

P24

BRAIN OPIOID RECEPTOR REGULATION IN THE DEVELOPING ANIMAL. J. Habas and A. Tempel[2], [2]Dept. of Neuroscience Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A. [1]Dept. of Medicine SUNY/Downstate Medical Center, Brooklyn, NY 11203, U.S.A.

Chronic treatment with opiate agonists and antagonists have been shown to induce changes in brain opioid receptor density in adult rats. The development of these receptors and their regulation by exogenously administered opioids in perinatal pups has been less well characterized. We examined opioid receptor regulation in brains of neonatal rats after chronic agonist and antagonist treatment. Two regimens of drug treatment were used: naltrexone was administered to 1) mothers prior to conception or 2) pups of non-treated mothers starting on day of birth. Pups (ages 1-7 days) from each treatment group were sacrificed and brains assayed for specific opioid receptor types. Aliquots of brain homogenate (1.0 ml, .45 mg/ml protein) were incubated with various concentrations of [^{3}H]D-Ala^2,Gly-ol^5-enkephalin (μ specific opioid) in the presence or absence of a 1000-fold excess of levorphanol. Scatchard analysis revealed a 139% increase in brain opioidy receptor density on the day of birth in offspring of naltrexone-treated mothers. Naltrexone administration to offspring of non-treated mothers induced a 60% increase in brain u receptor number at day 7. In a separate experiment, young rats were treated with morphine sulfate (5 mg/kg). Results of [^{3}H] etorphine (a universal ligand) binding to brains of pups treated postnatally with morphine sulfate revealed a 30% decrease in receptor density after 8 days of treatment; subsequently, the number of receptors increased to control levels. These data indicate that neonatal rats exhibit the ability to regulate brain opioid receptors and that these receptors are in a plastic state both in utero and for the first two weeks postnatal. (This work is supported by NIH grant No. No. NS21973).

P45A

LIPID REQUIREMENT FOR OPIOID RECEPTOR BINDING. J. Hasegawa, N. M. Lee and H. H. Loh, Depts. of Pharmacology and Psychiatry, University of California, San Francisco, San Francisco, CA 94143, USA.

We have recently purified to homogeneity an opioid receptor from rat brain that shows selectivity for mu-type opioid ligands (Cho et al., Proc. Natl. Acad. Sci., 1986, in press). While this receptor is a protein, it requires lipid for expression of high opioid binding activity. In order to define this requirment precisely, we have tested the ability of various lipid components to reconstitute binding to the partially purified protein. Several fatty acids were able to restore binding, with linoleic (18:2), arachidonic acid (20:4) and linoleic (18:3) the most potent; stearic (18:0), and oleic (18:1) were inactive. Phospholipids were active if, and only if, they contained a) active fatty acids; and b) acidic polar head groups. Thus phosphatidic acid (PA) and phosphatidyl inositol (PI) containing linoleic acid (18:2) restored opioid binding, while dioleoyl PA and dilineoyl phosphatidylcholine (PC) did not. Confirming our earlier findings, no lipids tested exhibited significant opioid binding activity in the absence of the protein component.

These results indicate that mu opioids bind to a lipid-protein complex, and that both the polar head group and the fatty acid of the lipid component are critical to this binding. Because of the greater degree of specificity possible in a protein, and because several lipids can combine with the protein in an opioid binding complex, we believe that the opioid binding site itself is on the protein; the role of the lipids, then, would presumably be to stabilize a particular conformation of the protein. However, we cannot rule out the possibility that the roles of protein and lipid are reversed, with lipids binding the opioid and proteins ordering the lipids in a specific array. To decide between these two possibilities, it will be necessary to label the receptor covalently with an opioid ligand.

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LUMBAR INTRATHECAL MORPHINE AFFECTS PUTATIVE PAIN MODULATORY NEURONS IN THE RAT MEDULLA. M.M. Heinrincher, K. Drasner, F. Fang and H.L. Fields, Departments of Physiology, Anesthesiology and Neurology, University of California, San Francisco, CA 94143, U.S.A.

On- and off-cells are putative pain modulating neurons in the rostral ventromedial medulla (RVM) of the rat. On-cell activity increases, while that of off-cells decreases, immediately prior to the tail flick reflex (TF) elicited by noxious heat. We have previously shown that systemic administration and PAG microinjection of morphine produce an increase in activity of all off-cells and a decrease in activity of all on-cells concomitant with inhibition of the TF. The present experiments investigate whether spinal opiates similarly affect the activity of on- and off-cells in the RVM.

A lumbar intrathecal catheter was implanted under pentobarbital anesthesia. The animals were allowed to recover for 5 to 10 days, and were then used in the electrophysiological experiment. Activity of off- and on-cells was recorded with a microelectrode stereotactically placed in the RVM. Measures of spontaneous and TF-related activity were obtained before and after intrathecal administration of 10 or 15 µg of morphine sulfate in 5 µl of a balanced salt solution.

Intrathecally-administered morphine that inhibited the TF (10 s cut-off) differentially affected the spontaneous activity of on- and off-cells. Activity of all off-cells was increased and that of all on-cells ceased following morphine. The slowing of the off-cells and acceleration of the on-cells in response to heat were attenuated or abolished. These changes were reversed by naloxone (0.25-0.5 mg/kg, i.v.). Administration of the vehicle had no effect on the activity of these cells.

These results raise the possibility that intrathecally-administered opiates produce analgesia in part by activation of the sane brainstem neurons that are activated by opiates given systemically or in the PAG.

P46

IMMUNOHISTOCHEMICAL LOCALIZATION OF AMINOPEPTIDASE M IN RAT BRAIN: RELATIONSHIP OF ENZYME LOCALIZATION AND ENKEPHALIN METABOLISM. Louis B. Hersh and Stanley Watson, Department of Biochemistry, Universitv of Texas Health Science Center at Dallas, Dallas, Texas 75235 and the Mental Health Research Institue, University of Michigan School of Medicine, Ann Arbor, Michigan, 48109, U.S.A.

It is currently believed that the physiological action of synaptically released enkephalins is terminated by enzymatic degradation. Two peptidase activities have been identified in synaptic membrane preparations which could act as "enkephalinases" in vivo; a neutral metalloendopeptidase ("enkephalinase") and an aminopeptidase referred to as the "enkephalin degrading aminopeptidase". Recently aminopeptidase M activity has been found in brain synaptic membranes preparations. This aminopeptidase has been proposed to represent the true enkephalin degrading aminopeptidase. If aminopeptidase M could be shown to exist at enkephalinergic synapses its involvment in the degradation of synaptically released enkephalins would be more firmly established. Using a monospecific polyclonal antibody to aminopeptidase M the localization of the enzyme in several brain regions has been determined immunohistochemically. The results clearly demonstrate a localization restricted to blood vessels. The evidence that this aminopeptidase functions as an "enkephalinase" must thus be reconciled with its localization on blood vessels. A model which is suggested is one in which diffusion of enkephalins away from their receptors is a primary event in terminating the action of enkephalins. Distal to enkephalin receptors several peptidases, including aminopeptidase M, hydrolytically inactivate enkephalins. This action can serve two functions. First it maintains a high concentration gradient for diffusion of enkephalins away from the receptor, and secondly it prevents free enkephalins from acting distal to the site of their release.

P43

REGULATION OF GENE EXPRESSION OF OPIOID PEPTIDES. V. Höllt, N. Kley, J.Ph. Loeffler and C.W. Pittius, Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, D-8033 Martinsried and Department of Physiology, University of Munich, D-8000 München, F.R.G.

The concentrations of messenger ribonucleic acids (mRNAs) coding for the three opioid peptide precursors proopiomelauocortin (POMC), proenkephalin A and prodynorphin were measured in a variety of tissues by hybridization to cloned nucleic acid sequences complementary to the opioid precursor mRNAs. Regulation of opioid peptide gene expression at the mRNA level was investigated in the following sets of experiments:

1. The effects of chronic stress and/or pain on the levels of POMC mRNA in the pituitary and on proenkephalin A and prodynorphin mRNA in the spinal cord was studied in rats in vivo. An inductive effect of chronic stress/pain on the POMC mRNA levels in the anterior pituitary was found which could be mimicked by infusion of corticotropin-releasing factor (CRF) and by drugs which release CRF from the hypothalamus (e.g. morphine).

2. The influence of CRF and other secretagogues on POMC mRNA levels was analysed in vitro using cultured rat anterior and intermediate lobe cells. CRF induced an increase in POMC mRNA levels in both pituitary lobe cells - an effect which was found to be mediated by cAMP. The expression of POMC mPNA levels in intermediate lobe cells was negatively regulated by dopamine and GABA and positively by ß-receptor agonists. cAMP was found to be a critical mediator for these effects.

3. Proenkephalin A gene expression was studied in cultured bovine adrenal medullary cells. An increase in the levels of proenkephalin A mRNA was seen after activation of nicotinic receptors, after treatment with depolarizing agents (veratridine, K^+) and after treatment with agents that stimulate the adenylatecyclase system. These effects were inhibited by Ca^{2+} channel blockers indicating that, besides cAMP, Ca^{2+} plays a major role in mediating proenkephalin A gene expression.

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FEEDBACK INHIBITION OF CARBOXYPEPTIDASE H. V.Y.H. Hook and E.F. LaGamma*, Laboratory of Cell Biology, NIMH, Bethesda, MD. 20892 and *Division of Developmental Neurology, Cornell University Medical College, New York 10021, USA.

Carboxypeptidase H (CPH) is one of several processing enzymes required for the conversion of peptide hormone and neuropeptide precursors into the smaller active peptides. CPH removes COOH-terminal lysine and arginine residues to result in the formation of peptides such as (Met^5)enkephalin (ME), ACTH, vasopressin, and others. It has been refered to as 'carboxypeptidase B-like' and 'enkephalin convertase' but has now been designated 'carboxypeptidase H' ("H" for Hormone, Enzyme Nomenclature Committee of IUB) to distinguish it from other carboxypeptidases. We demonstrate in this study that CPH activity can be regulated by its products by a feedback inhibition mechanism. CPH activity in bovine adrenal medulla chromaffin granules and in rat adrenal medulla homogenates, which contain enkephalin peptides, was inhibited by ME with an inhibition constant, K_i, of 12.0 ± 0.8 ($\bar{x}$ ± s.e.m.) mM. The known intragranular concentration forenkephalin peptides has been estimated to be in the millimolar range. Therefore, CPH may be inhibited by endogenous enkephalins as their concentration rises. In contrast to ME, the other proenkephalin-derived peptides (Leu^5)enkephalin (LE), ME-Arg^6-Gly^7-Leu^8, and ME-Arg^6-Phe^7 inhibited CPH with lower Ki values of 6.3 ± 0.4, 7.4 ± 0.6, and 4.4 ± 0.9, respectively. One proenkephalin precursor-molecule contains 4 copies of the sequence ME, but contains only one copy of each of the sequences LE, ME-Arg^6-Gly^7-Leu^8, and ME-Arg^6-Phe^7. Thus, the single copy peptides are present within the granule at lower concentrations than ME. The difference in Ki values for the single copy compared to the multiple copy sequences demonstrates that CPH can be inhibited by lower concentrations of LE, ME-Arg^6-Gly^7-Leu^8, or ME-Arg^6-Phe^7 compared to ME. It appears that the final products that result from complete processing of proenkephalin can equivalently alter CPH activity by feedback inhibition.

P122

METHAQUALONE: SPECIFIC BINDING IN CENTRAL TISSUES.

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Tritiated analogs of methaqualone having high specific activity were prepared for use in binding studies. Specific binding of $[^3H]_3$-MQ (70 Ci/mmolel was found in synaptosomal preparations of mouse, rat, and bovine brain with an apparent dissociation constant (Kd) of 0.18-0.39 μM and a Bmax of 5.6 pmoles/mg protein. This binding was satdrable, reversible, temperature sensitive and exhibited subcellular and regional variations. The specific binding was inhibited by drugs that share the pharmacological actions of methaqualone but was not inhibited by those in unrelated classes. The ability of various classes of opioid peptides to displace $[^3H]_3$-MQ binding will be discussed.

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PREPROENKEPHALIN mRNA LEVELS IN TESTIS UNALTERED BY HYPOPHYSECTOMY. R.D. Howells, B.D. Shivers, P. Morris and S. Udenfriend, Dept. Neuroscience, Roche Institute of Molecular Biology, Nutley, NJ 07110, U.S.A. and The Rockefeller University, New York, NY 10021, U.S.A.

We have reported recently that the proenkephalin gene is expressed in rat testis (Kilpatrick et al., PNAS 82:7467-7469, 1985). Since the major physiological functions of the testis, viz. production and release of androgens and spermatogenesis, are dependent on luteinizing hormone and follicle-stimulating hormone derived from the anterior pituitary gland, we have compared the steady state levels of preproenkephalin mRNA in hypophysectomized and sham-operated rats. Four week old rats were hypophysectomized (n = 7) then sacrificed nine days later with age-matched, sham-operated controls. Hypophysectomy was verified by observing a lack of weight gain over the nine day period and by visual inspection of the sella turcica following sacrifice. ETestes were removed, frozen in liquid nitrogen and total RNA was isolated following CsCl centrifugation. Steady state levels of preproenkephalin mRNA were compared between groups using Northern blot analysis with ^{32}P-labeled rat preproenkephalin cDNA as probe (Howells et al., PNAS 81:7651-7655, 1984). Hypophysectomy did not alter the levels or the size of the mRNA in testis, which is longer by about 500 bases than preproenkephalin mRNA extracted from other tissues. Thus, the expression of the proenkephalin gene in testis is not influenced by gonadotropins or androgens. Currently, we are determining the cellular localization of this mRNA in testis using in situ hybridization and enriched cell populations maintained in culture conditions. Knowing which cell type expresses the proenkephalin gene should provide a better understanding of the function of peptides derived from proenkephalin in the male reproductive tract.

P100

AGONIST ACTION OF THE AGONIST/ANTAGONIST ANALGESIC BUTORPBANOL ON MESOLIMBIC DOPAMINE METABOLISM IN THE RAT. S. Iyengar, H.C. Kim and P.L. Wood, Neuroscience Research, Research Dept., Pharmaceuticals Division, Ciba-Geigy Corp., Summit, N.J. 07901, U.S.A.

Agonist/antagonist (Ag/Ant) analgesics have been shown to exhibit bell-shaped dose-response curves with regard to analgesia and nigrostriatal dopamine metabolism (Wood et al., Life Sci. 33(1):759-762, 1983). Throughout this bell -shaped curve, these agents act as mu and delta opioid antagonists and demonstrate a lack of cross-tolerance between mu, delta and ag/ant drugs. Studies with both local drug injections as well as parenteral drug after hemisection showed that this phenomenon was not the result of auto-inhibition. To determine the action of butorphanol on mesolimbic and mesocortical DA neuronal pathways, various doses of the drug were administered intraperitonially and dopamine metabolism was studied by gas chromatography- mass fragmentography. Areas studied included pyriform cortex, prefrontal cortex, cingulate cortex, entorhinal cortex, olfactory tubercle, nucleus accumbens and striatum. The effect of 16 mg/kg morphine was studied in these regions as well. Butorphanol had no effect on the cortical regions as well as the olfactory tubercle, even though morphine caused potent increases in three of the cortical regions and olfactory tubercle. Thus, this drug had no mu opioid action in these regions. The previously observed bell shaped dose-response curve was reconfirmed in the striatum. However, in the nucleus accumbens, this drug exhibited a dose-dependent linear increase in dopamine metabolism. It thus has an agonist effect on a mesolimbic DA pathway which seems to be an action of this drug that is different from that in the nigrostriatal dopamine pathway. The receptor affinities of this action need to be elucidated.

053

ANTINOCICEPTIVE ACTIVITIES OF MORPHINE (A MU AGONIST) AND OF U 50,488 H (A KAPPA AGONIST) IN VARIOUS STRAINS OF MICE. J.J.C Jacob, G Michaud, M.A Nicola and N. Prudhomme, Unit of Pharmacology, Pasteur Institute, F75724 Paris Cédex 15, France.

Previous studies have shown, in mice, genetic variations in the latencies of some nociceptive reactions and in the amplitude, duration and naloxone sensitivity of stress induced analgesias. Differences were also found in the amounts of tritiated naloxone or EKC bound to their brain extracts. In this work, range of doses of two agonists, Morphine (Mo) and U 50,488 H, were studied in 4 strains (CXBH, CXBK, C3H and $C57B1_6$); doses-and times-effects curves were drawn and ED50 calculated. With the hot plate test (response: licking) Mo had much greater effects in two strains (CXBH - C3H) then in the two others (CXBH - CXBK) U 50,488 H was the most effective in a different pair of strains (C3H - $C57B1_6$) Results were different with the tail flick test. CXBK mice were still low responsive to Mo but $C57B1_6$ mice were than high responsive. The latter strain in contrast was hardly responsive to U 50,488 H (whereas it was much more in the hot plate test). The tail flick test was also used in spinalectomized mice (C_5-C_8). Surprisingly, effects of 10 mg s.c. of Mo and of U 50,488 H were almost similar, the pair CXBH-C3H being more responsive than the two other strains. Assuming that Mo triggered mu receptors and U 50,488 H kappa ones, it appears that in the hot plate test, opioid mu receptor systems (including related efferent and modulatory pathways) would be more efficient in CXBH-C3H mice than in $C57B1_6$-CXBK whereas kappa opioid system(s) would be more in C3H-$C57B1_6$ than in CXBH-CXBK. When considering the tail flick test in intact animals different mu system(s) would be involved, at least in $C57B1_6$, a strain which would then on the contrary lack a kappa system. The lumbo-sacral opioid system appeared to be more developed in CXBH and C3H but no mu or kappa functional selectity was observed; it cannot be excluded that in this latter case, the dose used was no more selective. These observations add a new link to previous ones.

P91

EFFECT OF ADRENERGIC BLOCKERS ON THE DEVELOPMENT OF ANALGESIC TOLERANCE TO MORPHINE IN MICE. H. Kaneto and T. Kihara. Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852, JAPAN.

A single dose of reserpine dose-dependently reduced the brain content of catecholamines and antagonized the analgesic effect of morphine. At the peak of the effect, 24 hr after 2.5 mg/kg i.p., the brain content of norepinephrine was lowered to 30 % of the control Level, and the analgesic effect of morphine was also reduced to 40 % of that in naive animals. The reduced effect of morphine was maintained almost the same level during 10 daily repetitions. Low dose, 0.1 mg/kg, of reserpine neither affected the brain content of norepinephrine nor modified the analgesic effect of morphine. Daily treatment of animals with this low dose of reserpine did not induce any appreciable changes in brain norepinephrine level during 10 days, but effectively blocked the development of tolerance to morphine. Similarly, α- and β-blockers, phentolamine and propranolol, at the dose of 0.5, 1 and 10 mg/kg, did not affect morphine analgesia but concomitant administration of 1 or 10 mg/kg of each blocker with 10 mg/kg morphine suppressed the development of tolerance to morphine analgesia. These results clearly indicate that the suppression of the development of tolerance to morphine analgesia is not attributed to the reduction of brain narepinephrine content by reserpine. Maintenace of analgesic effect of morphine in reserpinized animals without development of tolerance during repeated administration may support our previous finding that morphine analgesia and tolerance is separable from each other by underlying mechanisms. Effects of other adrenergic blockers are now under investigation.

P23

MU-1 OPIATE RECEPTOR: DO DISPLACEMENT EXPERIMENTS INDICATE ITS EXISTENCE IN THE BRAIN? A. Kenner & Y.Sarne Department of Physiology and Pharmacology, Tel-Aviv University, Tel-Aviv 69-978 Israel.

Displacement from brain membranes of labeled opiate alkaloids by low concentrations of enkephalins and of labeled enkephalins by low concentrations of alkaloids , has been previously explained by the existance of a common high affinity site termed mu-1 (Wolozin & Pasternak, PNAS 78,6181, 1981). An alternative interpretation of the same results is that the trough seen in the low concentration zone of the displacement curves represents cross binding of mu and delta opioid ligands to delta and mu receptors, respectively. That such cross binding takes place is suggested by our experiments in which the same low concentrations of [3H]-morphine bind to NG-108 membranes which contain a pure population of delta receptors. In two sets of experiments with brain membranes , the size of the trough (previously interpreted as mu-1) is shown to be dependent on the labled ligand used ; the ratio between the size of troughs seen with [3H]-D-ala-D-leu-enkephalin (DADLE) and [3H]-morphine varies with experimental conditions (storage of membranes at 4°C for 72h) and with the ratio of mu:delta receptors (e.g. in thalamus and cortex which are enriched in mu and delta sites, respectively). These results cannot be explained by a common high affinity site , but rather by binding of [3H]-DADLE to mu and of [3H]-morphine to delta opioid receptors.

P149

COMPARISON OF THE INHIBITORY EFFECTS OF CORTICOTROPIN-RELESING FACTOR (CRF) AND OPIOID PEPTIDES ON THERMAL INJURY IN RATS.

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Increased exudation of plasma proteins, as measured by Evans Blue leakage into the innervated pawskin, can be produced by antidromic stimulation of the saphenous nerve in the anesthetized rat. This condition, termed neurogenic plasma extravasation (NPE), is inhibited by opioid agonists, dynorphin (1-13), and CRF (Europ. J. Pharmacol. 114:111, 1985). Inhibition of NPE has been attributed to interference with the actions or release of a putative neurotransmitter, substance P, from sensory nerve endings and it has been shown by Saria (Brit. J. Pharmacol. 82:217, 1984) that administration of a substance P antagonist or depletion of substance P by pretreatment of neonatal rats with capsaicin can also reduce thermal injury to the rat's paw. In this study, edema and protein extravasation was produced by exposure of the anesthetized rat's paw to 48-58°C water for 5 min. CRF, injected intradermally or intravenously at microgram doses, dramatically inhibited these indices of thermal injury. By contrast, the effects of opioid peptides and morphine were less clearcut. These results suggest that pharmacological modification of the activity of sensory nerve endings may have potential therapeutic benefit for certain forms of skin injury. Supported in part by grant USPHS NIDA-00091.

P143

INCREASED MESOLIMBIC DOPAMINE METABOLISM IN THE RAT AFTER INTRAVENTRICULAR BETA-ENDGRPHIN. H.S. Kim, P.L. Wood and S. Ivengar. Neuroscience Research, Research Dept., Pharmaceuticals Division, Ciba Geigy Corp., Summit, N.J. 07901, U.S.A.

Intraventricular (ivt) injections of beta-endorphin are known to increase nigrostriatal dopamine metabolism in the rat (Wood et al., J. Pharmacol. Exp. ther. 215:697-703, 1980). We now report the effects of beta-endorphin on rat mesolimbic dopamine metabolism in the N. accumbens and olfactory tubercle. The levels of dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured via gas chromatography-mass spectrometry.

Beta-endorphin (0.5 to 20 ug, ivt , 60 min) produced potent, dose-dependent increases of DOPAC and HVA, up to 175% and 155% of control in the N. accumbens and olfactory tubercle, respectively. These actions were antagonized by both naloxone (5 mg/kg, ip) and the long acting narcotic antagonist WIN 44,441-3 (5 mg/kg, ip).

These data suggest that mesolimbic dopamine projections also possess an opioid receptor modulation. Selective tolerance experiments are still required, however, to determine if the actions reported involve a unique epsilon receptor.

P115

CHRONIC κ-ANTAGONIST TREATMENT RETARDS DEVELOPMENT OF HYPERTENSION IN THE SPONTANENEOUSLY HYPERTENSIVE RAT (SHR). K. Kraft, J. Diehl and K. O. Stumpe, Medizinische Poliklinik, 5300 Bonn, Wilhelmstr. 35 - 37, FRG.

In general, acute application of κ-receptor agonists lowers blood pressure in several species. The involvement of the κ-receptor in the development of hypertension is still unknown. Therefore male SHR rats at 6 weeks of age and their normotensive controls (n=11 per group) were implanted with osmotic minipumps chronically delivering the κ-antagonist MR 2266 (10 µg/h) or solvent. Systolic blood pressure (SBP) was recorded twice a week for 4 weeks. In normotensive rats development of SBP was not affected by treatment, in SHR rats MR 2266 significantly ($p<0.01$) retarded the increase of SBP, the effect starting at 8 weeks of age (167.1 ± 3,5 vs. 178.7 ± 2.3 mm Hg (control) x±SEM). At 10 weeks of age SBP reached 189.1 ± 3.8 and 201.6 ± 3.7 mm Hg respectively. MR 2266 did not influence the body weight development in all groups.

It is concluded that the increase of SBP in young SHR is retarded by chronic κ-antagonist treatment. This indicates a possible influence of κ-receptors in the development of hypertension in SHR.

P114

THE DIFFERING PLASMA ß-ENDQRPHIN AND LEUCINE-ENKEFHALIN CONCENTRATIONS IN NORMOTENSIVE AND HYPERTENSIVE PATIENTS ARE EQUALIZED BY CLONIDINE. K. Kraft, R. Theobald, K. O. Stumpe, Medizinische Poliklinik, 5300 Bonn, Wilhelmstr. 35 - 37, FRG.

Cpiate antagonists e.g. naloxone have been shown to block acutely the antihypertensive effect of clonidine (C) in hypertensive (H) rats and patients, but not in normotensive (N) controls. Thus, endogenous opioids may participate in the antihypertensive action of C in H patients. To determine whether there are differences in the secretion pattern of endogenous opioids in H and N patients, plasma ß-endorphin (ß-E) and leucine-enkephalin (L-Enk) concentrations of N and mild essential H males (n=7, 22-35 years of age) were measured hourly from 9 p. m. to 8 a.m. after treatment with placebo or C (2x150 µg) for 14 days. Blood pressure and heart rate were significantly reduced after C treatment in both groups. R-E was significantly lower in the placebo H group than in the N group (7.7 ± 1.8 vs. 18.7 ± 1.9 pg/ml, $\tilde{x}$ ± SEM) from 9 p.m. to 2 a.m., but not-during the R-E peak (2 - 8 a.m.>. In the placebo H group also the L-Enk concentration was lower (7.8 ± 0.5 vs. 11.9 ± 0.7 pg/ml). After 14 days of C, R-E concentration was increased in the H patients (18.4 ± 2.8), but not in the N group (19.9 ± 4.6; 9 p.m. -2a.m.) The shape of the ß-E peak was not influenced by C in both groups. Plasma L-Enk was significantly reduced in N patients but not altered in the H group (9.3 ± 0.8 vs. 9.0 ± 1.3). In conclusion, ß-E and L-Enk plasma concentrations in young H males are reduced. Treatment with C does not only normalize blood pressure, but also equalizes plasma concentrations of ß-E and L-Enk. This indicates a possible involvement of endogenous opioids in the maintenance of mild essential hypertension.

020

DERMORPHIN BINDING IN RAT BRAIN AND HEART

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Competitive inhibition of [^{3}H]diprenorphine (DPN) binding in rat brain by dermorphin, represented by multiphasic plots, suggests multi-site interaction for dermorphin. Detailed displacement studies using selective tritiated opioid ligands revealed that dermorphin interacts preferentially with μ receptors. In competition with [^{3}H][D-Ala^2, MePhe^4, Gly(ol)^5]-enkephalin (DAGO), dermorphin (IC_{50} = 2 nM) appeared equipotent with DAGO (IC_{50} = 3 nM) in Tris buffer. The μ-preferring characteristics prevailed in competition with [^{3}H]ethylketocyclasocine (EKC) in phosphate buffer. The displacement of [^{3}H]EKC by dermorphin fit a 2-site model with approximate IC_{50} values of 2 nM and 1 μM. At the addition of 0.1 μM DAGO, the major portion of [^{3}H] EKC binding was eliminated at very low concentrations of dermorphin (> 0.1 nM). Additional portions of [^{3}H] EKC binding were displaced by the presence of 1 μM [D-Ala^2, D-Leu^5)enkephalin (DADLE) and large concentrations of dermorphin (>10 μM). However, a considerable portion of [^{3}H]EKC binding, as indicated by the displacement with unlabeled EKC, was not displaced by large concentrations of dermorphin, indicating that dermorphin interacts very poorly, if at all, with κ receptors. In competition with ^{3}H DADLE, dermorphin displaced the &preferring ligand according to a 1-site model with IC_{50} = 75 nM, while the homologous displacement by DADLE was represented by a 2-site model with an overall IC_{50} value of 3.5 nM. Dermorphin displaced [^{3}H]diprenorphine binding in both atria and the right ventricle but overall not as effectively as unlabeled diprenorphine.

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P150

IN VITRO PRODUCTION AND RELEASE OF OPIOID PEPTIDES IN THE TOOTH PULP INDUCED BY BRADYKININ.

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It has been suggested in in vivo experiments that bradykinin (BK) might be a trigger in an increase of met-enkephalin(ME)-like peptide content in the rat incisor pulp induced by noxious stimulation, and in contrast, ME-like peptides in the pulp might inhibit BK release from the pulp which was noxiously stimulated in a mode of negative feedback mechanism. In the present study, a possible relationship between ME-like peptides and BK in the rat incisor pulp was examined in in vitro experiments using whole pulp, in order to clarify the suggestion mentioned above. ME-like peptide content in the pulp was increased by BK at a concentration of 1 nM, but not in higher concentrations, while the release of ME-like peptides from the pulp into the incubation medium was increased dose-dependently by BK(1 - 100 nM). These effects of BK were inhibited by Des-Arg^{9}-[Leu^{8}]-BK, a potent BK-antagonist, suggesting that the effects of BK were mediated through a specific BK-receptor, B1-receptor which might be generated de novo in the noxiously stimulated tissue, in the pulp. On the other hand, high K^{+}(40 mM) did not induce any increased release of ME-like peptides from the pulp and the BK-effects were influenced neither in Ca^{++}-free medium nor in the presence of ouabain(1 mM). These results suggested that ME-like peptide releasing effect of BK was not due to depolarization of the pulp cell membrane and was not active. In addition, kyotorphin(10 µM), an enkephalin releaser, the effect of which was Ca^{++}-dependent, could not only elicit a release of ME-like peptides from the pulp, but also a marked increase of the peptide content in the pulp. However, a combination of BK(1 µM) and kyotorphin(10 µM) attenuated the effect of BK or kyotorphin each other. These results suggested that there might be two kinds of mechanisms of ME-like peptide production in the pulp, one of which was Ca^{++}-independent and another of which was Ca^{++}-dependent, and those mechanisms might interfere mutually. From these findings, a physiological role of ME-like peptides in the pulp will be discussed.

P19

CHARACTERIZATION AND SOLUBILIZATION OF OPIATE RECEPTORS FROM NCB-20 CELLS. L. Kushner, M. Eufemio, S.R. Zukin and R.S. Zukin, Depts. of Neuroscience and Psychiatry", Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

The neuroblastoma x Chinese hamster brain hybrid cell line NCB-20 is known to contain the δ opiate receptor and benzomorphan-specific binding sites (West, et al., 1982). The various opiate receptors present in this cell line have been further elucidated. The specific binding of [^{3}H]ethylketocyclazocine (EKC) in the presence of 109 nM D-Ala^2, NPhe^4, Gly-ol^5-enkephalin (OAGO) and 100 nM D-Ala^2, D-Leu^5-enkephalin (DADLE) revealed a linear Scatchard plot, The receptor affinity (K_d) of 5.2 nM and density (B_{max}) of 92 fmol/mg were very similar to the values observed for rat bain K receptors. The rank order of potency of a series of opioids and related drugs was determined to be ketocyclazocine>cyclazocine>EKC at this site, whereas phencyclidine (PCP), TCP, and (+)SKF-10,047 do not compete for binding. The binding site for the σ specific ligand [^{3}H]TCP was characterized by a K_d of 99.7 nM and B_{max} of 8863 fmol/mg. The rank order of potency of a series of opiates, opioid peptides, and related drugs was determined to be TCP>dexoxadrol>(-)SKF-10,047, cyclazocine>(+)SKF-10,047>1evoxadrol>PCE, PCP at this site, whereas DAGO, DADLE, and normorphine do noT compeTe for binding. Binding sites for the δ-specific ligand [^{3}H]D-Pen^2, D-Pen^5-enkephalin (DPDPE) have a K_d of 6.5 nM and a B_{max} of 167 fmol/mg. Specific binding could not be obtained using the u-specific ligand DAGO. Thus, NCB-20 cell membranes appear to have δ_1, K, and σ receptors (no μ receptors are evident), and would seem to be an excellent source for the purification of multiple opiate receptors. Solubilization of opiate receptors from NCB-20 cell membranes was achieved using glycerol (50%) and the zwitterionic detergent CHAPS (10 nm); a 20% protein yield and a two-fold enrichment of receptor binding activity in the soluble protein fraction was obtained. The opioid receptor affinity in the solubilized fraction was very similar to that observed in membrane preparations of the cells. Thus, the procedure appears to maintain the native conformation of the opioid receptor.

P20

AUTORADIOGRAPHIC ANALYSIS OF KAPPA (^{3}H-U-69593) AND MU (^{3}H-dihydromorphine) BINDING IN GUINEA PIG BRAIN SLICES

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Receptor binding studies have been conducted in a series of coronal and sagittal sections from Guinea Pig brain using the highly selective kappa ligand ^{3}H-U-69593 (Lahti, et al., Eur. J. Pharmacol. 109: 281 (1985)) and in closely adjacent slices using the selective mu ligand ^{3}H-dihydromorphine. Use of the two ligands demonstrated the clear difference in localization of kappa and mu opioid binding.

Analysis of binding results obtained with ^{3}H-U-69593 demostrate that kappa sites are located in specific brain areas. Highest concentrations of kappa binding were noted in cortical areas, nuc. accumbens, olf. tubercles, and substantia nigra reticulata. On the other hand, mu binding had its highest concentration of sites in the striata, piriform cortex, amygdala, hypothalmus and the substantia nigra.

Autoradiograms of coronal and sagittal sections will be presented as will quantitative data on selected brain areas.

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YOHIMBINE PRODUCES OPIOID WITHDRAWAL-LIKE REACTION IN ADDICTS.

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Yohimbine HCI, an alpha$_2$ adrenergic receptor antagonist, or placebo was administered orally (0.43 mg/kg and 0.21 mg/kg) to methadone addicts and normal volunteers to test whether opiate addicts have dysregulated noradrenergic system homeostatic mechanisms and, specifically, to determine whether antagonism of the alpha$_2$ feedback system produces an accentuated noradrenergic response. Yohimbine or placebo was given 30 min after the patients' usual daily oral methadone dose (38 mg) under double-blind conditions. Blood pressure, heart rate, skin temperature, respiration, other signs and symptoms of opiate withdrawal and changes in mood were measured. After the high dose of yohimbine, the addicts experienced shakiness, restlessness, loss of appetite, tension, chills and hot flashes, stomach cramps, urge to urinate, twitching, muscle cramps, sweating ng, nausea, dlarrhea, difficulty concentrating, anxiety and irritability. Addicts had greater blood pressure increase and skin temperature drop after yohimbine compared to controls. The addicts characterized the symptoms as similar to opiate wfthdrawal. Clonidine, an alpha$_2$ agonist, suppresses noradrenerglc hyperactivity in opiate withdrawal. Addicts, even after their usual dose of methadone, demonstrate symptoms consistent with opiate withdrawal upon exposure to an alpha$_2$ antagonist. Opiate addicts and patients with affective disorders (panic disorder, depression) may share similar defects in locus ceruleus activity Important for malntaining homeostasis In the noradrenerglc system.

P156

DOES NALOXONE ANTAGONIZE THE ANALGESIC EFFECT OF THE PARTIAL OPIATE AGONIST BUPRENORPHINE IN MAN?

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In human volunteers the thermal pain threshold was determined by use of a Marstock thermode through which heat was applied to the thenar of either hand. The temperature at the thermode-skin interface was measured by a thermocouple glued to the thermode surface and continuously recorded by a pen recorder. Subjects manipulated the stimulus temperature by turning a potentiometer until they experience a "slightly painful" sensation which was referred as thermal pain threshold. The partial opiate agonist buprenorphine (0.2-0.4 mg s.1.) significantly elevated the thermal pain threshold. Naloxone alone administered as i.v. bolus (0-1-10 mg) did not significantly change pain threshold. Naloxone pretreatment with 0.1 mg i.v. still completely blocked the analgesic effect of buprenorphine but a 200 fold higher dose was required to antagonize the established analgesic effect of buprenorphine. The antagonism, however, was readily established when suprahigh doses of naloxone were administerd. These data do not necessarily support the concept of a "slow dissociation" of buprenorphine from its receptor which is often referred as reason for buprenorphines resistance to naloxone antagonism. It is suggested that either a high receptor occupancy or a "facilitated association" of buprenorphine with its opiate receptor accounts for its relative naloxone resistance.

024

THE EFFECT OF ANALGESIC DOSES OF MORPHINE ON CELLULAR ACTIVITY IN PAIN RELATED STRUCTURES: A 2-DEOXYGLUCOSE AUTORADIOGRAPHIC STUDY. R.M. Levy, M.P. Stryker, M.M. Heinricher and H.L. fields, Deoartments of Neurological Suroery, Neurology and Physiology, University of California, San Francisco, CA 94143, U.S.A.

Desoite reports that morohine (MS) diffusely decreases regional cerebral glucose metabolism (RCGM), the effect of opioids on RCGM in behaviorally documented analgesic states has not been investipated. We studied RCGM using high resolution 2-deoxyglucose (2-DG) autoradiography in rats during naloxone-reversible MS-induced analgesia. Tail flick latency (TF) to a noxious heat stimulus was tested in 12 ligntly anesthetized adult male rats. TF testings was repeated 2 min after MS (5 mg/kg IV). All animals had inital TF of 3-4 sec: after MS, TF increased to cutoff (> 10 sec). 14C-2-DG. 100 uCi/kg IV, was then adminis-tered and arterial glucose and radioactivity measured at logarithmic intervals. 45 min later, rats received 2 mg/kg naloxone IV and in all cases, TF returned to baseline. Animals were then sacrificed and standard autoradiography was oerformed on 20 micron tissue sections. Sections were analyzed using computer assisted digital techniques and RCGM calculated from tissue and plasma curves. Evaluation of RCGM after MS administration in pain related structures. including periaqueductal pray, nucleus reticularis paragiganto-cellularis and nucleus raphe magnus, reveals that while there is a trend toaward increased RCGM in these structures. there is no statistically significant change in RCGM as compared to non-pain related structures and to saline treated controls. These results suggest that, within the limits of resolution of this technique (200 microns), these pain related structures contain several cell oaoulations with heter-ogeneous responses to ooiates in doses which produce analpesia.

P56

REGULATION OF THE CONCENTRATION OF DYNORPHIN A (1-8) IN THE STRIATO-NIGRAL PATHWAY BY THE DOPAMINERGIC SYSTEM. S. Li, S. P. Sivam, J. F. McGinty* and J. S. Hong, LBNT, NIEHS, NIH, Research Triangle Park, NC, *Dept. Anatomy, East Carolina Univ., Greenville, NC, U.S.A.

It has been reported that the dopaminergic system exerts potent influence on the metabolism of [Met^5]-enkephalin and Substance P in the basal ganglia. Recent reports indicate that dynorphin-like immunoreactivity (DN-LI) is present in the striato-nigral pathway and is found in high concentration in substantia nigra. However, the regulation of dynorphin in this pathway is not known. The purpose of this study was to explore the dopaminergic control of the striato-nigral dynorphin system by measuring the levels of DN-LI after repeated injections of dopaminergic agonist and antagonist. Rats received different doses of apomorphine (0.5 mg-5.0 mg/kg, s.c. twice a day), haloperidol (1 mg/kg s.c. twice a day) or in combination for different periods and were decapitated 16 h after the last injection. Concentration of DN-LI was determined by radioimmunoassay using a specific antiserum against dynorphin A (1-8). Seven daily injections of apomorphine (0.5, 1.0, 2.5 and 5.0 mg/kg) caused a dose-related increase of DN-LI in the striatum (26, 34, 63, 85% over control at each corresponding dose). Similar increases were observed in the substantia nigra (22, 52, 50 and 62% over control). Immunocytochemical study revealed an increase in DN-LI, but not [Met^5]-enkephalin-like immunoreactivity in cell bodies located in striatal patches. An increase in fiber staining of DN-LI in the substantia nigra was also observed. In another experiment, rats received 5 mg/kg of apomorphine for 1, 3, and 7 days. There was a time-related increase in DN-LI both in the striatum (37, 50 and 85% over control at each corresponding period) and in the substantia nigra (32, 78 and 62%). In order to examine whether apomorphine-elicited increase in DN-LI in the basal ganglia is mediated by the dopaminergic receptors, haloperidol was injected alone or in combination with apomorphine for 7 days. Haloperidol alone failed to alter the striatal DN-LI, but completely blocked the apomorphine-induced increase in DN-LI. These results suggest that dopamine exerts potent influence on the metabolism of dynorphin in the basal ganglia.

CLEAVAGE OF PROENKEPHPALIN BY A CHROMAFFIN GRANULE TRYPSIN-LIKE PROCESSING ENZYME. I.Lindberg and G. Thomas, Dept. Biochem., L.S.U.M.C, 1901 Perdido St., New Orleans, LA 70112, and I.A.B.R., Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201 USA.

Proteolytic processing enzymes with trypsin-like specificity are required for the maturation of prohormones and neuropeptide precursors into active peptide products. We have been interested in the use of cDNA expression systems as a means to generate native prohormones which can then be used to study processing enzyme candidates. Proenkephalin was produced by infect ion of African Green Monkey Kidney cells with vaccinia virus containing cDNA for human proenkephalin. Intact proenkephalin secreted into the medium was partially purified by passage over a Peptide B affinity column followed by gel filtration chromatography. This material was used as the substrate for the chromaff in granule trypsin-like protease (Lindberg et al, BBRC 106, 186, 1982). Following incubation at 37°C for varying amounts of time the reactions were stopped by boiling, and then further incibated with pancreatic carboxrpeptidase B (in order to remove carboxyl terminal basic residues), Digestion products were analyzed by radioimmunoassay using antiserum to met-enk-arg-gly-leu, an internal sequence in proenkephalin. The production of met-enk-arg-gly-leu-immunoreactive peptides from proenkephalin was dependent on the length of incubation with the trypsin-like enzyme. In addition, no immunoreactivity was observed in the absence of additional carboxrpeptidase B digestion. Gel filtration of digestion products revealed the presence of low molecular weight immunoreactive met-enk, leu-enk, and met-enk-arg-gly-leu in incubated samples but not in control reactions which were stopped immediately. HPLC identification of low molecular weight immunoreacttve products revealed the presence of authentic met-enk, met-enk-arg-gly-leu, and their sulfoxides. Taken together, the results indicate that 1) the chromaffin granule protease possesses the correct specificity to act as an enkephalin-generating enzyme in vivo; and that 2) cDNA expression systems represent a useful method for the production of prohormone substrates for processing enzymes.

03

PARTIAL PURIFICATION OF THE MU OPIOID RECEPTOR IRREVERSIBLY LABELED WITH [^{3}H]β-FUNALTREXAMINE. L.-Y. Liu-Chen, C. A. Phillips, S. W. Tam, and L. G. Davis, Central Research and Development Department and Biomedical Products Department, E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, Delaware 19898, U.S.A.

The mu opioid receptor in bovine striatal membranes was irrevessibly labeled with high specificity by incubation with 5 nM [^{3}H]β-funaltrexamine (β-FNA) at 37°C for 90 minutes in the presence of 100 mM NaCl. The specific irreversible binding of [^{3}H]β-FNA, as defined by that blocked by 1 μM naloxone, was approximately 60% of total irreversible binding. The specific irreversible binding was saturable, stereo-specific, time-, temperature-, and tissue mass-dependent. Mu opioid ligands were much more potent than delta or kappa ligands in inhibiting the specific irreversible labeling. SDS polyacrylamide gel electrophoresis of solubilized membranes in the presence of 2-mercaptoethanol yielded a major radiolabeled broad band of MW 68-97K daltons, characteristic of a glyco-protein band. This band was not observed in membranes labeled in the presence of excess unLabeled naloxone or β-IFNA. The glycoprotein nature of the [^{3}H]β-IFNA-labeled opioid receptor was confirmed by its binding to a wheat germ agglutinin (WGA)-Sepharose column and its elution with N-acetylgluco-samine. Following this lectin column chromatography the mu opioid receptor became the only protein labeled by [^{3}H] β-IFNA in the absence of naloxone; no labeled protein was observed in the presence of naloxone. WGA column eluate was digested with pronase and the resultant mixture was subjected to high performance liquid chromatography using C_{18} reverse phase column. Three radiolabeled peptide peaks were obtained and purified to apparent homogenity. Works are currently under way to obtain sufficient material for sequence determination.

094

CONTROL OF PROOPIOMELANOCORTIN (POMC) GENE EXPRESSION BY cAMP AND CALCIUM. J.Ph. Loeffler, N. Kley C.W. Pittius and V. Höllt, Department of Neuropharmadology, Max-Planck-Institut für Psychiatrie, D-8033 Martinsried and Department of Physiology, University of Munich, D-8000 Miinchen, F.R.G.

The role of the second messengers cAMP and Ca^{++} in the control of POMC gene expression was investigated. Using hybridization with cloned cDNA probes, we measured POMC mRNA level in primary cultures of intermediate (IL) and anterior (AL) rat pituitary cells maintained in serum free medium.

8 Bromo-cAMP (1 mM) but no 8 bromo-cGMP (1 mM) induced a 2-fold increase in IL POMC mRNA levels. Furthermore, forskolin (1 µM) which directly stimulates the catalytic unit of the adenylate cyclase (AC), and cholera toxin (100 ng/ml) which activates the stimulatory subunit (Ns) of the AC, induced an increase in cAMP levels and a two-fold increase in POMC mRNA levels in IL and AL cells after two days of treatment.

In another set of experiments, we analyzed the role of Ca^{++} in POMC gene expression. When Ca^{++} entry was blocked with D_{600} (50 µM) and nifedipine (100 nM), POMC mRNA levels decreased to 50% of the control levels. Similar decrease was obtained when Ca^{++} free medium was used. The Ca++channel agonist BAYK 8644 (100 nM) stimulated POMC gene expression and this effect was additive with forskolin. Taken together, these data suggest that cAMP and Ca^{++} are implicated in the regulation of POMC gene expression in both intermediate and anterior cells of the rat pituitary.

P13

EFFECTS OF PHORBOL ESTERS ON OPIOID RECEPTOR ACTIVITY IN NEUROBLASTOMA X GLIOMA NG108-15 HYBRID CELLS. A. K. Louie, P. Y. Law and H. H. Loh, Departments of Pharmacology and Psychiatry, University of California, San Francisco, CA, 94143, U.S.A.

Incubation of neuroblastana x glioma NG108-15 hybrid cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) decreased opioid inhibition of cAMP accumulation in intact cells. In contrast, basal and prostaglandin E_1-stimulated cAMP accumulation were unaltered by TPA treatment. The effect on opioid inhibition was dose- and time-dependent and TPA abolished up to 4% of opioid activity. The potency order of phorbol esters and apparent K_{act} (90nM) for TPA were consistent with phorbol esters mediating this attenuation through the stimulation of protein kinase C. TPA also decreased the inhibition of cAMP accumulation seen with muscarinic and $alpha_2$-adrenergic agonists.Scatchard analysis of saturation binding of these membrane receptors demonstrated that TPA decreased the number of opioid and muscarinic receptors but not the number of $alpha_2$-adrenergic receptors. These data suggested that TPA attenuation of receptor activity might be at the site of the inhibitory-nucleotide-binding protein (N_i). When N_i was quantified by measuring the ADP-ribosylation of its 41 kDa alpha subunit by pertussis toxin and $[^{32}P]NAD^+$, TPA treatment decreased the maximal labeling without a change in the NAD K_m. In contrast, ADP-ribosylation of the 45 kDa alpha subunit of the stimulatory-nucleotide-binding protein (N_s) by cholera toxin was unchanged by TPA treatment. The functioning of the remaining N. protein was assessed by measuring their activation by guanine nucleotides. Studies on the effects of GTP and its stable analogue GppNHp on membrane adenylate cyclase activity were consistent with TPA inducing a decrease in N_i activation compared to control. We conclude that TPA attenuated opioid inhibition of adenylate cyclase and decreases in the number of opioid receptors and N_i proteins were identified as potential mechanisms for this action.

035

AUTORADIOGRAPHIC VISUALIZATION OF SIGMA RECEPTORS WITH [^{3}H]1,3 DI-O-TOLYL-GUANIDINE (DTG). S. McLean, J. F. W. Keana[2] and E. Weber[3], Laboratory of Neurophysiology, NIMH, Bethesda, MD 20892, [2]Dept. Chemistry, Univ. Oregon, Eugene, Oregon 97403, and [3]Institute for Advanced Biomedical Research, Oregon Health Science University, Portland, Oregon 97201, USA.

The existence of sigma receptors, which appear to mediate the psychotomimetic effects of certain opiates, was proposed by Martin et al. in 1976. Recently, a new compound, DTG has been synthesized (Weber et al., this meeting) that exhibits selectivity and specificity for the sigma receptor.

Using receptor autoradio raphy we have investigated the neuroanatomical distribution of [^{3}H]DTG binding. Biochemical studies using slide mounted tissue sections were carried out to establish binding conditions. Sections of guinea pig and rat brain were incubated for 45 min at 22° C in 50mM Tris (pH 8.0) containing 1mg/ml BSA and 2nM [^{3}H]DTC, washed (4x2min) in ice-cold 10mM Tris(pH 7.4) with 1mg/ml BSA, blown dry and placed in x-ray cassettes with 3_H sensitive film. Blanks incubated with 10 uM haloperidol indicated specific binding of 90 percent.

[^{3}H]DTG binding sites were heterogeneously distributed throughout the guinea pig and rat brain. In the forebrain, dense binding was present in the diagonal band of Broca, the paraventricular nucleus of the hypothalamus, and along the border of the medial and lateral habenula. Moderate densities were present in olfactory and retrosplenial cortex, pyramidal and granule cell layers of the hippocampal formation, and throughout the hypothalamus, septum, zona incerta, and midline nuclei of the thalamus. Low densities of diffuse binding were present in cortex, striatum, and thalamus. In midbrain a high density of binding was localized to the subcommissural organ, red nucleus and oculomotor nuclei, a moderate density in the periaqueductal gray, and low levels in the colliculi and substantia nigra. In the hindbrain dense binding was found in the locus coeruleus with moderate levels in the nucleus of the facial nerve and cerebellum.

These results demonstrate a distinct distribution of sigma receptors that is different from that of the mu, delta, or kappa opiate receptor subtypes.

P139

INVESTIGATION OF THE INHIBITORY EFFECT OF HIGH FREQUENCY ELECTRICAL STIMULATION ON THE GUINEA PIG ILEUM. L. R. McMartin, J. E. Olley and W. Hope, Department of Pharmacology, Monash University, Clayton, Victoria, Australia, 3168.

High frequency electrical stimulation (> 5Hz) of the isolated ileum of guinea pig elicits a post-stimulation inhibition of twitch responses at 0.1 Hz - a major component of which is considered to be opioid (Puig et al, 1978). Experiments were performed to investigate this inhibition. Naloxone (10 nmol/l) significantly reduced the inhibitory response from 76.0% inhibition (s.e.m.= 4.2: n = 61 to 51.6% (s.e.m.=1.8; n = 6) ($P < 0.051$. Mr 2266 [(-)2-(3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan] (10 nmol/ll also significantly reduced the inhibition from 84.5% (s.e.m. = 3.1; n = 5) to 36.3% (s.e.m. = 8.0; n = 5)($P < 0.05$). These data indicate a possible involvement of both μ κ opioid receptor ligands. The purinergic antagonist 8-phenyltheophylline (10 μmol/l) did not significantly affect the inhibition (n = 5, $P > 0.051$. Since the slow offset and resistance to washing resembled properties of the potent endogenous κ ligand dynorphin, the bathing fluid was examined for the presence of dynorphin following 3 min high frequency stimulation. Preliminary evidence was found for the presence of dynorphin-(1-13). dynorphin-(1-17) and a number of other as yet unidentified inhibitory components using reverse phase liquid chromatography techniques. Spasmogenic activity was also found. It is suggested that the high frequency stimulation-induced inhibition comprises several components, some of which may be the long-acting opioid dynorphins.

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051

MIDBRAIN STIMULATION-PRODUCED ANTINOCICEPTION (SPA) IN THE FREELY-MOVING RAT: EVIDENCE FOR MEDIATION BY ß-ENDORPHIN(ß-EP) AND µ-OPIOID RECEPTORS:

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Electrical stimulation of the ventral midbrain periaqueductal grey(PAG) of the rat evokes an antinocicention, in the mediation of which opioids play a role. The particular opioid peptide and receptor type responsible is, however, unknown. We have, thus, addressed this question employing freely-moving rats.

Unilateral stimulation of the ventral PAG elicited an antinociception strongly attenuated by a low dose of naloxone. The locomotor effects (ipsilateral turning) were unaffected. Destruction of central ß-endorphinergic neurones, which project from the hypothalamus to the PAG, greatly depleted ß-EP from brain and strongly reduced SPA, but not the locomotor effects. Pharmacological blockade of pituitary secretion of ß-EP into the plasma was ineffective. Chronic perfusion with a low dose of naloxone selectively blocked the antinociception induced by a µ-agonist (morphine) but not a κ-agonist (U50,488). Following pump removal, morphine but not U50,488 antinociception was enhanced, reflecting a supersensitivity of µ-receptors. As with morphine, SPA was reduced in the presence of naloxone and potentiated upon its removal. Recurrent stimulation led to a tolerance of SPA: tolerant rats showed a reduced antinociceptive action of morphine but not U50,488.

We suggest that the antinociception against noxious pressure and heat evoked by stimulation of the ventral midbrain PAG is mediated by central ß-EP and µ-opioid receptors. Whether ß-EP acts directly on µ-receptors remains to be clarified. In addition, a possible significance of any 'ε'-receptor, for which ß-EP has been postulated to be a ligand, remains open.

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KAPPA-TYPE OPIOID RECEPTOR IN HUMAN PLACENTAL MEMBRANE.

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We have reported that kappa opioid receptor in rat brain possesses a different coupling mechanism from those of mu and delta receptor between receptor binding site and GTP-binding protein. In human placental membrane, ^{3}H-EKC bound to a single class of binding site (Kd:0.75nM, Bmax:60fmol), and ^{3}H-DHM and ^{3}H-DADLE showed no binding activities. The EKC binding was strongly displaced by U-50488H, dynorphin A (1-13) and bremazocine, but not by morphine and DADLE. These data suggest that the EKC-binding site in human placental membrane is a homogeneouse kappa receptor.

The inhibitory effect of Gpp(NH)p in high concentration (1mM on the EKC-binding was very weak in the presence or absence of 100mM NaCl, in a similar manner as kappa receptor in rat brain. The effects of SH-group brocking reagent and pertussis toxin on the EKC-binding and post-receptor mechanisms will be discussed.

036

IN VIVO AUTORADIOGRAPHY: MATING-PRODUCED CHANGES IN OPIATE RECEPTOR OCCUPANCY ASSESSED BY [^{3}H] DIPRENORPHINE AND [^{3}H]CYCLOFOXY BINDING IN BRAIN. N. L. Ostrowski, K. Rice and A. Pert, Biological Psychiatry Branch, NIMH and Laboratory of Chemistry, NIADDK, NIH, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

Opiate receptors are found in hypothalamic regions that regulate neuroendocrine and reproductive functions in rats and hamsters. Using the procedure of Seeger et al., 1984 autoradiographic experiments were conducted to determine whether mating (a physiological stimulus Known to affect the hypothalamic-pituitary-gonadal [H-P-G] axis) produced changes in opiate receptor occupancy, an index of endogenous opioid release. Male hamsters were selected for testing because they show dense opiate receptor binding in the sexually dimorphic nuclear complex of the hypothalamus and their mating behavior is reliable, precisely timed and resistant to disruption by the administration of opiate antagonists. Males were injected (IV) with [^{3}H]cyclofoxy (CF) or [^{3}H]diprenorphine (DP) after: a) the 5th ejaculation of a mating session, b) exposure to a receptive female wearing a vaginal mask, or, c) exposure only to their home cage. Twenty min after the injection, animals were sacrificed, their brains removed, rapidly frozen and processed for film autoradiography. Computer assisted densitometric analysis of the binding showed a decrease in the accumulation of both [^{3}H]DP and [^{3}H]CF in the cingulate cortex sexually dimorphic nuclear complex and in the anterior but not caudal striatum of brains from mated males relative to control-mated males. Liquid scintillation counting of adjacent brain sections verifled high brain to cerebellar accumulation ratios, a lack of difference in cerebellar (i.e., non-specific) binding among groups and the ability of naloxone, when co-administered with the radioligands, to completely block brain accumulation of labeled drug. These data suggest that mating produces an Increase in opiate receptor occupancy, presumably by releasing endogenous opioids in discrete brain regions. This technique may prove valuable for investigating the neuroanatomical loci of opioid peptide-regulation of the H-P-G axis in living animals.

P40

MU AND KAPPA RECEPTORS ARE COUPLED TO Ca^{++} TRANSPORT IN REGULATING TEMPERATURE RESPONSE. N. P. Pillai and D. H. Ross, Molecular Pharmacology Division, University of Texas Health Science Center, San Antonio, TX 78284-7764, U.S.A.

The effect of opiates on body temperature has been shown to depend on both dose and route of administration. Additionally, Mu and Kappa agonists have recently been shown to increase and decrease respectively basal temperature in unrestrained rats (Geller et al, 1993). Opiates in vitro and in vivo have been shown to alter Ca^{++} content, binding and transport in brain membranes. Temperature regulation has previously been shown to be influenced by Ca^{++} ion movements in the hypothalamus. We have evaluated two opiate agonists, morphine (Mu) and U50-488H (Kappa) for their effect on temperature and Ca^{++} ATPase in hypothalamus. Morphine (15 mg/kg s.c.) produced hyperthermia (+ 1.5°C) at 2 hr. Ca^{++} ATPase in synaptic membranes was inhibited 24%. Hyperthermia and enzyme inhibition were both reversed by naloxone (2-5 mg/kg). Ca^{++} ATPase activity was decreased in P_2 fractions of hypothalamus (18%) and cortex (20%) ($p<.05$) but not cerebellum. Ca^{++} channel antagonists nimodipine (1 mg/kg), verapamil (2.5 mg/kg) or diltiazem (5 mg/kg) reversed hyperthermia and Ca^{++} ATPase inhibition. Administration of U50-488H (3-20 mg/kg s.c.) produced a decrease in basal temperature in unrestrained rats. This hypothermia was accompanied by an enhancement of Ca^{++} ATPase in synaptosomal P_2 fractions from hypothalamus but not cortex or cerebellum. Naloxone reversed the hypothermia and enhanced enzyme activity while nimodipine, verapamil and diltiazem potentiated the hypothermic effects and stimulation of enzymic activity. These results suggest that opiate Mu and Kappa agonists may increase and decrease respectively basal temperature by a mechanism which alters intracellular Ca^{++} levels. Changes in Ca^{++} ATPase activity in the hypothalamus together with blockade of Ca^{++} influx may contribute to altered intracellular Ca^{++}. These biochemical changes may be differentially coupled to Mu and Kappa receptors. Supported in part by grant from Upjohn Company.

076

BINALTORPHIMINE: A HIGHLY SELECTIVE, KAPPA OPIOID RECEPTOR ANTAGONIST. P. S. Portoghese,* A. W. Lipkowski,* and A. E. Takemori, Departments of Medicinal Chemistry* and Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

The concept of multiple opioid receptors has created a need for selective antagonists for the identification of different receptor types and for the investigation of agonist selectivity. Although selective opioid antagonists are available for mu and delta receptor types, the development of highly selective kappa antagonists has been elusive. A key problem has been the inability to eliminate the mu antagonist component. Here we report on the utilization of the "bivalent ligand" approach to designing an extraordinarily selective kappa antagonist, binaltorphimine (BNI), that is devoid of significant mu antagonist activity. The chemical structure of BNI is 17,17'-bis(cyclopropylmethyl)-6,6',7,7'-tetradehydro-4,5α:4',5'α-diepoxy-6,6'-(methylimino)[7,7'-bimorphinan]-3,3',14,14'-tetrol. In the guinea pig myenteric plexus longitudinal muscle preparation (GPI), BNI (20 nM) was a potent antagonist of the kappa-selective agonist, ethylketazocine (EK) (IC_{50} ratio, 165 ± 30). No significant antagonism of the mu-selective agonist, morphine, was observed (IC_{50} ratio, 1.2 ± 0.3). In the mouse vas deferens preparation, BNI (20 nM) weakly antagonized the effect of the delta-selective antagonist, [D-Ala^2,D-Leu^5]enkephalin (IC_{50} ratio = 4.5 ± 0.7). In mice, BNI (20 mg/kg IV) exhibited a selectivity profile similar to that in the GPI, with sc ED_{50} ratios (treated/control) for EK, U50488H, and morphine of 5.9, 14.6, and 0.85, respectively. These data indicate that BNI possesses no demonstrable mu antagonist activity and is the most highly selective kappa opioid receptor antagonist reported. Moreover, a comparison of the κ/μ selectivity ratio of BNI indicates that its kappa selectivity is over 800-times greater than that of naltrexone. The fact that BNI contains two opioid antagonist pharmacophores (a "bivalent ligand") raises the possibility that its high selectivity and potency are due to the simultaneous occupation of two vicinal recognition sites on the kappa receptor system. The significance of these results and the possible organization of vicinal kappa recognition sites will be discussed. (supported by NIDA grants)

P107

CNS INTERACTION OF OPIOIDS AND STEROIDS IN BLOOD PRFSSURE REGULATION. L.A. Reiser and E.E. Hahn, Rockefeller University, 1230 York Ave., New York, NY 10021, U.S.A.

Considerable experimental evidence has now been obtained that endoyenous opioids are involved in the regulation of blood pressure. Data from our laboratory have demonstrated that hypertensive rats have higher levels of opiate receptors relative to normotensive controls. This increase, which is dependent on age, coincides with the appearance of elevated blood pressure. In other studies, we showed that opiate receptors in SHR rats exhibited an abnormal sensitivity to salt which suggests that the impact of sodium on blood pressure may be related to its effect on the conformation of the receptor. Evidence has also accumulated that a CNS component may participate in the biochemical mechanisms by which steroid hormones regulate blood pressure. This activity may be mediated by specific hormones which are synthesized in situ in localized brain regions, or alternatively by those which enter the CNS by crossing the blood brain barrier. The discovery of an interrelationship between the opioid and endocrine systems led us to evaluate whether such an interaction in specific central sites may participate in blood pressure regulation. In studies on the metabolism of androstenedione in the CNS of rats we have shown that 19-hydroxylation of androqens greatly exceeds aromatization, and that 19-hydroxy and 19-oxo androstenedione, both of which are hypertensinoqenic agents, accumulate in quantities five times greater than estrogens. Using a radiometric procedure we have demonstrated that CNS production of these 19-oxygenated androyen metabolites was greater in SHR compared to age matched WKY rats. When naloxone or naltrexone was injected subcutaneously (0.3-1.0 mg/kg) in CD or WKY rats an increase in CNS androyen 19-hydroxylase activity was observed. The impact of opiates on this biotransformation was reinforced by in vitro studies in which preincubation of hypothalamic preparations with morphine in concentrations ranging from 10^{-9} to 10^{-6} M inhibited the activity of the 19-hydroxylase. In conjunction with our report that administration of opiate antagonists to either WKY or SHR rats increases blood pressure, the results support the hypothesis that an interaction in the brain between endoyenous opioids and 19-oxygenated androqen metabolites may be involved in blood pressure regulation.

04

PURIFICATION OF THE HUMAN ERYTHROCYTE OPIOID RECEPTOR.
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Previous studies from this laboratory demonstrated that opiates reduce human red blood cell deformability by a mechanism that can be correlated with the prevention of Ca^{++} efflux through inhibition of Ca^{++} ATPase. Moreover, the inhibiting effect is dose-dependent, stereospecific and naloxone reversible. The rank order of potency of various opiates and opioids in inhibiting cell deformability as well as their binding affinities suggest the involvement of an opiate receptor with kappa-like properties. In order to isolate this receptor, erythrocyte ghosts were solubilized in 0.5% Triton X-100 and purified by application to a wheat germ lectin-agarose column and elution with n-acetylglucosamine. When the eluate was applied to a dynorphin A (1-13)-Sepharose affinity column and eluted with nalbuphine, one major band was obtained following SDS polyacrylamide gel electrophoresis. Further purification by HPLC on Spherogel-TSK columns yielded a protein peak which after two dimensional gel electrophoresis exhibited a relative pI of 5.8 and an apparent molecular weight of 60,000. The calculated degree of purification was 11,250 fold which is close to the theoretical value of 11,500. That this apparently homogeneous material is the receptor responsible for opioid binding and action was substantiated by: 1) the lack of change in affinity for 3H-diprenorphine and 3H-dynorphin(1-8) after purification, 2) other opioids compete for the 3H-diprenorphine binding with the same affinity, and 3) cross linking 3H-dynorphin(1-8) to the isolated material with bifunctional reagents labeled a 60,000 Dalton protein.

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093

HALOPERIDOL INCREASES PROENKEPHALIN MRNA LEVELS IN THE CAUDATE-PUTAMEN OF THE RAT: A QUANTITATIVE STUDY USING IN SITU HYBRIDIZATION. G.J.Romano, B.D.Shivers, R.E.Harlan, R.D.Howells* and D.W.Pfaff. The Rockefeller University, New York, N.Y. 10021 and *The Roche Institute for Molecular Biology, Nutley, N.J. 07110 USA

Chronic haloperidol (HAL) treatment increases the levels of proenkephalin (PE) peptides and mRNA in the caudate-putamen (CPu) (Tang, et al. PNAS 80:3841,1983). To determine whether this increase in PE gene expression is exhibited by all enkephalinergic neurons of the CPu or only by a subpopulation, we have examined this effect on PE mRNA levels at the cellular level using in situ hybridization. Male rats were injected ip with 2mg/kg HAL or vehicle (VEH) daily for 2 wks,decapitated 24hr after the final injection, and CPu were dissected and stored at -80°C.A cDNA probe complementary to rat preproenkephalin, (Howells, et al. PNAS 81:7651,1984), was nick-translated with [32P] or [3H] nucleotides. In situ hybridization was carried out following the method of Shivers, et al. (Meth. Enz.V124,1986). Emulsion autoradiograms of cryostat sections hybridized with a [3H] probe were subjected to grain counting to quantitate the autoradiographic signal. Labelled neurons were uniformly distributed throughout the CPu. HAL treated rats (n=6) had 3.0-fold more grains/labelled neuron than VEH-treated rats (n=5). The total number of labelled neurons in the CPu did not increase following HAL treatment. Film autoradiograms of sections which were hybridized with a [32Pl labelled probe also showed a 3.0-fold increase in the signal from the CPu after HAL treatment. For dot blot analysis, total RNA was isolated from individual CPu tissue blocks. The autoradiographic signal intensities of the dots from HAL-treated animals (n=7) were 2.7x greater than the dots from VEH-treated animals (n=5), concurring with the results of northern blots.(Tang, et.al., ibid.) These findings suggest that dopamine exerts a tonic inhibitory effect on PE gene expression in all enkephalinergic neurons of the CPu. The agreement between the in situ hybridization results and dot blot analysis supports in situ hybridization as a method for quantitative studies of gene expression in the brain.

067

TEBULET, A NEW HIGHLY POTENT AND SELECTIVE LIGAND FOR δ OPIOID RECEPTORS. B.P. Roques, G. Gacel, C. Seguin, V. Daugé, J. Belleney and P. Delay-Goyet, Département de Chimie Organique, U 266 INSERM, UA 498 CNRS, UER des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'observatoire 75006 Paris, France.

Two types of compounds are commonly used for the biological and the pharmacological characterization of 6 opioid receptors ; DTLET, a linear hexapeptide (1) and D-Pen^2-Pen^5 enkephalin a cyclic pentapeptide (2). However both ligands are not perfect, since the former is highly potent ($K_i\delta$ = 1.35 ± 0.15 nM) but its cross-reactivity with μ sites remains relatively high (Ki δ / K_i μ = 0.053), whereas the latter is very selective ($K_i\delta$/ K_i μ = 0.0125) but displays a somewhat low affinity ($K_i\delta$ = 10.9 ± 1.2 nM) (3). From the proposed model (4) of selective recognition of μ or δ sites and extensive conformational studies of linear and cyclic peptides, new ligands belonging to the linear hexapeptide series were designed in order to increase the δ selectivity without loss of affinity. The must efficient compound Tebulet (5) characterized by a constrained structure exhibits a $K_i\delta$ = 4.9 ± 0.6 nM and a discriminatory factor Ki δ / K_i μ = 0.0122. This new proble was triticted and is now proposed as reference ligand for studies of δ receptors.

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021

AN EXAMINATION OF THE OPIATE RECEPTOR TYPE5 LABELED BY THE OPIATE ANTAGONIST, [^{3}H]CYCLOFOXY: A LIGAND SUITABLE FOR POSITRON EMMISION TOMOGRAPHY. R. B. Rothman[1], S. McLean[2], V. Bykov[1], A. E. Jacobson[3], R.A. Lessor[3] and K. C. Rice[3], [1]Laboratory of Preclinical Pharmacology, St. Elizabeths Hospital, NIMH, Washington DC 20032. [2]Laboratotory of Neurophysiology, NIMH, Bethesda, MD, 20205. [3]Laboratory of Chemistry, NIADDK, Bethesda, MD, 20205. U.S.A.

The major goal of this study was to define the types of opiate receptors labeled in vitro by the novel opiate antagonist [^{3}H]cycloFOXY synthesized by Rice and associates. The three approaches used were: 1. quantitative 1 igand binding studies utilizing the technique of binding surface analysis; 2. selective site-directed alkylating agents BIT (mu-selective), FIT (delta-selective), and beta-FNA (mu-selective) ; 3. receptor autoradiography.

Receptor binding assays were conducted using frozen lysed-P2 preparations of rat brain. The assay conditions were 4 to 6 hr incubations at 4° C in 50 mM TRIS-HCl, 100 mM NaCl, pH 7.4, containing the protease inhibitors bacitracin, bestatin, chymostatin, and leupeptin.

Pretreatment of membranes with FIT did not significantly reduce C[^{3}H]cycloFOXY binding, whereas pretreatment with BIT or FNA reduced binding by 20 and 25 % respectively. Binding surface analysis of the interaction of DAGO and dynorphin (1-8) with [^{3}H]cycloFOXY binding resolved into 2 binding sites. The Kd's (nM) of DAGO and dynorphin were: 7.1 and 480 for DAGO, and 2912 and 141 for dynorphin. Autoradiographic studies are in progress.

The data presently available suggests that [^{3}H]cycloFOXY labels mu and kappa binding sites in rat brain.

P66

UNEXPECTEDLY HIGH LEVELS OF OPIOID FEPTIDES IN RAT BRAIN MEMBRANES. R. B. Rothman,[1] J.A. Danks[1] and M. Iadarola[1]. [1]Laboratory of Preclinical Pharmacology, St. Elizabeths Hospital, NIMH, Washington DC 20032, U.S.A.

An important experimental paradigm is the measurement of neurotransmitter receptors *in vitro* following *in vivo* manipulations of the animal. The possibility that observed changes reflect changes in receptor occupancy by endogenous ligands must be carefully ruled out.

These considerations led us to directly measure using radioimmunassay the levels of dynorphin (1-8), CCK-8, and MERGL in lysed P2 membranes (group I), membranes incubated 60 min at 25° C (group II) in 50 mM TRIS-HCl at pH 7.4, and membranes incubated 60 min at 25° C in TRIS buffer containing 0.4 M NaCl (group III).

	pmol/mg protein		
GROUP	DYN(1-8)	CCK-8	MERGL
I	.253+.031	1.727±.221	.983±.081
II	.108_+.009	1.015±.224	.585±.090
III	.106_+.029	.179+±.007	.240±.060

Similar results were obtained when substance-P was measured. Additional experiments indicated that membranes rapidly degraded MERGL added to the homogenate. Freincubation of membranes with 0.4 M NaCl failed to increase the levels of [^{3}H]etorphine binding, indicating that frozen lysed-P2 membranes do not have endogenous ligands occupying opiate receptors. Detailed comparisons of the two binding sites labeled by [^{3}H]naloxone revealed changes in binding parameters induced by preincubation with NaCl. Additional studies are required to determine the significance of apparently sequestered neuropeptides in this preparation of rat brain membranes.

0:8

Purified mu-opioid receptor exhibits phosphatase activity. S.Roy, N.M. Lee and H.H. Loh. Deparment of Pharmacology,University of California, San Francisco, CA. 94143.

A mu-opioid receptor protein has been purified by this laboratory (Cho et al., 1986). The purified receptor is a glycoprotein with a molecular weight of 58,000 as determined by SDS-polyacrylamide gel electrophoresis. Preliminary investigations show that endogenous phosphatase-like activity is assosciated with this protein. Further studies show that the pH optimum for the enzymatic activity was at pH 9 with a Km= 9uM when para-nitro phenol phosphate was used as the substrate. Analysis of gel slices following SDS-gel electrophoresis show that the major peak of phosphatase activity is assosciated with a band of apparent molecular weight 60,000,which corresponds to the molecular size of the purified mu-opioid receptor protein. Our results also show that the enzymatic activity is activated by Mg^{2+}, Mn^{2+} and Ca^{2+}. Further characterization of the enzymatic activity will be presented.

022

HUMAN NEUROBLASTOMA SK-N-SH CELLS: PHENOTYPIC VARIATION OF μ AND δ OPIOID RECEPTORS, MUSCARINIC CHOLINERGIC RECEPTOR AND THE UPTAKE$_1$ SYSTEM.

W. Sadée[1], V.C. Yu[1], M.L. Richards[1], F.M. Brodsky[2] and J.L. Biedler[3]. [1]School of Pharmacy, University of California San Francisco, CA 94143, [2]Becton Dickinson Immunocytometry Systems, Mountain View, CA 94039, and [3]Memorial Sloan-Kettering Cancer Center, New York, NY 10021, U.S.A.

Neuroblastoma cells grown in culture can undergo phenotypic changes between epitheloid and neuroblast forms. We have established the presence of μ and δ opioid receptors, muscarinic cholinergic receptors and an effective uptake$_1$ transport sytem for norepinephrine in the human SK-N-SH cell line. In order to address the question of the expression of these systems during phenotypic modulation, phenotypically stable subclones of SK-N-SH were used that exist either in the neuroblast state (SY-5Y), an intermediate state (IN), or the epitheloid state (EP). While both the neuroblast SY-5Y and the intermediate IN subclones expressed all three systems to similar degrees,epitheloid cells were devoid of these systems. This result documents that the coordinate expression of multiple receptor systems correlates with the phenotypic switch from epitheloid to neuroblast status. In contrast there was no change of the expression of clathrin heavy and light chain subunits with the different phenotypes. Further, the light chain clathrin subunit was of the peripheral tissue-type rather than the brain-type, which is consistent with the peripheral source of neuroblastoma tissue samples. Supported by DA 01095 from NIDA, Bethesda, MD.

P61

ACTIVATION OF MEDULLARY OPIOID-PEPTIDERGIC SYSTEMS BY FORMALIN-INDUCED PERSISTENT PAIN AND ITS PHYSIOLOGICAL ROLE. M. Satoh, M. Sugimoto, Y. Kuraishi and H. Takagi, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

The nucleus reticularis gigantocellularis (NRGC) including nucleus reticularis paragigantocellularis is known to be an important site of analgesic actions of opioids. Kuraishi et al. have demonstrated an increased release of immunoreactive Met-enkephalin (iME) from the nucleus following injection of formalin to the hind-paw in rats. In the present experiments, we further studied the physiological role of opioid-peptidergic system and the content of iME in the rat NRGC. 1) Durations of licking response were measured at periods of 0-10 min (P-A), 10-30 min (P-B), 30-60 min (P-C) and 60-120 min (P-D) after formalin injection into the hind-paw of the rat. Naloxone injected systemically (using miniosmotic pumps) or into the 4th ventricle (30 nmol/rat), but not intrathecally (up to 300 nmol/rat), significantly increased the duration at P-C. On the contrary, injections of enkephalin-degrading enzyme inhibitors (thiorphan plus bestatin; 50 µg each/rat) into the 4th ventricle significantly suppressed the duration at P-C. 2) The region of NRGC was punched out from the freezed slices of lower brain stem of rats. The content of iME in the NRGC increased after treatment with thiorphan plus bestatin. This increase was augmented by formalin injection into the hind-paws, while treatment with cycloheximide blocked such an augmentation. The iME content measured after digestion of NRGC extracts with trypsin and carboxypeptidase B was elevated by formalin injection in rats given thiorphan plus bestatin.
These findings suggest that formalin-induced persistent pain stimulates the biosynthesis of ME in the NRGC and is inversely attenuated by the stimulated MEergic system.

054

THE ROLE OF SPINAL KAPPA-OPIATE RECEPTORS IN ANALGESIA. C. Schmauss and A. Herz, Max Planck Institute for Psychiatry, Munich, F.R.G.

Male Spraque Dawley rats were implanted with a chronic intrathecal catheder extending into the lumbar subarachnoid space. The antinociceptive potency of intrathecally administered morphine and the putative kappa-opiate receptor ligands bremazocine, Dyn 1-17, Dyn 1-13, U50488H and trifluadom was estimated in the tail flick (TF) and tail pressure (TP) nociceptive tests. Morphine equipotently inhibited both TF and TP nociceptive responses. The kappa-ligands Dyn 1-17, Dyn 1-13, trifluadom and U50488H blocked TP but not TF nociceptive responses at doses which did not impair motor function. Only bremazocine was more potent in blocking TF than TP nociceptive responses. Morphine-tolerant rats did not exhibit tolerance to the analgesic effects of U50488H and trifluadom on the TP response, indicating that these ligands act upon a spinal opiate receptor which is distinguishable from the receptor through which morphine exerts its analgesic effect. In line with these results is the observation that the effects of bremazocine on the TF, but not on the TP nociceptive response, show cross-tolerance to morphine. These data suggest that the spinal kappa-opiate receptor is involved in the mediation of TP analgesia. Further support is obtained for the suggestion that spinal opiate receptor subtypes differentially associate with different nociceptive input.

012

A MONOCLONAL ANTI-IDIOTYPIC OPIOID RECEPTOR ANTIBODY. R.Schulz[1] and C. Gramsch, Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, Martinsried; [1] Institut für Pharmakologie, Toxikologie und Pharmazie, Universität München, FRG.

The 3-E7 monoclonal antibody, which recognizes the N-terminal sequence of endogenous opioid peptides, has been employed to raise monoclonal antibodies against opioid receptors via an anti-idiotypic route. Culture supernatants were screened for immunoglobulins that interacted with $F(ab)_2$ -fragments of the 3-E7 antibody coated to the wells. Their presence was identified by an anti-IgM-F_c -peroxidase test(ELISA). A second screening procedure involved binding to opioid receptors in rat brain membranes, using ^{125}I-ß-endorphin as tracer. Positive master wells were cloned by limiting dilution. Supernatants of posltive clones were tested in the radio receptor assay (^{125}I-ß- endorphin as tracer). Positive samples were then purified by anion-exchange chromatography and the purified material was subjected to SDS-gel electrophoresis. This technique revealed the presence of an immunoglobulin, showing characteristics of a reference mouse-IgM. Furthermore, purified antibodies were submitted to affinity-chromatography (3-E7 linked to Sepharose). The gel retained the active principle responsible for the interaction with opioid receptors. The highly purified antibodies were employed for detailed receptor binding studies on rat brain membranes. The antibody was most powerful in competing with ^{125}I-ß-endorphin, followed by ^{3}H-D-Ala_2,-D-Leu_5-enkephalin and ^{3}H-ethylketazocine; it showed a much lower competition with ^{3}H-dihydromorphine. In contrast, the antibody failed to interfere with the benzodiazepin ^{3}H-flunitrazepam. The data suggest the successful generation of a monoclonal anti-idiotypic antibody of the IgM-type with affinity for both the 3-E7 monoclonal antibody (antigen) and membrane-bound opioid receptors.

019

MECHANISM BY WHICH ASCORBIC ACID ABOLISHES THE ETORPHINE INDUCED COMPENSATORY INCREASE IN INTRACELLULAR CYCLIC AMP LEVELS IN NG108-15 HYBRID CELLS

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In NG108-15 hybrid cells ascorbic acid suppressed the delayed etorphine induced compensatory increase in the levels of cyclic AMP. It had however, no effect on the early response of the cells to etrophine, as manifested in 2 transient decrease in the levels of cyclic AMP (1,2). The mechanism of action of ascorbic acid is not understood. In this communication we report on the following observations. (1) The effect of ascorbic acid on transient inhibition of adenylate cyclase activity in the homogenates of NG hybrid cells. (2) Binding of (15,16-^{3}H) etrophine to monolayer cultures of NG hybrid cells in the presence or absence of ascorbic acid. (3) Adenylate cyclase and guanine nucleotide regulatory protein activity of homogenates of NG hybrid cells which were grown with etrophine in the presence or absence of ascorbic acid.

(1) Shail K. Sharma and Navin C. Khanna. Biochem. J. 208, 43-46 (1982).

(2) Navin C. Khanna and Shail K. Sharma, Life Sciences, 33, 401-404 (1983).

062

BETA-ENDORPHIN ACUTELY STIMULATES SUPEROXIDE PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES: COMPARISONS TO MORPHINE AND FMLP.

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We have recently shown that β-endorphin (β-END), dynorphin and morphine rapidly elicit chemiluminescense from human polymorphonuclear leukocytes (PMN) in vitro due to the production of superoxide (O_2^-). The present work evaluated the opiate receptor involved in the acute PMN response to β-END. Using a ferricytochrome C reduction assay to detect significantly stimulated O_2^- production by PMN from normal donors (N = 5) at concentrations between 10^{-14}-10^{-8}M. The peak response, 14.3 ± 1.3 (mean ± SE) $nM_2O_2^-$/mg protein, occurred at a physiological concentration (10 . N-acetyl-β-END did not affect O_2^- production and (-)-naloxone 10^{-12}M completely abolished the stimulation by β-END 10^{-12}M, whereas (+)-naloxone showed minimal inhibition (<16%). (-)-Morphine sulfate (MS) produced a similar dose-response profile to β-endorphin, although peak PMN O_2^- accumulation was significantly greater with β-endorphin ($p<.05$). Unlike β-endorphin, MS was ineffective at 10 M. N-formyl-methionyl-leucyl phenylalanine (FMLP), a peptide which stimulates PMN chemotactic activity and O_2^- production, was tested for comparison. FMLP-stimulated O_2^- accumulation was first detectable at 10^{-12}M; in contrast to β-END and MS, the response to FMLP increased directly with concentration. Both (+) and (-)-naloxone were equipotent against FMLP 10^{-8}M, resulting in only 30% inhibition which was not significant. H_2O_2, the immediate product of O_2^- metabolism, was also measured. Significant H_zO_2 release by PMN followed stimulation by β-endorphin 10^{-12}M. This was markedly reduced (92%) by nitroprusside, an inhibitor of superoxide dismutase. We conclude that: a) β-endorphin acutely stimulates O_2^- and H_2C_2 production by PMN at peptide concentrations present in the systemic circulation; b) a stereoselective, μ -like opiate receptor mediates the action of β-endorphin. Thus, stress-peptides and exogenous opiates may modulate host defense.

P129

QUATERNARY OPIOID ANTAGONISTS : AN APPRAISAL, J.S. Shaw, N.J.W. Russell and R. James, ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. SK10 4TG, U.K.

There have been several recent reports on the action of opioids at receptor sites outwith the blood-brain barrier. Such studies have relied on the use of standard opioids quaternised on the basic nitrogen which are believed to have limited access to the CNS.

In the present study, receptor affinities have been determined for five quaternary antagonists and their parent compounds (naloxone, naltrexone, nalorphine and levallorphan). This has been related to their ability to reverse μ and k agonist effects in an abdominal constriction test modified to demonstrate peripheral opioid actions (Bentley, Newton and Starr, Brit. J. Pharm. 73, 325, 1981) and in conventional antinociceptive assays.

In all cases quaternisation reduced the affinity for both μ and k-receptors and the extent of this reduction varied considerably between compounds. Taking this into account in subsequent in-vivo studies, N-methyl-naloxone showed no peripheral selectivity. In contrast, the potencies of N-methyl-levallorphan, N-allyl-levallorphan, N-methyl-nalorphine and N-methyl-naltrexone were substantially lower (10-100 fold) in hot plate and conventional abdominal constriction tests than could be accounted for by loss of affinity. This indicates limited CNS penetration. However, attempts to demonstrate peripheral antagonism with these compounds in the Bentley, Newton and Starr test were only successful with N-methyl-nalorphine and N-methyl-naltrexone. The two levallorphan analogues were ineffective at doses up to 30mg/kg S.C.

These results highlight the need for caution in the use of quaternary opioid antagonists as research tools.

P140

BIPHASIC MOTIVATIONAL PROPERTIES OF PARTIAL OPIOID AGONISTS: μ-RECEPT0RS MEDIATE THEIR REINFORCING EFFECTS. T.S. Shippenberg, R.F. Mucha and A. Herz, Dept. of Neuroparmacoloqy, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18a, D-8033 Martinsried, F.R.G. and Addiction Research Foundation of Ontario, 33 Russell Street, Ontario, Canada.

The positive reinforcing effects of opioids have been attributed to agonist actions at μ-receptors, whereas activation of κ-receptors may underlie their dysphoric or aversive effects. The motivational properties of the opioid agonist-antagonist analgesics are little understood and the nature of their interactions with particular opioid receptor subtypes remains ill-defined. The present study employed a place conditioning procedure to characterize the reinforcing/aversive properties of these druqs and the receptor mechanisms mediating their effects. Rats were exposed to one of two distinctive environments following injections of drug or vehicle. After six such training sessions, preference for a particular environment was assessed by allowing rats free access to both settings and measuring the time spent on each. Pure p-agonists such as morphine produced clear preferences for the drug-paired place, while the selective κ-agonists, U-69593 or U-50488, produced dose-related aversions. Of the partial agonists tested, pentazocine was reinforcing at low doses while nalorphine was aversive. Nalbuphine and buprenorphine produced biphasic motivational effects. Preferences were observed with low doses while higher doses resulted in significant aversions for the drug-paired place. Constant infusion of naloxone via minipumps during training, atadose sufficient to blocky-receptors but not κ-receptors, antagonized the preferences induced by morphine and the partial agonists, but was ineffective in modifying their aversive properties or those of U-69593. These data strongly indicate that the partial agonists pentazocine, nalbuphine and buprenorphine, in addition to their purported κ-agonist and μ-antagonist like actions, can function as agonists at the μ-opioid receptor and that activation of this receptor underlies their reinforcing effects.

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090

CELLULAR LOCALIZATION OF PROENKEPHALIN mRNA-CONTAINING NEURONS IN RAT BRAINSTEM AND SPINAL CORD. B.D. Shivers, R.E. Harlan, G.J. Romano, *R.D. Howells and D.W. Pfaff, The Rockefeller University, New York, NY 10021 and *Roche Institute of Molecular Biology, Nutley, NJ 07110, U.S.A.

Previously, we reported the location of proenkephalin mRNA-containing neurons in rat forebrain (Harlan et al., Society for Neuroscience 11:143, 1985), and we have now extended the description of the location of these neurons to brainstem and spinal levels. We performed in situ hybridization on frozen, paraformaldehyde-fixed tissue sections probed with a tritiated 435 b.p. cDNA complementary to rat proenkephalin mRNA encoding amino acids 56-200 (Howells et al., PNAS 81:7651, 1984).Autoradiograms were exposed 40 days. Hybridizations following RNase pretreatment, or with probe diluent alone yielded no labeled cells. Competition with a 25-fold excess of unlabeled probe greatly reduced the autoradiographic signal. Sections probed with tritiated rat pro-opiomelanocortin cRNA (gift of B. Schachter) yielded a different labeling pattern than that observed with proenkephalin cDNA. Labeled cells were found in the lateral geniculate nucleus, periaqueductal gray, midbrain reticular formation, ventral tegmental area of Tsai. inferior colliculus, dorsal and ventral tegmental nuclei of Gudden, dorsal and ventral parabrachial nuclei, pontine and medullary reticular formation, some of the raphe nuclei, nucleus of the solitary tract, nucleus of the spinal trigeminal tract, ventral and dorsal cochlear nuclei, medial and spinal vestibular nuclei, cuneate and external cuneate nuclei, gracile nucleus, superior olive, nucleus of the trapezoid body, some deep cerebellar nuclei, Golgi II neurons of the cerebellum, and most laminae of the spinal cord, especially I and II. Generally, there was good agreement between regions shown previously to contain immunoreactive enkephalin neurons and those regions which we found to have neurons containing proenkephalin mRNA; however, in many of these regions, more neurons were found to contain proenkephalin mRNA than were shown previously to contain immunoreactive enkephalins. The results are consistent with the concept that opioid peptides derived from proenkephalin participate in the integration of motor and sensory information.

P68

ISOLATION AND SEQUENCING OF OPIOID PEPTIDES FROM PORCINE BRAIN EXTRACTS USING A [^{3}H]-DAGO BINDING ASSAY TO FOLLOW THE PURIFICATION. Mark Sonders, Bruce Campbell, Charles Jimenez, Marjorie Shih, and Eckard Weber. Institute for Advanced Biomedical Research, The Oregon Health Sciences University, Portland, Oregon 97201, U.S.A.

One of the current projects in our laboratory concerns the isolation of a putative endogenous sigma receptor ligand using a binding assay to follow the purification (see our presentation elsewhere in this meeting). Binding assays have rarely been used to isolate and characterize endogenous neurotransmitter or neuromodulator candidates and it is therefore unclear whether the use of binding assays to purify endogenous receptor ligands will yield substances of physiological relevance. In order to determine whether binding assays can be used to follow the purification of endogenous receptor ligands yielding physiologically meaningful compounds, we used an opioid receptor binding assay to follow the purification of putative opioid active peaks in chromatography fractions from separated acid acetone extracts of 5 porcine brains (513 g). The mu-opioid receptor-specific radioligand [^{3}H]-DAGO was used in the binding assay. At least eight major peaks of [^{3}H]-DAGO displacement activity were observed in porcine brain extracts that had been separated on a preparative reverse phase C-18 column. Five of the peaks were purified to homogeneity using various reverse phase HPLC separation systems. The homogenous peaks were subjected to gas phase sequencing and/or amino acid composition analysis using microanalysis methods established in our laboratory. The five compounds were unambiguously identified as known opioid peptides from the proenkephalin and prodynorphin precursors. [Met^5]-enkephalin and alpha-neoendorphin represented the largest peaks of [^{3}H]-DAGO displacement activity. These studies demonstrate that radioligand binding assays can be used to follow the purification of endogenous neurotransmitter or neuromodulator candidates and that this method can yield physiologically meaningful endogenous receptor ligands.

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075

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF AN ENDOGENOUS COMPOUND THAT COMPETITIVELY AND REVERSIBLY INHIBITS [^{3}H]-DTG FROM BINDING TO THE HALOPERIDOL-SENSITIVE SIGMA RECEPTOR. Mark Sonders, Bruce Campbell, and Eckard Weber, Institute for Advanced Biomedical Research, The Oregon Health Sciences University, Portland, Oregon 97201, U.S.A.

The development of [^{3}H]-DTG as a novel, selective ligand for the haloperidol-sensitive sigma receptor (see our presentation elsewhere in this meeting) has enabled us to screen brain extracts for the presence of endogenous material that can displace [^{3}H]-DTG from its brain membrane binding sites. Acid extracts from whole cow brains were purified by cation exchange chromatography. These partially purified extracts contain a small molecular weight compound which competitively and reversibly inhibits the binding of [^{3}H]-DTG to the haloperidol-sensitive sigma receptor in guinea pig brain membrane preparations. The substance is not a peptide. Its physicochemical properties [chromatographic behavior in various systems, solubility properties in polar and nonpolar solvents etc.] will be presented. Whether or not this compound is a physiologically relevant, endogenous ligand for the haloperidol sensitive sigma receptor can only be determined after the compound has been purified to homogeneity and its chemical structure has been determined. These studies are underway.

Supported by NIMH grant MH40303.

074

EVIDENCE FOR THE EXISTENCE OF ENDOGENOUS LIGANDS FOR SIGMA OPIOID RECEPTORS IN THE BRAIN ("SIGMAPHIN"): STUDIES FROM BINDING ASSAYS. T.-P. Su and S. Y. Yeh, National Institute on Drug Abuse, Addiction Research Center, Baltimore, Maryland 21224, U.S.A.

SKF-10047 was the prototypic drug for the sigma opioid receptors which was purported to mediate the psychotomimetic effects of certain opioids and other drugs. Sigma opioid receptors were later identified in tHe guinea-pig brain homogenates in a binding assay using [^{3}H]SKF-10047 (Su, J. Pharmacol. Exp. Ther. 223:284, 1982). Ligand selectivities of the sigma opioid receptors indicated that sigma opioid receptors do not represent any other known receptor systems in the brain and may therefore represent a new psychotomimetic pathway. The unique ligand selectivities of the sigma opioid receptors and the fact that its prototypic ligand SKF-10047 is psychotomimetic in humans suggest a possibility of the existence of endogenous ligand for this receptor. This report presents evidence that endogenous ligands for sigma opioid receptors ("SIGMAPHIN") may exist in the brain. Two endogenous ligands which interacted preferentially with the sigma opioid receptors were identified from the guinea-pig extract in a Sephadex G-50 fractionation. These two ligands were more potent in the sigma receptor assay than in the mu, kappa and delta receptor assays. In the phencyclidine receptor assay, two ligands were almost inactive. Incubation of those ligands with trypsin destroyed at least 50% of the activities of those ligands in the sigma receptor assay. Both ligands inhibited sigma binding in a dose response manner. The inhibitions were competitive. The inhibitions could be eliminated when the ligands were removed from assay media by extensive washings. It is concluded therefore that sigma opioid receptors are not phencyclidine receptors and that endogenous ligands for sigma opioid receptors may exist in the brain.

P154

MOOULATION BY ENKEPHALIN ANALOGS AND NEUROLEPTICS OF APOMORPHINE-INDUCED STEREOTYPY AND TURNING BEHAVIOR IN RATS.

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Some of the most characteristic behavioral effects of classical opiates and opioid peptides are similar to those elicitable by neuroleptics (Bloom et al., Science, 194, 630, 1976; Jacquet and Marks, idem 194, 632, 1976). Thus the question arises whether also opioids inhit the central dopaminergic tone. The neuroleptics are known to antagonize the motor effects of apomorphine (A) at specific low dose levels. In our previous study (Sineger et al., Eur., J. Pharmacol., 80, 359, 1982) s.c. injected morphine (M) and a potent enkephalin analog (2-D-Met,5-Pro-enkephalinamide, EA) only partially inhibited the A-induced stereotypy (S) and turning behavior (TB) in rats with unilateral nigral lesion. Nevertheless, the inhibition by EA of A-induced S was statistically significant already at subanalgesic dose levels. The aim of the present investigations was to examine that which brain areas might mediate the anti-A effect of opioids. As the first step of these examinations the substances under study were injected either i.c.v. or into the nucleus accumbens (NA) i.e. a mesolimbic region rich in dopamine. Substances examined were M a μ-selective opiate, 2-D-Ala,5-Nle--enkephalin sulfonic acid (ES) a delta-selective opioid, EA a highly potent non-selective opioid peptide, furthermore, haloperidol (H) and chlorpromazine (C). As for the inhibition of A-induced TB the order of potency was: EA>H>M>ES≅C if injecting the substances i.c.v. but EA>M>H≅ES≫C when administering them into the NA. Examining the suppression of A-induced S the order of potency was EA≫H≅M>ES≅C upon i.c.v. administration and EA≫H>M≅ES>C upon injecting them into the NA. The data show that certain endogenous opioids might inhibit the brain dopamine activity via the NA.

P60

KYOTORPHIN AS A NEUROREGULATOR; EVIDENCE FOR ITS BIOSYNTHESIS, RELEASE, RECEPTOR AND POST RECEPTOR MECHANISM H. Takagi, H. Ueda and Y. Yoshihara, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, - Kyoto 606, Japan

Kyotorphin (Tyr-Arg) is distributed unevenly throughout the brain regions and concentrated in the synaptosomes. Following studies have demonstrated that kyotorphin produces an analgesia via a release of met-enkephalin from the brain. In the course of studying the mechanism of met-enkephalin release, we found the facilitatory effects of kyotorphin on the Ca^{2+} entry into synaptosomes. Thus it has been postulated that kyotorphin plays a physiologically significant role as a neuroactive peptide. In the present study, we reports the further evidence indicating the role of kyotorphin as a neuroregulator. BIOSYNTHESIS Kyotorphin is formed from tyrosine and arginine in the brain synaptosomes. The partially purified kyotorphin synthetase was characterized. UPTAKE AND-RELEASE Kyotorphin was actively taken up into P_2 (crude synaptosomes). Kyotorphin preloaded into synaptosomes was released by high KCl stimulation in a Ca^{2+} dependent manner. KYOTORPHIN SPECIFIC RECEPTOR ^{3}H-kyotorphin specific binding activities were obtained using the brain membrane preparations. Leu-Arg showed a potent inhibition of the binding. POST RECEPTOR MECHANISM We observed the kyotorphin-induced mobilization of the plasma membrane-bound Ca^{2+} into cytosolic space, determined with the measurement of the fluorescence of Quin-II and chlortetracycline, and $^{45}Ca^{2+}$ entry into synaptosomes. The kyotorphin-induced increase of the $^{45}Ca^{2+}$ entry was completely abolished by Leu-Arg (a putative kyotorphin antagonist). ANALGESIA Kyotorphin (i.cisternal)-induced analgesia in mice was significantly attenuated by co-administration of Leu-Arg.

P50

THE EFFECT OF HALOPERIDOL ON PRO-DYNORPHIN END PRODUCTS IN THE RAT STRIATUM AND SUBSTANTIA NIGRA. R. Tandon, R. Day, J.E. Kelsey, S.J. Watson and H. Akil, Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109-0720, U.S.A.

While the nigro-striato-nigral loop has been extensively studied in the context of interactions between dopamine, acetylcholine and GABA, little is known about the role of the recently discovered striatonigral-dynorphin pathway. Pro-dynorphin (Pro-Dyn) perikarya are localized in the caudate-putamen and some of these neurons are thought to be the source of dynorphin innervation to the pars reticulata of the substantia nigra (SN). On the other hand, dopaminergic cell groups within the SN give rise to ascending projections to the striatum (STR) via the nigrostriatal pathway. Pharmacological manipulation of this system using the dopamine receptor antagonist haloperidol (HAL) was used to examine Dyn-dopamine interactions. Male Sprague Dawley rats were injected i.p. over 4 consecutive days, and sacrificed on the fifth, with either saline or HAL at doses of 0.25, 0.5, 1.0 or 2.0 mg/kg/day. The STR and SN were collected and extracted in methanol:0.1N HCl (1:1 v/v). Dyn A 1-8 and Dyn A 1-17 content was measured using specific RIAs. In both regions, Dyn A 1-8 levels increased at the lower doses of HAL treatment (0.25 and 0.50 mg/kg), but returned to control values or lower than control values at the higher doses of HAL. However Dyn A 1-17 content in STR and SN progressively decreased from control values with increasing doses of HAL. A comparison of the Dyn A 1-17:Dyn A 1-8 ratio shows an increase in the conversion of Dyn A 1-17 to Dyn A 1-8 in STR and SN in rats treated with HAL. The sum of these observations may be explained by alterations in rates of biosynthesis, processing and/or release of Pro-Dyn end products. Our results demonstrate the regulation of the striatonigral dynorphin system by dopamine. In turn, the changes in Pro-Dyn end product ratios could subsequently modify nigrostriatal dopaminergic function. This work was supported by the T. Raphael Research Fund and NIMH Grant # MH39717.

P16

DETERMINATION OF SIZES OF THE OPIOID RECEPTORS IN THEIR MEMBRANE ENVIRONMENT BY RADIATION INACTIVATION.

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Opioids, like other drugs, are thought to initiate their effects by associating with their specific receptors. However, very little is known about the opioid receptor as a molecular entity. The binding components have been solubilized in detergent and purified by different approaches, but the molecular size of soluble opioid receptor complexes reported by different groups varied from 23,000 to 750,000. In this study, the technique of radiation inactivation by gamma ray was used to investigate the apparent size of the opioid receptor in the rat brain membranes under different conditions. The molecular sizes of opioid receptor complexes were egstimated as 313,000 in the presence of [D-Ala^2,D-Leu^5] enkephalin, NaCl and Gpp(NH)p; as 165,000 in the presence of NaCl only or of both NaCl and Gpp(NH)p; as 217,000 in the presence of Gpp(NH)p only: and as 286,000 in the presence of $MgCl_2$ only. A simple model has been proposed to explain these different apparent target sizes of opioid receptor obtained under different conditions.

P117

ROLE OF THROMBOXANE A, IN THE MORPHINE INDUCED POTENTIATION OF THE GASTROULCEROGENIC EFFECTS OF INDOMETHACIN IN RATS.

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Morphine has been shown to potentiate the ulcerogenic activity of indomethacin in a dose dependent manner following subcutaneous administration in rats. This potentiation is blocked partially by anticholinergics and cimetidine and completely by naloxone thus showing the involvement of opiate receptors in its aetiology. We have further studied the effect of OKY-046, a specific thromboxane A_2 synthesis inhibitor; BM 13177, a thromboxane A_2 receptor antagonist and sodium cromoglycate, a mast cell stabilizing agent on the morphine induced potentiation of the gastroulcerogenic activity of orally administered indomethacin in rats. Our results show that thromboxane A_2 is significantly involved in this action, whereas the involvement of histamine released through the disruption of mast cells is completely ruled out as pretreatment with sodium cromoglycate does not affect the potentiating action of morphine. It would be interesting to study as to how thromboxane A_2 is involved in the opiate receptor mediated potentiation of indomethacin's ulcerogenic effect.

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THE ROLE OF μ- AND δ-RECEPTORS IN GASTROINTESTINAL PROPULSION IN RATS. A. Tavani, M. Sbacchi, A. La Regina, P. Petrillo, Istituto di Ricerche Farmacologiche "Mario Negri", via Eritrea 62 - 20157 Milan, Italy.

To study the role of μ- and δ-receptors in gastrointestinal propulsion, overnight fasted rats received i.p. injections of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGO) or morphine or [D-Ala^2, D-Leu^5]enkephalin (DADLE) respectively 1, 5 and 5 min before a charcoal meal and the percentage of small intestine traversed by charcoal was measured 5 min after the meal. The doses inhibiting gastrointestinal transit by 50% compared to controls (ED_{50}), obtained from the dose-response curve, were 6.20, 15.19 and 23.03 μg/kg respectively for DAGO, morphine and DADLE. In the presence of naloxone (50 μg/kg i.p. 5 min before DAGO and 1 min before morphine or DADLE), the dose-response curves of DAGO and morphine were parallel but shifted to the right of the respective curve in the absence of the antagonist; the ED_{50} were respectively 153 and 239 μg/kg. Conversely, the dose-response curve of DADLE with the same dose of naloxone was flat and doses from 15 to 1000 μg/kg of DADLE produced about 50% of the maximal effect. In the presence of the δ-selective antagonist ICI 174,864 (1 mg/kg i.p. 5 min before DAGO and 1 min before morphine or DADLE), the dose response curves of DAGO and morphine were the same as in the absence of the antagonist. Conversely, the dose-response curve of DADLE in presence of ICI 174,864 was different, although the antagonist shifted it only very weakly. It thus appears that μ-receptors may be involved in the inhibition of gastrointestinal propulsion induced by DAGO and morphine and both μ- and δ-receptors in the inhibition induced by DADLE. The effects of the selective δ-compound [D-Pen^2, D-Pen^5] enkephalin are discussed too.

D34

μ, δ & K OPIOID RECEPTOR DISTRIBUTION IN RAT BRAIN USING IN VITRO LIGHT MICROSCOPY AUTORADIOGRAPHY. A. Tempel and R. S. Zukin, Dept. of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

We have used quantitative in vitro autoradiography at the level of the light microscope to determine the precise neuroanatomical distribution of the μ, δ, and K opioid receptors. Adjacent sectons of frozen rat brain were thaw-mounted on glass slides, incubated with radiolabelled opioid in the presence or absence of nonlabelled ligand in order to control for nonspecific binding of the radioligand, and apposed to tritium-sensitive film. The neuroanatomical pattern of μ receptors was determined using D-Ala^2,N-Me-Phe^4, Gly-ol^5-enkephalin (DAGO μ-specific opioid). At the level of the anterior commissure, discrete patches of densely-labeled opiate receptors were observed, surrounded by areas of diffusely-organized receptors. At the level of the diencephalon dense labeling of μ receptors was seen to overlie the molecular layers of the hippocampus, particularly the pyramidal field. Dense μ receptor labeling was also observed in specific thalamic nuclei, layers I and III of the neocortex and the locus coeruleus of the pons. In contrast, receptors labeled by ^{3}H[D-Pen^2, D-Pen^5]enkephalin (DPDPE specific ligand) exhibited a diffuse distribution in the striatum with few visible patches. In the neocortex, selected localization was observed in layers I & II and V & VI. Nuclei of the thalamus, hypothalamus midrain and brainstem were notably lacking in δ receptors. K receptors (labeled using ^{3}H-ethylketocyclazocine in the presence of μ and δ blockers) exhibited a distribution similar to that of μ receptors in the striatum and hippocampus, but differed markedly in midbrain and hindbrain areas. High K receptor density was observed in the midline thalamic nuclei, central gray area, substantia nigra reticulata, interpeduncular nucleus, the locus coeruleus and cerebellum. Thus, pronounced differences in the neuroanatomical distributions of the μ, δ and K receptors may account in part for the diverse pharmacological profiles, of μ, δ and K opioids. [Supported by NIH grants DA 01843 & DA 00069 (to R.S.Z.) and NS 21973 (to A. T.)].

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STRUCTURAL REQUIREMENTS LEADING TO AGONIST AND ANTAGONIST ACTIVITY IN 4-ALKYL-4-m-(HYROXY)-PHENYLPIPERIDINES. L. Toll, J. A. Lawson, W. Polgar, E. Uyeno, G. Frenking, G. H. Loew, Life Sciences, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025, U.S.A.

The family of 4-(m-OH)-phenylpiperidines is a flexible fragment of the morphine/benzomorphan fused-ring opioid family. Analogs in this family were synthesized with varying 4-alkyl substituents, increasing in bulk from H, through methyl and n-propyl, to t-butyl, each with the three N-substituents, methyl, allyl, and phenethyl. These twelve compounds were evaluated for analgetic agonism and antagonism in mice and for binding affinities using five ^{3}H-labelled ligands. Energy conformation calculations on these structures were also carried out in order to better under stand how the 4-alkyl substituents modulate receptor binding affinities and agonist/antagonist potency ratios through equatorial/axial conformers. The results obtained show that the 4-alkyl-4-(m-OH)-phenylpiperidines vary as agonists with a range of activity from weak to morphine-like and that only a few analogs have antagonist activity. Combined results of energy conformation calculations and in vivo and in vitro analysis suggest that binding in a phenyl axial conformation will lead to agonist activity, while phenyl equatorial binding will lead to antagonist activity. Furthermore, specific interactions with 4-n-propyl and N-phenethyl moieties can induce phenyl axial and equatorial interactions, respectively. None of the analogs, showed fused-ring-like N-substituent modulation of agonist/antagonist activity, even though the 4-t-butyl analogs exist essentially completely in the phenyl axial conformation. These compounds are the first simple 4-alkyl substituted 4-phenylpiperidines to be reported with a strongly preferred phenyl-axial conformation.

P59

IMMUNOCYTOCHEMICAL LOCALIZATION OF PRO-OPIOMELANOCORTIN -DERIVED PEPTIDES IN THE ADULT RAT SPINAL CORD.

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A dispersed descending pro-opiomelanocortin (POMC) fiber system has been demonstrated by PAP immunocytochemistry in the adult rat spinal cord. (β-endorphin, ACTH, α-MSH and 16K immunoreactive fibers exist in the spinal cord from cervical down to sacral level. Descending fibers running in parallel in the dorsolateral and lateral funiculus send collaterals ventromedially or medially to terminate in the gray matter surrounding the central canal, where nociceptive neurons have recently been located, in addition to those nociceptive cells in the dorsal horn. After spinal transection at lower thoracic level, POMC peptide immunoreactivities disappeared below the lesion. Moreover, no POMC cell bodies were found in the spinal cord. Therefore, the descending fibers are most likely of supraspinal origins.

050

THE ANIMAL PHARMACOLOGY OF A NOVEL OPIOID ANALGESIC DRUG. XOHPHANOL

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Xorphanol is a novel morphinan opioid drug, which has been shown to have analgesic activity in man[1]. In vitro experiments suggest that xorphanol is a potent opioid partial agonist, with good agonist selectivity for the κ-receptor. Thus in the field-stimulated, isolated, guinea-pig ileum, it showed the low maximum agonist activity (E_{max} 40%; IC_{20} = 0.2nM) which is typical of a partial agonist. It was not antagonised by the μ-selective antagonist β-funaltrexamine; but its dose-response curve showed a large rightwards shift after effective κ-receptor antagonism with the irreversible antagonist β-chlornaltrexamine, in the presence of μ-receptor protection[2]. Xorphanol behaved as an antagonist in both the rabbit vas deferens (κ-receptors; pA_2 for antagonism of ethylketocyclazocine = 9.04) and rat vas eferens (μ-receptors; pA_2 for antagonisn of D-Ala^2, $MePhe^4$, $gly(ol)^5$ enkephalin = 8.44) which, together with the guinea-pig ileum data, suggests partial agonist activity at the κ-receptor and antagonist activity at the μ-receptor. Xorphanol produced potent antinociceptive activity in the mouse abdominal constriction and guinea-pig paw pressure tests (ED_{50} values = 0.05mg/kg s.c. and 0.035mg/kg s.c. respectively), but was inactive in the mouse hotplate test and only weakly active in the rat paw pressure test. This profile is characteristic of a low efficacy κ-agonist[3]. Xorphanol, 0.1-0.3mg/kg s.c., also showed potent and long-lasting antinociceptive activity in the dog toothpulp stimulation test. As expected for a partial κ-agonist, xorphanol produced only low maximum respiratory depression and inhibition of gastrointestinal motility in the mouse (E_{max} = 20% and 46% respectively at 3mg/kg s.c.) and produced no significant sedation in the rotarod test in the mouse at 0.1-9mg/kg s.c. Some short duration sedation was seen with xorphanol, 0.1-3mg/kg s.c., in the marmoset. These animal data predict that xorphanol should be a potent and efficacious analgesic in man, with very low side-effect potential.

(1) Bloomfield, S S et al (1984) IUPHAR 9th International Congress of Pharmacology, Abst. 1737P. (2) Sheehan M J et al (1985), Br.J.Pharmac; 84, 68P. (3) Hayes A G et al (1985), Br.J.Pharmac; 86, 703P.

We would like to acknowledge H.G. Pars, Pharma.Labs.Inc., for supplies of xorphanol mesylate.

042

EVIDENCE FOR THE PRESENCE OF PRESYNAPTIC AUTO-REGULATION OF MET-ENKEPHALIN RELEASE IN THE BRAIN AND -EFFECTS OF KAPPA OPIOID ANTAGONISTS THEREON. H. Ueda, N. Fukushima and H. TAKAGI Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan.

Presynaptic regulation of met-enkephalin release from slices of rat midbrain, pons plus medulla oblongata and its physiological role were studied. Amounts of met-enkephalin released from slices were measured by radioimmunoassay. The effects of several subtypes of opioids on Ca^{2+}-dependent and high K^{+} (30 mM)-evoked release of met-enkephalin in the presence of tetrodotoxin (0.3 μM) were determined. The IC 20 of the high K^{+}-evoked release by μ-type opioid agonists, morphine and DAGO were 1.2 and 0.9 μM, δ-type agonists, DTLET and DPLPE 1.5 and 0.7 μM and κ-agonists, U-50488, bremazocine, dynorphin (1-17) 0.06, 0.32 and 0.15 μM, respectively. On the other hand, opioid κ-antagonists (0.1-10μM) facilitated the evoked release. Effects of κ-opioid anatgonists on the nociceptive responses were observed in vivo. When very small doses (0.2-20ng/kg s.c.) of Mr2266 were administered into mice, it produced a significant analgesic effect with the tail flick test when administered intracisternally. To summarize, opioid κ-type antagonist produces an analgesic effect via blocking the autoreceptor on the nerve terminals of met-enkephalin neurons.

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P99

THE INVOLVEMENT OF ENHANCED Ca^{2+} CHANNEL ACTIVATION IN THE NERVE TERMINALS OF MORPHINE WITHDRAWAL ABSTINENCE IN MICE. H. Ueda, S. Tamura, M. Satoh and H. Takaqi, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan

When naloxone was administered intrathecally (i.th.) 24hr after the implantation of a morphine pellet into mice, withdrawal jumping behavior was precipitated and lasted for 5 min. Repeated (i.th.) injection of naloxone produced no more jumping behavior. The intrathecal pretreatment with IAP (5 µg) 24 hr before morphine pellet implantation completely abolished the naloxone-precipitated withdrawal jumping. Furthermore, intrathecal pretreatment with substance P antagonist, (D-Pro^4,D-$Trp^{7,9,10}$)-stbstance P(4-11) 5 min before the naloxone treatment, also completely abolished the precipitated jumping. High K^+-evoked ^{45}Ca entry into the synaptosomes of the spinal cord of the morphine dependent mice was enhanced by naloxone (10^{-4}M). Naloxone (10^{-4}M) enhanced maitotoxin (a Ca channel agonist)-induced ^{45}Ca entry into the spinal synaptosomes of dependent mice, but not of the intact mice. Naloxone at 10^{-4}M increased the c-AMP content in the synaptosomes of the spinal cord of dependent mice, but not of intact mice. Taking the previous report that intracellular c-AMP enhances the Ca^{2+} entry into synaptosomes of rats, the present findings suggest that in the morphine abstinent state, the Ca^{2+} channel may be activated via excessively formed c-AMP and then neurotransmitter release enhanced. In the withdrawal jumping of mice, excessive release of substance P from the spinal cord is possibly involved.

P11

CHARACTERIZATION OF OPIOIO RECEPTORS IN RAT BRAIN FOLLOWING LESIONS OF THE NUCLEUS ACCUMBENS. E.M. Unterwald, R.S. Zukin, and G.F. Koob*, Depts. of Neuroscience and Biochemistry, Albert Einstein College of Medicine, Bronx, NY, 10461 and *Res. Inst. Scripps Clinic, La Jolla, CA, 92037, U.S.A.

Autoradiographic studies have identified dense patches of mu and kappa opioid receptors and less dense patches of delta opioid receptors in the rat nucleus accumbens. In addition, the nucleus accumbens is rich in dopaminergic fibers as it receives extensive innervation from the mesolimbic dopamine tract. Anatomical and functional relationships between the opioidergic and dopaminergic circuitry of the brain are well established and considerable evidence supports the concept that opioid peptides act on presynaptic dopaminergic neurons to modulate dopamine release from their axon terminals. The present study investigates the topographical organization of opioid receptor types in the nucleus accumbens with respect to their pre- and postsynaptic localization and their relationship to dopaminergic nerve fibers.

Dopaminergic terminal fields in the nucleus accumbens of rats were destroyed by unilateral injection of 6-hydroxydopamine (2µl, 8µg/µl) into the nucleus accumbens. Fourteen days after the lesions, the fraction of each receptor type lost in the nucleus accumbens was determined using highly specific in vitro binding assays. Aliquots of homogenate from 6-hydroxydopamine-lesioned, sham-lesioned, and non-lesioned nucleus accumbens (.25 ml, 0.16-0.39 mg/ml protein) were incubated with selective [^{3}H]-ligands in the presence and absence of 10 µM levorphanol. The specific ligands used were [^{3}H]-D-Ala^2,N-Me-Phe^4.Gly-ol^5-enkephalin (DAGO) for mu receptor assays, [^{3}H]-D-Penicillamine^2,D-Penicillamine^5-enkephalin for the delta receptor assays, and [^{3}H]-ethylketocyclazocine in the presence of DAGO (100nM) and D-Ala^2, D-Leu^5-enkephalin (100nM) for kappa receptor assays. Results indicate substantial changes in opioid receptor binding, particularly a dramatic loss of mu receptors, in the nucleus accumbens following these lesions. These results suggest a neuromodulatory role for mu opioid peptides on dopamine release in the nucleus accumbens. (Supported in part by NIH grant DA 01843 and DA 00069 (R.S.Z.)).

P36

THE OPIOID RECEPTOR-GTPase-ADENYLATE CYCLASE SYSTEM IN NG108-15 CELLS: DISSOCIATION BETWEEN DESENTIZATION AND DOWN-REGULATION. L. Vachon, T. Costa and A. Herz, Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, D-8033 Martinsried, F.R.G.

Opioid receptor binding, low Km GTPase and adenylate cyclase were studied in membranes derived from NG 108-15 cells pre-treated with [D-Ala^2, D-Leu^5]enkephalin (DADLE) or morphine. Pre-treatment with DADLE results in a rapid loss of responsiveness of the opioid-dependent GTPase: this desensitization occurs as two time-dependent processes with t 1/2 of 10 min and 8 hr. Opioid binding sites, as assessed using [^{3}H]diprenorphine, also decay as two time-dependent processes upon exposure to DADLE. However, in the presence of Na^+ and GTP, only the slow component is observed. The inhibition of adenylate cyclase by opioids is also lost tine-dependently following pre-treatment with DADLE. However, significant responsiveness of the enzyme is still observed even after desensitization of GFPase has occurred. While the desensitization of GTPase is due to a pure reduction in the maximal effect of DADLE, desensitization of adenylate cyclase occurs throuqh both a loss of maximal effect and a decrease in the potency of DADLE. In contrast to DADLE, morphine induces only a minimal desensitization of the opioid-GTPase-adenylate cyclase system, and can antagonize the fast component of the desensitization induced by DADLE. These findings suggest that 1) desensitization of the opioid receptor-GTPase-adenylate cyclase system results, as a primary event, from an uncoupling between the receptor and the nucleotide-binding regulatory protein (N_i), and occurs before down-regulation of the receptor; and 2) the differential potencies of DADLE and morphine in desensitizing the opioid-GTPase-adenylate cyclase system in NG 108-15 cells cannot be accounted for by a mere difference in receptor affinity, but rather appears to be related to their respective full- and partial-aqonistic properties. (Supported by the Deutsche Forschunqsqemeinschaft, Bonn, L.V. is postdoctoral fells of the Fond de la recherche en Sante du Québec, Québec, Canada).

P63

METABOLISM OF [MET]ENKEPHALIN IN THE RAT SPINAL CORD: RESPONSE TO PEPTIDASE INHIBITORS. K.I. Van Alstyne and K. Jhamandas, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada, K7L 3N6.

Enkephalin metabolism in the brain is mainly a result of the activities of aminopeptidase and enkephalinase, and inhibition of these enzymes by pharmacological agents leads to analgesia. At present, little is known about the metabolism of enkephalins by the spinal cord, a source of significant levels of enkephalins and an important site of opioid analgesia. To study the relationship between analgesia and pharmacological inhibition of enkephalin metabolism in the same region, a system was developed to study the metabolism of [Metlenkephalin (ME) in the isolated rat spinal cord. The separation of ME from its putative metabolites, tyrosine (T), tyrosine-glycine-glycine (TGG) and des[Met]enkephalin, was performed by reverse-phase gradient HPLC using a C_{18} column and 0.01 M sodium acetate (pH 4.0) and acetonitrile as the mobile phase. Coronal slices of rat spinal cord (400 µM) were incubated with ^{3}H-ME for various time intervals over a 60-min period. The radiolabelled metabolites were separated by HPLC. The metabolism of ^{3}H-ME by spinal cord tissue yielded mainly ^{3}H-T and ^{3}H-TGG, reflecting the activities of aminopeptidase and enkephalinase or angiotensin-converting enzyme respectively. In the absence of peptidase inhibitors, cervical, thoracic and lumbar regions of the cord showed similar metabolic profiles. Metabolism of ^{3}H-ME by dorsal and ventral regions of the lumbar cord also yielded similar metabolic profiles. Bestatin, an aminopeptidase inhibitor (1×10^{-7} - 2×10^{-5} M), inhibited the formation of "H-T and augmented the formation of ^{3}H-TGG from ^{3}H-ME. Thiorphan, an enkephalinase inhibitor (1×10^{-8} - 1×10^{-6} M), did not appear to inhibit the formation of ^{3}H-TGG from ^{3}H-ME. This model could be useful in making correlations between inhibition of ME metabolism and production of analgesia by peptidase inhibitors.

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P92

INCREASE IN MORPHINE ANALGESIA BY CORTICOSTERONE: A CENTRAL EFFECT ?

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Evidence has accumulated that opioids influence the secretion of adrenal steroids. Conversely, little is known about the modulation of the opioid system by glucocorticoids. According to Holaday et al., corticosteroids modulate opioid effects through a mechanism peripheral to the neuroaxis. In a recent study, we have shown that corticosterone (CT) potentiates the effect of enkephaline in the hippocampus in vitro, thus indicating an interaction at a central level. The possible behavioral correlates of such interaction was investigated in the present work. Mice received either saline or morphine hydrochloride (1 or 3 mg/kg s.c.); they were then immediately injected intracerebroventricularly with either CT (10^{-4} M or 10^{-5} M; 20 µl/animal) or its vehicle (ethanol 0.5% or 2%). Groups of 16 animals were used. Licking latencies (sec) were measured 30 min after ICV injection (hot plate test; T° of the plate 55°C). Licking latencies were as follows(vehicle was in Exp. I and II ethanol 0.5%, in Exp. III ethanol 2%)

	Exp.I	Exp.II	Exp.III
Vehicle	12.25 ± 0.7	15.1 ± 1.5	14.5 ± 1.4
CT 10^{-4} M	13 ± 1.1	11 ± 1.1	12.8 ± 1.4
Mo 1 mg	16.5 ± 1.7		
Mo 3 mg		17.8 ± 1.74	14.4 ± 1.1
CT 10^{-4} M	-		
+ Mo 1 mg	19.5 ± 2.45		
+ Mo 3 mg		22.25 ± 2.7	18.4 ± 2.1

CT alone has no significant effect; morphine I or 3 mg/kg s.c. had no or slight effects. The associations CT 10^{-4} + Mo had always greater effects than those produced by Mo and vehicle. The differences were hardly significant when considereing each experiment separately but they were significant (for $p < 0.05$) when all results were gathered. Further work is in progress.

P38

KAPPA OPIATE AGONISTS INHIBIT ADENYLATE CYCLASE AND PRODUCE HETEROLOGOUS DESENSITIZATION IN RAT SPINAL CORD CELLS. Z. Vogel and B. Attali, Neurobiology Department, Weizmann Institute of Science, Rehovot 76100, Israel.

As with other receptor ligands which regulate adenylate cyclase activity, opiate agonist binding in spinal cord is regulated by GTP and Na^+, suggesting a coupling to the adenylate cyclase system. The present communication investigates the exact conditions in which opiate inhibition of the spinal adenylate cyclase can be observed. Furthermore, the opioid regulation of the enzyme has been studied in rat spinal cord primary cultures following chronic agonist treatment. Using crude P_2 preparations of rat spinal cord, we were able to show that μ (DAGO 10μM) and δ (DADL 10μM) agonists did not affect the basal cyclase activity (3892±11 pmoles cAMP/15min/mg protein, n=3), whereas the K selective opiate agonist U50488 significantly and dose-dependently inhibited this activity (30% inhibition at 10μM, $p<0.02$), Levorphanol at 10μM inhibited by 20% the basal enzyme levels. The regulatory action was stereospecific since dextrorphan was ineffective. The K selective antagonist MR2266 (10μM) fully antagonized the K agonist response. In all cases GTP(10μM) and NaCl(100mM) were required for the expression of the agonist inhibitory action. The inhibitory effect of K agonists was also observed when the cyclase activity was stimulated by forskolin (100μM). These data suggest that in the spinal cord, opiate receptors of the Kappa type are coupled to adenylate cyclase. Following chronic exposure to etorphine (1μM for 4 days without wash) of rat spinal cord primary cultures, the K agonist U50488 lost its ability to inhibit the cyclase, the basal as well as the forskolin stimulated activities remaining identical to those of paired control cultures. Furthermore, this desensitization process seems to be heterologous, since the α_2 adrenergic agonist, norepinephrine, and the muscarinic agonist, carbachol, exhibit significant lower potency for inhibiting the enzyme activity following exposure to etorphine, suggesting that the site of tolerance induced by opiates in the spinal cord is related to post receptor regulatory components.

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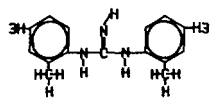
[^{3}H]1,3-Di-O-TOLYL-GUANIDINE ([^{3}H]-DTG) , A NOVEL, SELECTIVE LIGAND FOR THE HAMPERIDOL SENSITIVE SIGMA RECEPTOR. Eckard Weber. Mark Sonders, Merrit Quarum, Sovitji Pou*, John F. W. Keana*, Institute for Advanced Biomedical Research, The Oregon Health Sciences University, Portland, Oregon 97201 and *Department of Chemistry, University of Oregon, Eugene, Oregon 97403, U.S.A..

Sigma receptors are thought to mediate the hallucinogenic effects of benzomorphan-type opiate drugs. The characterization of the sigma receptor by biochemical methods has been difficult because of the lack of selective and potent ligands. During a systematic search for potential sigma receptor active drugs we discovered that symmetrically substituted guanidines interact with the sigma receptor as judged by their capability to displace [^{3}H](+)SKF 10,047 from guinea pig brain membrane binding sites. We have synthesized a [^{3}H]-labeled derivative of the most potent one of these symmetrically substituted guanidines. This compound has the following structural formula:



[3H]1,3-di-o-tolyl-guanidine ([3H]-DTG)

Radioligand binding assay experiments have shown that this novel, [^{3}H]-labeled compound binds with high affinity [K_D = 30 nM] to a single population of binding sites [B max = 80 pmol/gram brain tissue] in guinea pig brain membrane preparations. The drug selectivity profile of the [^{3}H]-DTG binding site was identical to that described for the haloperidol-sensitive sigma receptor (1,2). The potential applications of this novel sigma receptor ligand to the characterization of the sigma receptor in biochemical and pharmacological experiments will be discussed.

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2.) Tam, S.W. and Cook, L., PNAS 81, 5618 (1984).

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EVIDENCE THAT THE MAJOR IMMUNOREACTIVE MORPHINANS IN MAMMALIAN BRAIN ARE MORPHINE AND CODEINE. C. J. Weitz, L. I. Lowney, R. W. Barrett and A. Goldstein, Addiction Research Foundation, Palo Alto, CA 94304, U.S.A.

Partially purified extracts of bovine hypothalamus and rat brain contain two major compounds recognized by antisera to morphine and resolved by reversed-phase high pressure liquid chromatography (HPLC). Both are found consistently in bovine hypothalamus and rat brain, but the pattern and concentrations are highly variable in bovine adrenal. In addition, a third minor immunoreactive (ir) morphinan is present in hypothalamus. Extractions and purification procedures were carried out in sequestered glassware. A "tissueless" blank was run through the entire system prior to each tissue work-up and shown to be free of significant immunoreactivity on HPLC analysis.

The two major ir-morphinans in bovine hypothalamus, rat brain, and (variably) bovine adrenal appear to be morp ine and codeine by the following criteria: 1) co-lution with ^{3}H-morphine and ^{3}H-codeine in two different reversed-phase HPLC systems; 2) crossreactivity with two antimorphine antisera of contrasting specificities; and 3) absolute crossreactivity in a quantitative immune-removal system (bovine hypothalamus).

These results support previous work reported from our laboratory (A Goldstein et al, Proc Natl Acad Sci USA 82: 5203, 1985) in that ir-morphinans (one of which is morphine) are detected consistently in mammalian brain. In addition, we can now tentatively identify our previously-reported "peak 4" as codeine; the minor ir-morphinan in hypothalamus was reported previously as "peak 5". These results differ from our previous work in that "peaks 2, 3, and 6" are not detected: we have been able to identify a source of laboratory contamination that probably accounts for them.

Supported by DA-1199 from the National Institute on Drug Abuse.

P-160

CALCITONIN AND CALCITONIN GENE-RELATED PEPTIDE INDUCED MODULATION OF CALCIUM FLUXES: CORRELATION TO ANALGESIC EFFECTS AND MODULATION OF OPIATES. S. P. Welch, C. Cooper and W. L. Dewey, Dept. of Pharmacology and Toxicology, Medical College of Virginia, Richmond, VA 23298 and University of Texas Medical Branch, Galveston, TX 77550 U.S.A.

Salmon calcitonin (CT) and Calcitonin Gene-Related Peptide (CGRP) were evaluated for analgesic activity in the tailflick, hotpleate, and p-phenylquinone writhing (PPQ) test in mice following intraventricular (ivt.) administration. Both CT and CGRP produce antinociception, however, CGRP is less potent than CT (ED_{50}=20ug and .36ug respectively in the PPQ test). CT produced peak analgesic effects at 2 hr., while the maximal effects of CGRP occurred at 1 hr. Naloxone reversed the analgesic effects of CT in the tailflick and hot plate tests, but not in the PPQ test. Naloxone reversed CGRP analgesic effects in the PPQ test. At 5 min post administration ivt. both CT and CGRP produced antagonism of subcutaneously administered (s.c.) morphine. However, CT produced a significant 2 fold shift in the morphine ED_{50}, while CGRP-induced shifts were not significant,

In the electrically stimulated guinea pig ileum (GPI) assay both CT and CGRP produced non-dose responsive, naloxone reversible inhibition of the ileum (25 and 51% respectively). 5 min. pretreatment of the ileum with CGRP produced an effect additive with that of morphine, while 5 min. pretreatment with CT antagonized morphine. However, pretreatment of the ileum for 1 hr. with CT slightly potentiated morphine. CT and CGRP both produced time-dependent alterations in calcium uptake to whole brain synaptosomes. Both CT and CGRP increased calcium uptake when incubated with synaptosomes for 5 min. However both decreased calcium uptake with 1 hr (CGRP) and 2 hr. (CT) incubations. Both CT and CGRP appear to have intrinsic agonist/antagonist effect which correlate well to the modulation of calcium fluxes in the central nervous system. (Supported by USPHS Grants DA01647 and T32DA07027).

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TREATMENT OF PAIN IN CANCER SUBJECTS BY INTRATHECAL ADMINISTRATION OF DYNORPHIN

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Dynorphin, an opioid peptide originally isolated from the pig pituitary, has been shown to have potent activity in the guinea pig ileum bioassay. In contrast to β-endorphin, this peptide when administered intracereberally to experimental animals showed weak to no analgesic activity. Anatomical studies have shown that the spinal cord contains a high concentration of inmunoreactive dynorphin and it has been rationalized that this organ may be the primary site of action for this peptide. In contrast to the early studies at the brain level, several groups have shown that the intrathecal administration of dynorphin can induce a long lasting analgesic effect in rats. In order to evaluate the potential application of this peptide in the treatment of pain in human subjects we have performed some preliminary experiments by administering very small doses of this peptide intrathecally into six cancer patients. Compared with the injection of saline, dynorphin 1-13 was able to reduce pain score by at least 150% between the dose of 7.5 to 60 µg. The mean duration of relief after the administration of dynorphin 1-13 was 4.3, 5.8, 4.5 and 5.3 hours at doses of 7.5, 15, 30 and 60 µg, respectively. The mean time of relief after saline was only 0.35 hour. Throughout the study only one to two cases of minor side effects such as headache and dizziness were recorded. Dynorphin 1-10 essentiallyproduced a very similar profile of response. Thus, the structural active canponent of the dynorphin molecule appears in this case to reside between amino acid 1 to 10.

This work was supported by a grant from the Lee Foundation of Singapore.

P45

ENKEPHALIN BIOSYNTHESIS AND QENE EXPRESSION ARE INCREASED IN HIPPOCAMPAL MOSSY FIBERS FOLLOWING SEIZURES. J.D. White, C.M. Gall and J.F. McKelvy, Dept. of Neurobiology and Behavior , SUNY, Stony Brook, NY 11794; *Dept.of Anatomy, Univ. California, Irvine, CA 92717, USA

The biosynthesis and post-translational proccessing of proenkephalin and the level of preproenkephalin mRNA were investigated in the mossy fiber system of the granule cells of the hippocampus in the presence or absence of a unilateral lesion of the hilus, a procedure which produces bilateral hippccampal seizures. In the mossy fibers contralateral to the lesion, increases in enkephalin immunoreactivity were detected by both immunocytochemistry and radioimmunoassay. By the technique of in *vivo* radiolabeling and high performance liquid chromatographic purification of identified radiolabeled peptides, we observed a 14-fold increase in incorporation of radiolabeled methionine into Met^5-enkephalin in lesioned vs. control animals, when Met^5-enkephalin was purified from the mossy fiber terminal fields. To examine the post-translatinal proteolytic processing of proenkephalin, the biosynthesis of five additional Met^2-enkephalin-containing peptides was also examined. We determined that the post-translational processing of proenkephalin did not yield exclusively penta-, hepta- and octa-peptides but larger opioid peptides as well in both control and lesioned animals; however, the ratio of the enkephalin peptides was not altered following the lesion Measurement of preproenkephalin messenger RNA levels in the granule cells by Northern analysis revealed a marked increase (24-fold) following the lesion. These date support the hypothesis that enkephalin biosynthesis in the granule cells is stimuleted as a consequence of seizure activity and that the mossy fiber system offers a model system to study the modulation of opiate peptide synthesis by neuronal activity.

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ALTERED METABOLISM OF β-ENDORPHIN AND DEVIANT BIOLOGICAL ACTIVITY OF γ-ENDORPHIN IN POST MORTEM BRAIN OF SCHIZOPHRENIC PATIENTS.

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The α- and γ-endorphins (αE, γE) are endogenous neuropeptides, enzymatically generated in the brain from β-endorphin (βE). In a variety of behavioral test systems αE and γE produce opposite effects, not related to their opiate like activity. The actions of αE resemble those of psychostimulant drugs, whereas γE displays neuroleptic-like properties. In view of the neuroleptic-like activity of γ-type endorphins, it was hypothesized that a reduced availability of γE-type activity in the brain is an ethiological factor in psychopathologies for which neuroleptics are beneficial, such as schizophrenia.

We have pursued this hypothesis, and investigated the concentration of αE, βE and γE in post mortem hypothalamic tissue of schizophrenic patients with combined HPLC and RIA techniques. In addition, we determined the activity of γE fractions from brain and pituitary tissue of such patients on the passive avoidance response in rats. In both lines of experimentation post mortem tissue of subjects without psychopathology was taken as control.

The concentration of αE and γE was found to be significantly higher in the hypothalamus of schizophrenic patients than in that of control subjects. No difference was found in the βE levels. In the passive avoidance test, γE fractions from hypothalamic or pituitary tissue of schizophrenic patients displayed an activity different from control and synthetic γE; they were inactive or induced the opposite response.

These results are suggestive of an altered metabolism of βE in brain tissue of schizophrenic patients, although it remains to be established whether these alterations are consequence of ante mortem drug treatment of the patients or are connected to the psychopathology itself. Moreover, our results indicate that γE in schizophrenics possesses deviant biological properties.

P72

ENKEPHALIN DEGRADATION IN LOCUS COERULEUS MEASURED ELECTROPHYSIOLOGICALLY. J.T. Williams, M.J. Christie, R.A. North and B. Roques*. Neuropharmacology Laboratory, M.I.T., Cambridge MA 02139, U.S.A. and *University Rene Descartes, Paris 75270, France.

The increase in potassium conductance caused by μ-opioid agonists was measured by intracellular electrodes in single locus coeruleus neurons in slices cut from rat brain. [Met^5] enkephalin (ME) was applied by superfusion for periods of 2-5 min, which was sufficient for its effect to reach steady-state. The effect measured was membrane hyperpolarization or, more commonly, the outward current under voltage clamp at -60 mV holding potential. Kelatorphan (up to 60 μM) had no effect on the LC neurons, but the amplitude and duration of the response to ME was increased when kelatorphan was in the superfusing solution; this potentiation was observed in all cells. The concentration-effect curve for ME was shifted to the left 3-30 fold by kelatorphan, the degree of shift being dependent upon the kelatorphan concentration (2-20 μM); further increase in kelatorphan caused no further increase in the response to ME. The minimum effective concentration of ME was reduced from about 300 134 to 30 nM by kelatorphan. The time course of action of kelatorphan was studied by applying the ME for longer periods of time (20-30 min) and during this period changing the superfusion solution to one which contained both ME and kelatorphan. The increase in the effect of ME occurred within 2 min and reversed 10-20 min after the kelatorphan was removed. Outward currents evoked by Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) and Tyr-D-Ala-Gly-Phe-D-Leu (DADLE)(which act on the same p-receptors) or by noradrenaline (which acts on α_2 receptors but opens the same potassium channels) were unaffected by kelatorphan. Noradrenaline responses were potentiated 10-30 fold by cocaine (which did not affect responses to ME); acetylcholine responses were potentiated 10-30 fold by physostigmine. The results indicate that a single brain slice (containing 300-500 LC neurones) avidly destroys a large proportion of ME superfusing it, that the action of kelatorphan is readily reversible, and that the degree of potentiation of response observed is very similar to that seen with catecholamines and acetylcholine.

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THE ENDOGENOUS KAPPA AGONIST, DYNORPHIN(1-13) DOES NOT ALTER NIGROSTRIATAL OR MESOLIMBIC DOPAMINE METABOLISM IN THE RAT. P.L. Wood. H.S. Kim and S. Iyengar. Neuroscience Research, Research Dept., Pharmaceuticals Division, Ciba-Geigy Corp., summit, N.J., 07901, U.S.A.

Dopaminergic pathways in the rat brain possess both mu and delta opioid regulatory inputs. In contrast, previous studies with kappa agonists have reported that these pathways appear to lack kappa receptor regulation. We therefore examined the actions of the endogenous kappa agonist, dynorphin(1-13) on nigrostriatal (striatum) and mesolimbic (N. accumbens dopamine metabolism in the rat. As a control, plasma corticosterone levels were also measured in all animals to assess the bioavailability of the test drugs. This is a useful control since serum levels of this hormone are under central mu, delta and kappa opioid control.

While parenteral morphine elevated both plasma corticosterone and dopamine metabolism (both striatum and N. accumbens), neither parenteral U 50488H nor intraventricular dynorphin(1-13) altered dopamine metabolism. However, both kappa agonists potently elevated plasma corticosterone levels indicating their bioavailability to the CNS.

In summary, our data support previous reports that mesolimbic and nigrostriatal dopaminergic neurons lack kappa opioid regulatory inputs in the rat.

P27

CHARACTERIZATION OF OPIOID RECEPTOR IN HAMSTER BRAIN.

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The distribution density of subtype of opioid receptor differs markedly in brain of different species . The nature of opioid receptor in hamster brain has been studied in this paper. The mu binding site was determined with the highly selective mu ligand, ^{3}H-DAGO. For the delta site, ^{3}H-DADLE was used in the presence of unlabelled 30 nM morphine to suppress binding to the mu site. For the estimation of kappa binding, the ^{3}H-etorphine was used in the presence of unlabelled 30 nM morphine and 100 nM DADLE to suppress bindings to the mu and delta sites. The non-Specific binding was determined with 10 μM levorphanol. The results of the experiments indicated that specific binding of the three ^{3}H-ligands is concentration-dependent and saturable. Scatchard analysis showed that the K_d and B_{max} for mu binding were 1.3 nM and 17.8 pmol/g protein, respectively. For delta binding 0.88 nM and 11.6 pmol/g protein, respectively. For kappa binding 0.52 nM and 33.8 pmol/g protein, respectively. Competition binding studies indicated that cold mu ligand, ohmefentanyl easily displaced the binding of ^{3}H-DAGO (K_I=26 pM). Relative selective δ-ligand, DADLE readily inhibited the binding of ^{3}H-DADL (K_I=1.0 nM). However, selective kap a ligand, U-50488 competed effectively the binding of ^{3}H-etorphine to kappa site (K_I=5.9 nM). Based in our experiments, we estimate that the hamster brain conteins 54% kappa sites, 28% mu sites and 18% delta sites. These results suggested that the binding of the high affinity to the kappa site is predominantly presented in the hamster brain. It markedly differs from the brain of rat or guinea-pig.

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STRESS-INDUCED CHANGES IN DIFFERENT MOLECULAR WEIGHT FORMS OF DYNORPHIN PEPTIDES IN RAT BRAIN AND SPINAL CORD. G. X. Xie and A. Goldstein, Addiction Research Foundation and Department of Pharmacology, Stanford University, Palo Alto, CA 94304, U.S.A.

The immunoreactivities of three peptide products of the preprodynorphin gene -- dynorphin A, dynorphin B and α-neo-endorphin -- were measured in single rat brains and spinal cords using specific antisera after Sephadex G-50 filtration. Three peaks of M_r about 7, 4 and 2 kDa for ir-dyn A and ir-dyn B, and one single peak of M_r less than 2 kDa for a-neo-endorphin were found both in the brain and in the spinal cord. The 7 kDa peak was recognized by a specific antiserum against the carboxyl terminus of dyn B, indicating it terminates in threonine-13 of dyn B. The relative amounts of 7 and 4 kDa materials to 2 kDa material were greater in spinal cord than in the brain, suggesting a different rate of processing or release in different regions of the central nervous system.

Swimming in 10°C cold water for 10 min produced a naloxone-blocked increase of tail flick latency, and also caused a striking reduction of 7 and 4 kDa dynorphins in spinal cord but little or no change in the small M, products of the preprodynorphin gene. The processing and release of dynorphin peptides in the spinal cord may be accelerated by stress.

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P48

CONVERSION OF β-ENDORPHIN TO γ-ENDORPHIN IN RAT PLASMA. Elizabeth A. Young and Huda Akil, Mental Health Research Institute. University of Michigan, Ann Arbor MI 48109 U.S.A.

With a number of stressors β--endorphin is released into plasma. The rate of disappearance of this released β--endorphin is rapid, with estimates of the half life in the 10 to 15 minute range. it is unclear if β-endorphin is coverted into any other biologically active products, nor is it clear if the rate or pathways of degredation is changed during chronic stress. To explore these issues, we incubated [^{3}H] β--endorphinh labelled in position 1 and 27 with plasma from normal and chronically footshocked rats and measured the rate of conversion of the label from β-endorphin size material to smaller size material. Initial separations were done using a G-50 molecular sieving column. with subsequent characterization and identification on BPLC. Using these methods, we see a time dependent formation of only one radioactive peak with G-50 column sieving. The size estimate of this peak is 16 or 17 amino acids. Since the [^{3}H] β-endorphin was labelled on both ends of the molecule, two radiolabelled fragments should be formed. This suggested that the molecule may be cleaved near the middle of the molecule resulting in two fragments of similar size. To confirm this hypothesis we used an HPLC separation of the pre-sieved fractions from the G-50 column, and looked for the co-migration of label with either α or γ endorphin as well as other unidentified peaks. We found two radioactive peaks on HPLC, one of which co-migrated with γ-endorphin. and the other peak which is unidentified currently but may well be β-endorphin 18-31. Interestingly. this enzymatic activity is induced by chronic stress so that the labelled β-endorphin shows a 2-3 fold faster conversion to this product. Whether the γ-endorphin remains stable after formation is less clear. but fragments of γ-endorphin such as des-tyrosine γ-endorphin and des-enbphalin γ--endorphin have been reported to be behaviorally active. We conclude that post-release conversion of β-endorphin into other biologically active fragments can occur, and that γ-endorphin is one of the fragments formed in plasma. Similar data have been reported in brain synaptasomes, and a common enzyme may be present in both tissues.

P18

EFFECTS OF DIFFERENTIATION ON CELLULAR AND BIOCHEMICAL PROPERTIES OF A μ RECEPTOR CONTAINING NEUROBLASTOMA CELL LINE. V.C. Yu, G. Hochhaus, M.L. Richards and W. Sadée. School of Pharmacy, University of California, San Francisco, San Francisco, CA 94143, U.S.A.

We have recently identified a human neuroblastoma cell line, SK-N-SH, that expresses μ and δ opioid receptors. Morphologically, SK-N-SH cells appear to consist of two distinct cell types (epithelial-like and neuroblast-like) that can be interconverted to one another. SH-SY5Y is a thrice-cloned cell line of SK-N-SH which displays an essentially homogenous population of neuroblast-like cells and can be grown to high cell density. The SH-SYSY clone, like the parent SK-N-SH cells, expresses both μ and δ opioid receptors, muscarinic cholinergic receptors, and a catechol uptake$_1$ system. The cells can be stimulated to release inositol triphosphate by acetycholine but not by opioids, bradykinin, ATP and Sub P. Six days treatment of SH-SY5Y cells with either nerve growth factor (50 ng/ml), dBcAMP (1 mM) or retinoic acid (20 μM) induces differentiation of the cells marked by extensive neurite outgrowth. The various receptor systems were differentially affected by the agents used to induce neuronal maturation on receptor expression and the cellular properties. Most strikingly, nerve growth factor and retinoic acid increased the opioid receptor density (on calculated per mg protein) by as much as two-fold, while dBcAMP decreased it by approximately 40%. The effects of the differentiating agents were less pronounced on the ACh receptor, while retinoic acid selectively increased the uptake system by three-fold. Supported by DA 01095 from NIDA, Bethesda, MD.

P148

NEONATAL TYR-MIF- ALTERS NOCICEPTIVE EFFECTS OF NEONATAL BETA-ENDORPHIN AND MORPHICEPTIN DURING DEVELOPMENT AND IN ADULTHOOD. James E. Zadina, Abba J. Kastin, Penelope K. Manasco, and Kent L. Nastiuk, Veterans Administration Medical Center and Tulane University School of Medicine, New Orleans, LA 70146, USA.

Male rats were injected SC once daily during the first week of life with beta-endorphin (BE), morphiceptin (a mu opiate receptor-selective peptide), a putative endogenous antiopiate Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), or one of the two opiate peptides in combination with Tyr-MIF-1. They were then tested during early development and in adulthood (4.5 months) for their responsiveness to thermal pain. Pups treated with neonatal BE removed their tails from a series of increasingly hot water baths significantly faster than control animals on day 9, confirming our earlier studies. In rats injected with both Tyr-MIF-1 and BE, however, the latency to remove the tail was significantly longer than in the group given BE alone, and was not different from control animals, indicating that Tyr-MIF-1 blocked the effect of BE. Randomly chosen subgroups of animals, which were statistically shown to adequately represent the groups from which they were drawn, were retested in adulthood for responsiveness to thermal pain using the hot plate. When placed on a 50°C surface, the rank order of the treatment groups in their latency to lick a hindpaw was similar to their ranking in the tail-flick test on day 9. The latencies were significantly longer in each of the 3 groups given Tyr-MIF-1 than in groups given BE alone, morphiceptin alone, or the control vehicle. None of the neonatal peptide treatments significantly affected the degree of analgesia shown when animals were exposed to footshock and retested on the hot plate. The mean tail-flick latencies of the animals on day 9 correlated well with adult baseline scores on the hot plate test, indicating that the effects of the peptides persisted into adulthood. These results support the role of Tyr-MIF-1 as an antiopiate and further illustrate the long-term effects of neonatally administered peptides.

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PHENCYCLIDINE RECEPTOR IN BLOOD VESSELS. H. Zhu, A.Z. Zhang, X.R. Xu* and W.L. Ye, *Dept. of Neurobiology, Shanghai Medical University, Shanqhai Institute of Materia Medica*, Shanghai, 200032, China

Binding studies of (3H) Phenclidine (PCP) to rabbit mesenteric blood vessel detected a specific, saturable and stereoselective binding site of which Kd=31.9±3.9 nM, Bmax=4.96±0.29 pmol/mg pr. IC_{50} of a series of ligands are: PCP 0.39±0.04, dextrosin 3.01±0.19, levorphanol 18.2 ± 1.2, dl-ketamine 0.17±0.02, dl-pentazocine 0.14±0.03, d-SKF10,047 9.77±0.48, 1-SKF10,047 85.1±2.5, d-cyclazocine 2.57±0.11 and l-cyclazocine 4.07±0.17 uM. Naloxone and etorphine failed to inhibit (3H) PCP binding at concentrations as high as 200 uM.

In vitro bioassay showed that PCP markedly enhanced the electrical field stimulated contraction (ESC) of rabbit central ear artery with a minimum eftective conc. of 0.1 uM, which was naloxone irreversible. ED_{50} were: PCP 7.52±0.94, d-SKF10,047 1.52±0.23, pentazocine 6.44 and ketamine 1.43±0.14 uM. Such effect could be abolished by 1 uM of phentolamine. HPLC detected a significant increase of NE content ($p<0.01$) in the bath medium after PCP administration.

Our finding of PCP and siqma opioid receptors in the blood vessel and their vascular function may at least partially explain the significant pressor effect of these drugs, and also propose a dual system, i.e. kappa (Neuropeptides, 1985,5;595) and PCP/sigma opioid receptors which cooperatively modulate NE release and the function of blood vessels.

P96

INVOLVEMENT OF ENDOGENOUS OPIOID PEPTIDES IN BURN-INDUCED SHOCK. Y. X. Zhu, C. H. Wang and B. C. Lin, Department of Physiology, Second Military Medical College, Shanghai 200433, China

192 male Sprague-Dawley rats weighing 200-250g were given III burn injury of 20% body surface area, and equally divided into 6 groups: control, naloxone, anti-β-endorphin, anti-dynorphin A, anti-met-enkephalin, and anti-leu-enkephalin. Each animal of these groups immediately postburn was intravenously administrated 50µl of normal rabbit serum, naloxone solution(1mg/kg), antisera against β-endorphin, antisera against dynorphin a, antisera against met-enkephalin and antisera against leu-enkephalin, respectively. There is no significant difference($P>0.05$) of survival rate at 6 hours postburn between the control(21%) and anti-met-enkephalin(31%) or anti-leu-enkephalin(2%) group. However, the survival rate of the anti-dynorphin A(61%) group at the same time postburn was much hi her, and those of the naloxone(42%) and anti-β-endorphin (44%) group were slightly higher than that of the control group($3<0.05$). The onset of hypotension, bradycardia, hypothermia and dyspnea were greatly delayed in the naloxone group, as compared with the control group. Blood pressure showed a 62% reduction at 180 minutes postburn in the naloxone group, while a 62% reduction was already seen at 90 minutes postburn in the control group. It is suggested that endogenous opioid peptides, especially dynorphin A and β-endorphin, might be involved in burn-induced shock, and naloxone might to be effective in the treatment of burn-induced shock.

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