Molecular Biology of Opioid Receptors

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INTRODUCTION

Morphine and its alkaloid derivatives are used extensively in the treatment of pain (Gilman et al. 1990). These compounds are the most potent class of analgesics used clinically. The high potency and specificity of morphine suggest that it may bind to specific receptors in the nervous system to induce its biological effects. In the early 1970s, several groups of researchers identified specific opioid receptors in brain and peripheral tissues (Pert and Snyder 1973; Simon 1991). While these receptors were highly sensitive to morphine, morphine is not endoge-nously expressed in the body and therefore could not be the endogenous ligand for these receptors. This led to the search for the endogenous neurotransmitters at the opiate receptors and, in the mid-1970s, several groups identified the enkephalins (Hughes et al. 1975), dynorphins (Goldstein et al. 1979), and beta-endorphin (Bradbury et al. 1975) as endogenous peptide ligands for these receptors.

Both the enkephalins and dynorphins are neurotransmitters in the brain involved in pain perception, cognitive functions, affective behaviors, and locomotion, and they are involved in the central control of certain endocrine functions such as water balance (Herz 1993; Jaffe and Martin 1990; Simon 1991). Both peptides are widely distributed in the central nervous system (CNS) but localized to discrete neuronal pathways (Khachaturian et al. 1983). Beta-endorphin is expressed at much lower levels in the brain and only synthesized in a few neuronal pathways in the CNS, in particular in those originating from hypothalamic nuclei. As a result, the enkephalins and dynorphins are considered the predominant central opioid peptide transmitters.

The opioids induce their biological effects by interacting with three major classes of receptors: the delta, kappa, and mu receptors (Herz 1993). Delta opioid receptors express low affinity for dynorphin, but

high sensitivity to enkephalin, indicating that enkephalin may be an endogenous ligand for this receptor. In contrast, dynorphin A is very potent at kappa receptors, whereas these receptors have low affinity for enkephalin, suggesting that dynorphin A may be the endogenous transmitter for the kappa receptor. The mu opioid receptor expresses high affinity for enkephalin and the distribution of mu receptors and enkephalin messenger ribonucleic acid (mRNA) are highly correlated, suggesting that enkephalin may interact with mu receptors under physiological conditions (Delfs et al. 1994). Beta-endorphin binds potently to both the delta and mu receptor but has relatively lower affinity at kappa receptors, suggesting that in peripheral tissues, where endorphin is more abundant than enkephalins or dynorphin, beta-endorphin may be an endogenous ligand for the delta and mu receptors.

The pharmacological properties of the three opioid receptor classes are distinct and can be clearly differentiated (Goldstein and Naidu 1989; Herz 1993; Lutz and Pfister 1992; Portoghese 1993; Schiller 1993; Simon 1991). Delta opioid receptors have high affinity for the agonists deltorphin II, [D-penicillamine^{2}, D-penicillamine^{5}] enkephalin (DPDPE),[D-Ser^{2},O-Leu^{5}]-enkephaly-Thr (DSLET), and the antagonist naltrindole. These compounds essentially do not bind to the kappa or mu receptors. The kappa receptor has high affinity for the agonists U50,488 and U69,593 and the antagonist nor-binaltorphimine (nor-BNI). These agonists do not bind to the delta or mu receptor and the antagonist is much less potent at these other opioid receptors. Mu opioid receptors have high affinity for the peptide agonist [D-Ala^{2}-MePhe^{4}, Gly-ol^{5}] enkephalin (DAMGO), morphine and its derivatives, and the antagonists [D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH_2] (CTOP) and naloxonazine. DAMGO and the antagonists do not bind to delta or kappa receptors, and morphine and its derivatives are much less potent at the delta or kappa receptors. All three opioid receptors are sensitive to the antagonist naloxone, although to varying degrees. Kappa and mu receptors have high affinity for naloxone, while delta receptors have lower affinity for this antagonist.

The development of pharmacological agonists and antagonists that can distinguish the opioid receptors has facilitated investigations into their distinct functional roles. Such agents were necessary because most tissues express more than one opioid receptor type, which has made it difficult to study each receptor individually to reveal any unique biological actions. This problem has now also been overcome by the recent cloning of the three opioid receptor classes, since each

receptor can now be expressed in a separate cell line and their pharmacological, functional, and biochemical characteristics can be studied independent of the other receptors.

Cloning of the Opioid Receptors

The first opioid receptor to be cloned was the delta receptor. Two groups, Evans and colleagues (1992) and Kieffer and colleagues (1992), simultaneously identified the delta receptor from cDNA libraries derived from NG-108 neuroblastoma cells by expression cloning. Using the fact that opioid and somatostatin receptors have some similar pharmacological properties, Yasuda and associates (1993) cloned the kappa receptor as well as the delta receptor by screening a mouse brain cDNA library with probes selective for the cloned somatostatin receptors. The probes were directed against transmembrane spanning regions of the somatostatin receptors, which are conserved between the somatostatin and opioid receptors. Similarly, the rat kappa receptor cDNA has been cloned and its predicted amino acid sequence is almost identical to the mouse receptor (Meng et al. 1993; Minami et al. 1993). At approximately the same time as the initial cloning of the mouse kappa receptor, Chen and colleagues (1993) cloned the mu opioid receptor using probes against conserved regions of the delta opioid receptor to screen a rat brain cDNA library. This was possible because of the high degree of amino acid sequence similarity between the delta and mu opioid receptors, in particular in the transmembrane spanning regions, which is where the probes of Chen and associates (1993) were directed. Others (Thompson et al. 1993; Wang et al. 1993) have cloned the rat mu opioid receptor with similar if not identical amino acid sequences to those reported by Chen and colleagues (1993).

The predicted amino acid sequences of the three opioid receptors are shown in figure 1. They are approximately 65 percent identical with highest similarity in the transmembrane spanning regions and intracellular loops. Regions that diverge the most are the N- and C-termini and the extracellular loops. All three receptors have the aspartate-arginine-tyrosine (DRY) sequence and aspartates in the second and third transmembrane spanning regions that are conserved among guanosine triphosphate binding protein (G-protein)-linked receptors. Each receptor has multiple potential glycosylation sites in its N-terminal region. It is likely that each receptor has carbohydrate moieties associated with at least one of these sites since the size of the native receptors identified from biochemical studies is considerably larger than the size predicted

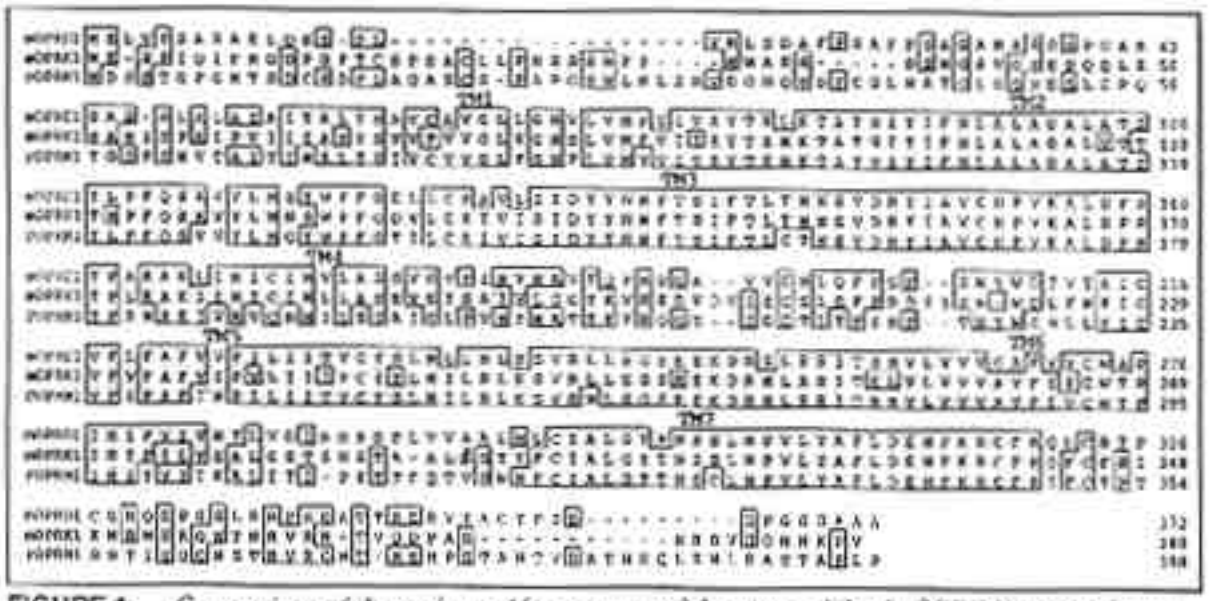

FIGURE 1. *Comparison of the amino acid sequences of the mouse delta (mOPRD1), mouse kappa (mOPRK1), and rat mu (rOPRM1) opioid receptors. The sequences are shown using the single-letter abbreviations for the amino acids. Residues identical in two or more of the receptors are boxed. Gaps introduced to generate this alignment are represented by dashes. The predicted transmembrane spanning regions are indicated by TM1 TM7.*

from the amino acid sequence of each cloned receptor. In fact, studies to covalently cross-link the cloned delta receptor expressed in CHO cells with ^{125}I-beta-endorphin reveals the size of the receptor as 55 to 60 kilodaltons (kDa), which is larger than the predicted size of 41 kDa (Raynor and Reisine, unpublished observations). Furthermore, the labeled receptor appears as a smear following SDS-PAGE and autoradiography, which is consistent with the glycoprotein nature of this receptor. Mutagenesis studies to knock out the glycosylation sites in these receptors may reveal the functional role of the carbohydrates on these receptors.

The three opioid receptors have high similarity to somatostatin receptors, particularly SSTR1, with approximately 40 percent amino acid sequence similarity (Reisine and Bell 1993; Yasuda et al. 1993). They have very low similarity with all other receptors. The similarity with somatostatin receptors is consistent with the pharmacological overlap of these receptors since some somatostatin analogs, including the clinically employed peptide SMS-201-995, which binds with moderate potency to mu opioid receptors (Maurer et al. 1982), and the extremely potent and selective mu antagonist CTOP, were developed using the structure of SMS-201-995 as its basis (Pelton et al. 1985).

PHARMACOLOGICAL AND FUNCTIONAL PROPERTIES OF THE CLONED OPIOID RECEPTORS

Following expression of the cloned receptors in COS-7, PC12, or CHO cells, the pharmacological and functional properties of these receptors were investigated (Kong et al. 1993*a*; Raynor et al. 1994*a*, 1994*b*; Reisine and Bell 1993; Yasuda et al. 1993). The pharmacological properties are described in table 1. The endogenous peptides, the enkephalins, potently bound to the cloned delta and mu receptors. Kappa receptors have very low affinity for these compounds. In contrast, dynorphin A potently bound to the kappa receptor, but it was less potent at the mu receptor and did not interact with the delta receptor. Both the delta and mu receptors have high affinity for beta-endorphin, whereas kappa receptors were relatively insensitive to this peptide. The binding of beta-endorphin to the receptors was dependent on the presence of the N-terminal tyrosine residue, consistent with the opioid nature of the receptors. The affinities of the cloned receptors for the endogenous peptide transmitters were similar to those reported for the native opioid receptors.

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors.

	κ Receptor [3H]U-69,593	δ Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Nonselective Compounds			
Dynorphin A	0.5	>1,000	32
Leuenkephalin	>1,000	4.0	3.4
Metenkephalin	>1,000	1.7	0.65
ß-endorphin	52	1.0	1.0
des-Tyr1-ß endorphin	>1,000	>1,000	>1,000
(-)naloxone	2.3	17	0.93
(+)naloxone	>1,000	>1,000	>1,000
Levorphanol	6.5	5.0	0.086
Dextrorphan	>1,000	>1,000	>1,000
(±)bremazocine	0.089	2.3	0.75
Ethylketocyclazocine	0.40	101	3.1
Etorphine	0.13	1.4	0.23
Pentazocine	7.2	31	5.7
Diprenorphine	0.017	0.23	0.072
ß-FNA	2.8	48	0.33
Naltrexone	3.9	149	1.0
Nalbuphine	39	>1,000	11
Nalorphine	1.1	148	0.97

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors (continued).

	κ Receptor [3H]U-69,593	δ Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Mu-Selective Compounds			
CTOP	>1,000	>1,000	0.18
Demorphin	>1,000	>1,000	0.33
Methadone	>1,000	>1,000	0.72
DAMGO	>1,000	>1,000	2.0
PLO17	>1,000	>1,000	30
Morphiceptin	>1,000	>1,000	56
Codeine	>1,000	>1,000	79
Fentanyl	255	>1,000	0.39
Sufentanil	75	50	0.15
Lofentanil	5.9	5.5	0.68
Naloxonazine	11	8.6	0.054
Morphine	538	>1,000	14

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors (continued).

	κ Receptor [3H]U-69,593δ	Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Kappa-Selective Compounds			
NorBNI	0.027	65	2.2
Spiradoline	0.036	>1,000	21
U-50,488	0.12	>1,000	>1,000
U-69,593	0.59	>1,000	>1,000
ICI 204,488	0.71	>1,000	>1,000
Delta-Selective Compounds			
DPDPE	>1,000	14	>1,000
D-Ala2-deltorphin II	>1,000	3.3	>1,000
DSLET	>1,000	4.8	39
BW 3734	17	0.013	26
DADL	>1,000	0.74	16
SIOM	>1,000	1.7	33
Naltrindole	66	0.02	64
NTB	13	0.013	12
BNTX	55	0.66	18

Binding to the cloned receptors was stereospecific. The opioid antagonist (-) naloxone bound much more potently to the receptors than the (+)isomer. Similar selectivity was observed with the agonist levorphanol versus its inactive stereoisomer dextrorphan.

Kappa receptor selective agonists specifically bound to the cloned kappa receptor. Furthermore, the antagonist nor-BNI is one hundredfold selective for the kappa receptor. The pharmacological profile of the kappa receptor is consistent with it being a $kappa_1$ subtype defined previously in studies on the endogenously expressed kappa receptors (Clark et al. 1989).

Mu receptor selective agonists and antagonists specifically bound to the cloned mu receptor. Furthermore, many of the clinically used opioids, such as morphine, methadone, codeine, and compounds with high abuse potential such as fentanyl specifically bound to the cloned mu receptor. This finding suggests that the therapeutic as well as the side effects of physical dependence and respiratory depression induced by these compounds are due exclusively to their interaction with mu receptors.

A comparison of the potencies of a large series of opioids at binding to the cloned kappa and mu receptors with their potencies at binding to the endogenously expressed receptors reveals a very high correlation. This indicates that the ligand specificities of the cloned and endogenously expressed receptors are similar. This finding is of interest considering that the artificial cellular environment of the cell membrane where the cloned receptors are expressed would be expected to be quite different from the environment of the endogenously expressed receptor.

In contrast to the kappa and mu receptors, little similarity exists in the pharmacological profile of the cloned delta receptor compared to the endogenously expressed delta receptors. One clue that may explain this disparity is the much higher affinity of the cloned receptor for the antagonist NTB (the benzofuran analog of naltrindole) than the antagonist 7-benzylidenenaltrexone (BNTX). These compounds have been described as subtype selective, with BNTX selectively binding to the $delta_1$ receptor subtype and NTB selectively interacting with the $delta_2$ subtype (Portoghese 1993; Portoghese et al. 1993; Sofuglu et al. 1991*a*, 1991*b*). Furthermore, the $delta_2$ selective agonists DSLET and deltorphin II are more potent at binding to the cloned receptor than the $delta_1$ selective agonist DPDPE. These findings suggest that the cloned receptor may correspond to the $delta_2$ receptor subtype.

Since the radioligands that have been used to examine native delta receptors in brain do not distinguish the subtypes, the pharmacological profile of the endogenously expressed delta receptor may consist of a mixture of potencies at the two delta receptor subtypes, whereas the findings on the cloned receptor may represent the ligand selectivities of only one of the subtypes. If this is the case, the findings suggest that either one more delta receptor subtype remains to be cloned, or subtypes expressed endogenously in the nervous system and peripheral tissues are created by differential processing of a common gene product.

Following expression in cell lines, all three cloned opioid receptors have been found to couple to pertussis toxin-sensitive G-proteins and to mediate the inhibition of adenylyl cyclase activity by opioids (Chen et al. 1993; Kong et al. 1993*a*; Reisine and Bell 1993; Yasuda et al. 1993). Furthermore, the cloned kappa receptor has been reported to couple to a Ca^{++} channel and can mediate agonist inhibition of an N-type Ca^{++} current (Tallent et al. 1993). Kappa receptors in the brain have been reported to couple to N-type Ca^{++} currents in neurons (Gross and MacDonald 1987; North 1993; Weisskopf et al. 1993), and this interaction may be important for the ability of kappa agonists and dynorphin to presynap-tically inhibit neurotransmitter release. All three opioid receptors have been reported to couple to Ca^{++} and K^{+} channels and can mediate the modulation of ionic conductance through these channels via pertussis toxin-sensitive G-proteins (North 1993).

Neurotransmitter receptors have been proposed to couple to G-proteins via their intracellular loops (Dohlman et al. 1988). The intracellular loops of the opioid receptors are very similar, suggesting that they may couple to similar G-proteins, consistent with the similar effector systems they regulate. However, the C-termini of the receptors differ significantly in amino acid sequence, and for some neurotransmitter receptors the C-terminus has been shown to be critical for G-protein and effector system coupling. This has been most clearly shown in the case of the prostaglandin receptors (Breyer et al. 1994; Namba et al. 1993) and one of the somatostatin receptors, SSTR2 (Reisine et al. 1994), in which alternative splicing creates two receptors with divergent C-termini and differing abilities to couple to adenylyl cyclase. Direct studies to identify the G-proteins and effector systems associated with each cloned opioid receptor will be critical in establishing the cellular mechanism of action of opioids acting via each receptor type.

Structure-Function Analysis of the Cloned Opioid Receptors

The cloning of the opioid receptors has now allowed for analysis of the structural elements in the receptors involved in their functioning. Many G-protein-linked receptors can be regulated by sodium ions (Horstman et al. 1990; Kong et al. 1993*b*; Kosterlitz et al. 1988; Limbird et al. 1982; Minuth and Jakobs 1986; Neve et al. 1990; Pert and Snyder 1974). In particular, receptors that mediate agonist inhibition of cyclic adenosine monophosphate (cAMP) formation can have their affinity for agonists reduced in the presence of sodium ions. Sodium ions were first reported to reduce the affinity of opioid receptors for agonists (Kosterlitz et al. 1988; Pert and Snyder 1974). To investigate the mechanisms by which Na^+ regulates agonist binding to $alpha_2$-adrenergic receptors, Horstman and colleagues (1990) mutated an aspartate in the second transmembrane spanning region of the cloned $alpha_2$-adrenergic 2_a receptor to an asparagine and found that Na^+ regulation of agonist binding was abolished. Similar results were obtained with a mutation of the conserved aspartate in the second transmembrane spanning region of the somatostatin receptor subtype SSTR2 (Kong et al. 1993*b*). For both of these receptors few changes in ligand binding properties were reported. These studies indicated that a conserved aspartate in the second transmembrane spanning region of several G-protein-linked receptors was necessary for Na^+ regulation of agonist binding.

To investigate the mechanisms by which Na^+ modulates opioid receptor properties, Kong and associates (1993*a*) mutated aspartate 95 in the cloned mouse delta opioid receptor to an asparagine (see figure 2), and like the adrenergic and somatostatin receptors found that Na^+ regulation of agonist binding was lost, indicating that this residue was necessary for the modulatory actions of Na^+ on the delta receptor. However, in contrast to the results reported with the other receptors, the ligand binding properties of the mutant delta receptor were also altered. Whereas antagonist binding to the mutant and wild-type receptors was similar, selective delta receptor agonists had greatly diminished potencies at binding to the mutant receptor. This finding suggests that selective agonists and antagonists bind differently to the delta receptor, possibly by interacting with distinct ligand binding domains. Furthermore, non-selective agonists that are able to potently bind to other opioid receptors besides the delta receptor bound with similar affinities to the wild-type and mutant delta receptor. This finding indicates that selective and nonselective agonists bind differently to the delta opioid receptor. The findings of these mutagenesis studies indicate that aspartate 95 is

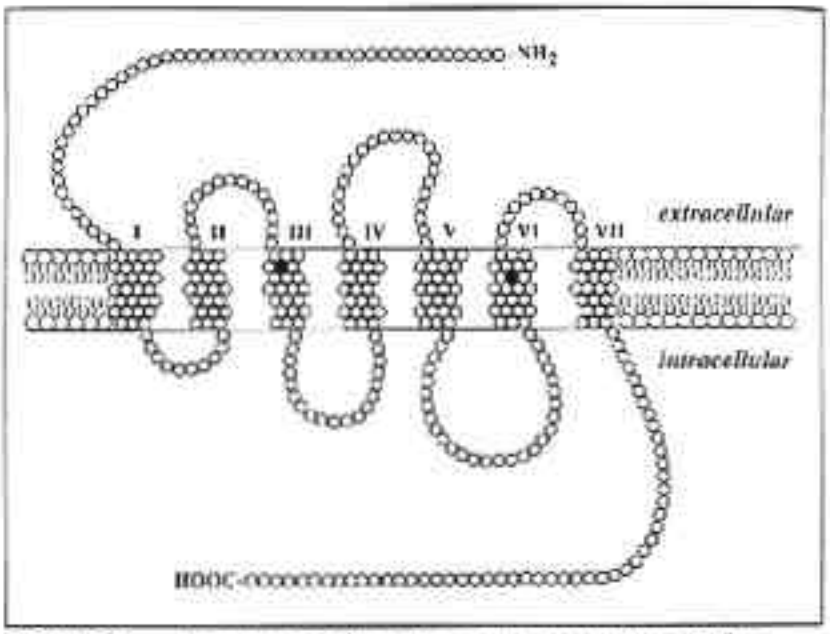

FIGURE 2. *Aspartates in the delta opioid receptor involved in ligand binding. The predicted amino acid sequence of the mouse delta opioid receptor is presented. The darkened circles represent aspartates that have been converted to asparagines by site-directed mutagenesis.*

necessary for Na^+ regulation of agonist binding to the cloned delta receptor. Furthermore, this amino acid residue appears critical for the high affinity binding of selective agonists to the delta receptor, but is not essential for the interaction of any other ligands with this receptor.

To further evaluate residues in the delta opioid receptor involved in ligand binding, Livingston and associates (submitted) mutated aspartate 128 in transmembrane spanning region 3 to an asparagine in the cloned mouse delta receptor (see figure 2) and tested the effects of this mutation on ligand binding. Previous studies on the beta-adrenergic receptor had shown that mutation of the aspartate 113 in the third transmembrane spanning region to an asparagine abolished high affinity antagonist binding to the receptor and modestly diminished agonist binding (Strader et al. 1987). It has been proposed that the aspartate provides a counterion to the cation of beta-adrenergic ligands and serves to stabilize binding through electrostatic interactions. Mutation of aspartate 128 of the delta receptor to an asparagine abolished high affinity agonist binding to the

receptor but did not alter antagonist binding. This finding differs from that reported on the beta-adrenergic receptor and indicates that agonists and antagonists bind differently to the adrenergic and delta receptors.

Both aspartate 95 and 128 of the delta receptor are necessary for high affinity binding of selective agonists. In contrast, only aspartate 128 is critical for nonselective agonist binding to the delta receptor. These findings indicate that different types of agonists at the delta receptor bind in fundamentally distinct manners. The aspartate 128 may serve as the counterion to the cation provided by charged nitrogens in opioid agonists. However, aspartate 95 contributes to the selectivity of recognition of specific delta agonists.

Interestingly, neither mutation of aspartate 95 nor 128 affected antagonist binding to the delta receptor. Antagonists also have positively charged nitrogens necessary for high affinity binding to opioid receptors. The lack of effect of mutation of the aspartates on antagonist binding suggests that some other residues within the delta receptor provide the negative counterion for antagonist binding. This finding supports the hypothesis that agonists and antagonists bind to the delta receptor via distinct molecular mechanisms, possibly to distinct ligand binding domains.

To more directly identify the ligand binding domains of the kappa and delta opioid receptors, Kong and associates (1994) have generated chimeric kappa/delta opioid receptor by exchanging the N-termini of each receptor and tested the mutant receptors for agonist and antagonist binding (see figure 3). Comparison of the predicted amino acid sequences of the kappa and delta opioid receptors reveals that the N-terminus is the region of each receptor that differs the most between each receptor and therefore is likely to contribute to variations in the ligand selectivity. A $kappa_{1-78}/delta_{70-372}$ bound delta selective agonists and antagonists in a similar manner as the wild-type delta receptor. Similar results were obtained with a truncated delta receptor lacking the N-terminal 70 residues. These findings indicate that the N-terminus of the delta receptor is not needed for ligand binding. It is likely that either the second and/or third extracellular loops of the delta receptor contain the ligand binding domains since these are the only other extracellular domains besides the N-terminus that differ in amino acid sequence between the delta receptor and the other opioid receptors. Mutagenesis studies to exchange these regions between the opioid receptor should help to test this hypothesis.

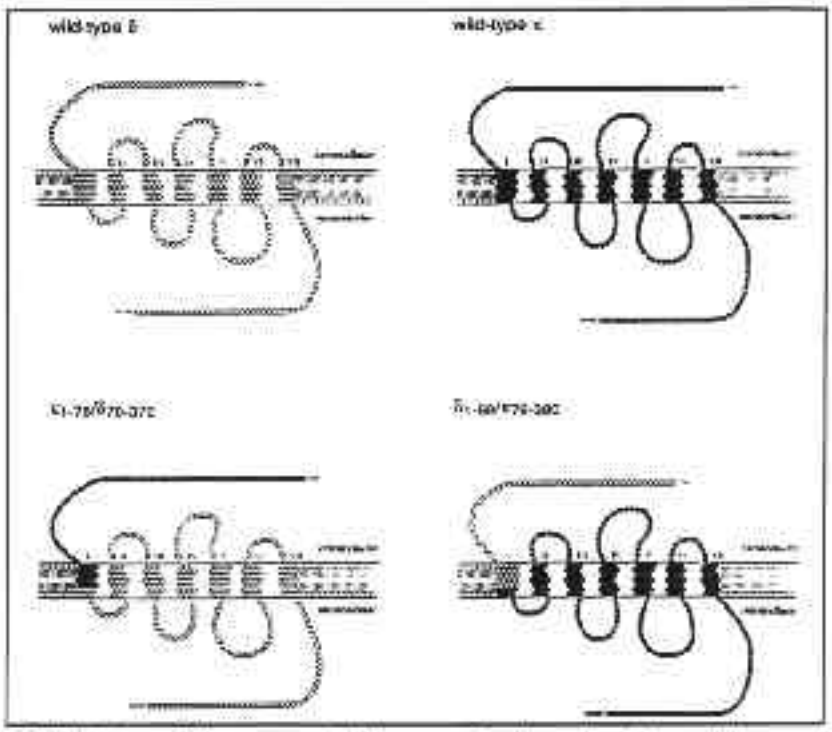

FIGURE 3. *Schematic of wild-type and chimeric delta and kappa opioid receptors.*

In contrast to the results with the mutated delta opioid receptor, the results of mutagenesis studies on the kappa receptor reveal that agonists and antagonists bind to this receptor in a manner fundamentally different from that found with any other neurotransmitter receptor (see figure 3). Kappa selective antagonists bound potently to the $kappa_{1-78}/delta_{70-372}$ chimera but kappa agonists did not. Conversely, kappa selective agonists interacted with a $delta_{1-69}/kappa_{79-380}$ chimera and a truncated kappa receptor lacking the N-terminal 78 amino acid residues. Kappa antagonists did not bind potently to these latter mutant receptors. These findings indicate that selective agonists and antagonists bind to clearly separable recognition sites in the kappa receptor with antagonists interacting with sites within the N-terminus and agonists binding to more C-terminal regions probably in either the second and/or third extracellular loops. The separation of the agonist and antagonist binding domains of the kappa receptor suggests that the N-terminus must be able to fold upon the receptor to allow the separate recognition sites to be in close proximity to allow for competi-tion of agonist and antagonist

binding. The identification of the specific residues in the kappa receptor involved in agonist and antagonist binding may facilitate the further development of therapeutically useful opioids. This will be particularly important in the case of the kappa receptor since kappa agonists have minimal abuse potential and do not cause respiratory depression, two major side effects of the use of mu receptor selective agonists. In contrast, kappa agonists are effective analgesics and useful diuretic agents.

Molecular Mechanisms of Tolerance Development to Opioids

A major limitation to the clinical use of opioids is the development of tolerance (Koob and Bloom 1992; Koob et al. 1992). With tolerance, increasing concentrations of an opioid are needed to maintain a constant therapeutic effect. All three opioid receptor types undergo tolerance development (Koob et al. 1992). The molecular basis of opioid tolerance is not established, although it has been suggested that tolerance development is related to opioid receptor desensitization (Childers 1991; Loh and Smith 1990; Nestler 1993). To investigate the molecular mechanisms of tolerance to kappa agonists, Raynor and colleagues (1994) expressed the cloned kappa receptor in mouse kidney fibroblast (COS) cells, treated the cells for increasing times with various kappa agonists, and showed that the kappa receptors desensitized. The desensitization was characterized by a reduced affinity of the receptor for agonists, an uncoupling of the receptor from G-proteins, and a diminished ability of the receptor to mediate agonist inhibition of adenylyl cyclase activity. The desensitization was reversible, blocked by opioid antagonists and induced in a stereoselective manner by agonists. Furthermore, the enzyme beta-adrenergic receptor kinase (BARK) (Benovic et al. 1989) appears to be involved in kappa receptor desensitization. This was suggested by experiments in which a BARK dominant negative mutant was cotransfected into COS cells with the kappa receptor cDNA; in those cells treatment with agonists did not cause receptor desensitization. The BARK dominant negative mutant has been shown to block the activity of wild-type BARK but can not catalyze the phosphorylation of substrates due to a single point mutation in its catalytic domain (Kim et al. 1993). These studies suggest that kappa agonists may stimulate BARK activity to catalyze the phosphorylation of the kappa receptor, thus causing an uncoupling of the receptor from G-proteins and effector systems. The intracellular domains of the kappa receptor have multiple potential phosphorylation sites that could act as substrates for BARK, and mutagenesis studies may reveal which one

of those sites is phosphorylated during desensitization and is critical for the desensitization of the kappa receptor.

Interestingly, the regulation of the three cloned opioid receptors by agonists differs considerably. Agonist pretreatment of COS cells expressing the cloned delta receptor results in rapid desensitization and downregulation of the receptor within an hour of treatment. This down-regulation of the delta receptor is not blocked by a BARK dominant negative mutant suggesting that different cellular mechanisms are involved in the regulation of the delta and kappa receptors. Furthermore, after 4 hours of agonist pretreatment, kappa receptors desensitize but do not downregulate, indicating that the sensitivity of the kappa and delta receptors to downregulation differs. This finding is of particular importance since the only intracellular regions of the two receptors that differ in amino acid sequence and that could represent domains of the receptors responsible for variations in agonist regulation are the C-termini. Mutagenesis studies to exchange the C-termini of the two receptors could test the hypothesis that these regions are involved in the regulation of opioid receptor sensitivity following agonist treatments and are critical for the development of tolerance of these receptors.

Prolonged treatment (4 to 16 hours) of COS cells expressing the cloned mu receptor did not desensitize nor downregulate this receptor. Since all three receptors were expressed in the same COS cell line, differences in sensi-tivity to agonist treatment cannot be explained by variations in cellular environment. Instead, these findings suggest that the three receptors are regulated differently at a cellular level. As a result, the cellular basis of tolerance to the three opioid receptors may be different. Tolerance to delta selective agonists may involve the desensitization and downregulation of the delta receptor. Tolerance to kappa selective agonists may involve the slow desensitization of the receptor via a BARK mediated mechanism. Tolerance to mu selective agonists such as morphine could involve postreceptor events such as changes in intermediate early gene expression or other cellular events. The fundamentally different adaptive responses of the opioid receptors to prolonged agonist treatment may reveal strategies to overcome tolerance development, which is a major limiting factor in the continuous use of opioids in the treatment of chronic pain.

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