Mu Opioid Receptors: Cellular Action and Tolerance Development

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INTRODUCTION

Opioids are some of the most effective pain-relieving drugs used in the clinical management of pain (Gilman et al. 1990). In addition to their analgesic effect, opioid peptides and alkaloids also affect a number of physiological functions including hormone secretion, neurotransmitter release, feeding, gastrointestinal motility, and respiratory activity (Pasternak 1988). Extensive physiological, behavioral, and pharma-cological studies have defined at least three major types of opioid receptors designated mu, kappa, and delta (Corbett et al. 1993; Goldstein 1987; Wood and Iyengar 1988). Although there is substantial overlap in their tissue distribution and pharmacological profiles, each opioid receptor type maintains a unique pattern of expression while displaying characteristic binding affinities for various subtype-selective ligands. The delta receptors that bind the enkephalin peptides are expressed most predominantly in the basal ganglia, striatum, and cerebral cortex (Mansour et al. 1988; Wood 1988). Although delta receptors have been implicated in spinal analgesia (Porreca et al. 1984; Yaksh 1981), recently it has been suggested that specific delta receptor subtypes may also be involved in supraspinal analgesia (Pasternak 1993). The kappa receptors are most highly expressed in cortex, striatum, and hypothalamus (Mansour et al. 1987), with various subtypes identified by autoradiography using subtype-selective ligands (Nock et al. 1988; Unterwald et al. 1991). With the development of more highly subtype-selective ligands, these receptors have been shown to mediate both spinal and supraspinal analgesia (Pasternak 1993).

The mu receptor represents the third major class of opioid binding sites. Named after morphine, the mu opioid receptor is the physiological target of such potent analgesics as morphine and fentanyl, as well as the endogenous opioid peptides, ß-endorphin, enkephalins, and dynorphins (Wood and Iyengar 1988). Opioid drugs with high abuse liability such as morphine, methadone, and fentanyl all bind the mu receptor with high affinity. In addition, heroin (diacetylmorphine), a semisynthetic derivative of morphine, crosses the blood-brain barrier much more readily than morphine due

to its increased hydrophobicity. Once in the brain, heroin is rapidly hydrolyzed to morphine, which acts at the mu opioid receptor and results in a euphoric effect, thus conferring the reinforcing properties of the drug and contributing to the development of addiction. Because of the high affinity of these opioid narcotics at the mu receptor, it is considered the main cellular mediator in the development of tolerance (Loh et al. 1988) and opioid addiction (Di Chiara and North 1992).

Analgesia and the development of opioid tolerance, dependence, and addiction have been the subject of extensive studies (Collin and Cesselin 1991). Several schemes, including receptor-mediated modulation of membrane conductance, have been proposed for the acute and chronic actions of opioids in the central nervous system (Johnson and Fleming 1989). One scheme involves protein phosphorylation by various kinases as a possible way to regulate opioid-induced cellular processes. The molecular mechanism of such regulation, however, has not been clearly delineated.

MOLECULAR CLONING OF MU OPIOID RECEPTORS

To begin exploring the molecular basis for opioid tolerance, the authors sought to clone the cDNAs encoding mu opioid receptors. Using a strategy of low-stringency hybridization for isolating opioid receptors related to the mouse delta opioid receptor (Evans et al. 1992; Kieffer et al. 1992), the authors first reported the molecular cloning of a mu opioid receptor from the rat brain (Chen et al. 1993a), and at the same time, isolated cDNAs encoding a kappa opioid receptor (Chen et al. 1993b) and a novel member of the opioid receptor gene family (Chen et al. 1994). Using the rat mu opioid receptor as a hybridization probe, a cDNA for the human mu opioid receptor has also been isolated (Mestek et al. 1995). The human and rat mu opioid receptors are very homologous, with 94percent similarity at the amino acid sequence level (figure 1). The mu opioid receptors are also closely related to the delta and kappa opioid receptors, with 62percent and 58 percent similarities to the rodent delta (Evans et al. 1992; Fukuda et al. 1993; Kieffer et al. 1992) and kappa (Chen et al. 1993b; Liet al. 1993; Meng et al. 1993; Minami et al. 1993; Yasuda et al. 1993) opioid receptors, respectively. Figure 1 displays the alignment of the human mu opioid

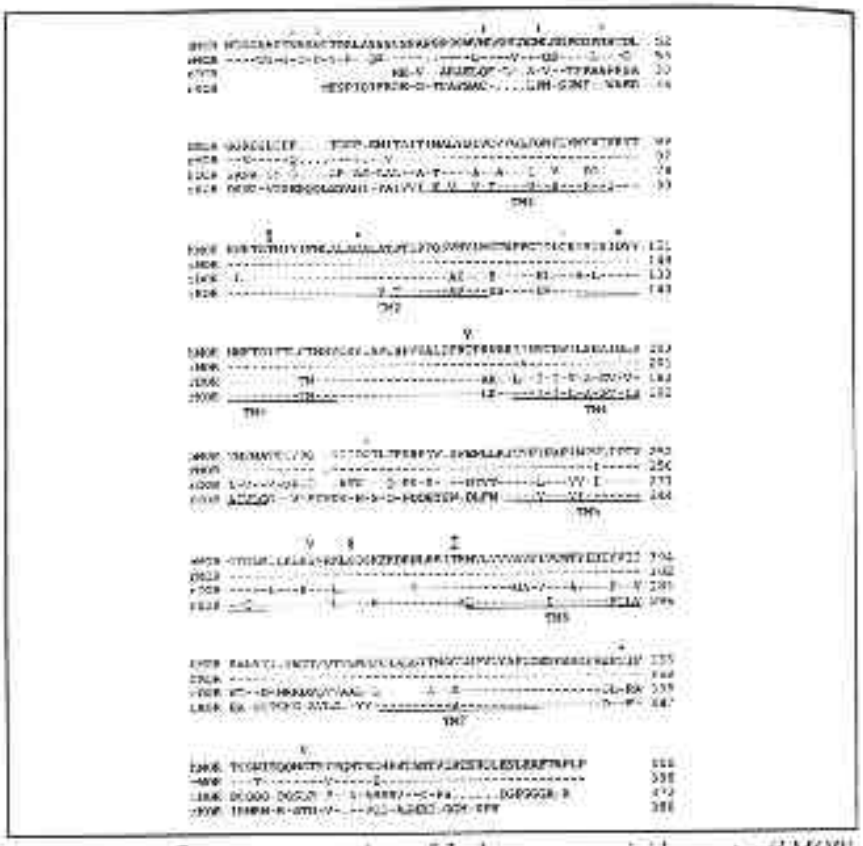

FIGURE 1. *Sequence comparison of the human mu opioid receptor (hMOR) and the rodent mu, kappa, and delta opioid receptors (rMOR, rDOR, and rKOR). Seven putative transmembrane domains are underlined and numbered TM1 through TM7.*

KEY:
- (-) = amino acids identical to those in human mu opioid receptor;
- (.) = gaps introduced for sequence alignment;
- (‡) = putative N-linked glycosylation sites;
- (§) = potential site for phosphorylation by multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II);
- (♦) = conserved aspartic acid residues proposed to interact with the amine group of ligands;
- (=) = conserved cysteine residues that may form a disulfide bond;
- (▽) = potential sites for phosphorylation by protein kinase C (PKC);
- (⧖) = putative phosphorylation site for PKC or CaM kinase II;
- (⁀) = potential phosphorylation site for CaM kinase II or cAMP-dependent kinase A;
- (.) = potential palmitoylation site.

receptor sequence against the rodent mu, kappa, and delta opioid receptor sequences. The regions of greatest divergence among the different types of opioid receptors include the N-and C-termini, the fourth and sixth trans-membrane domains, and the second and third extracellular loops. Several potential sites for posttranslational modification are present (figure 1). The N-terminus contains five potential N-linked glycosylation sites that remain conserved between the human and rat mu opioid receptors. Aspartic acid residues that occur in other guanosine triphosphate binding protein (G-protein)-coupled receptors and have been shown to interact with the protonated amine group of various ligands (Dohlman etal. 1991) are also present in the putative transmembrane (TM) domains TM2 and TM3 of the mu receptor, and two conserved cysteine residues believed to be involved in disulfide bonding (Dixon et al. 1988) occur in the first and second extra-cellular loops. There are also potential phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC), as well as the multifunctional calcium/calmodulin-dependent protein kinase (type II Ca2+/calmodulin-dependent kinase, CaM kinase). One of these sites, conserved among all opioid receptor types, occurs in the third intracellular loop. This region between TM5 and TM6 is often referred to as the G-protein loop because of its importance in G-protein coupling (Dohlman et al. 1991). The conser-vation of this site suggests that phosphorylation may play a role in modula-ting signal transduction of all the opioid receptors. There is also a cysteine residue in the C-terminus that is conserved among many G-protein-coupled receptors and that may serve as a target for palmitoylation (Collins et al. 1991).

To characterize the pharmacological profile of the mu opioid receptors, cDNA fragments containing the protein coding region of each of the mu opioid receptors were subcloned into a mammalian expression vector containing the human cytomegalovirus promoter. Using these plasmids, the mu opioid receptor was either transiently expressed in COS-7 cells or stably expressed in Chinese hamster ovary (CHO) cells. Membranes from these cells were prepared and saturation binding was performed using either [3H]diprenorphine, a nonselective opioid antagonist with high affinity for all three types of opioid receptors, or [3H] [D-Ala2-Me Phe4, Gly-ol5]-enkephalin (DAMGO), a mu-selective agonist. The mu opioid receptors display high affinity binding to both [3H]diprenorphine and [3H]DAMGO, with dissociation constant (Kd) values in the nano-molar (nM) range. Also, various unlabeled opioid ligands were used in competitive displacement experiments. Several therapeutic opioid ligands, including morphine, methadone, and fentanyl, all displayed nM affinities at the mu opioid receptors, indicating that these highly potent analgesic drugs are mu-selective. In addition to these exogenous opioids,

all three types of the endogenous opioid peptides, ß-endorphin, Met- and Leu-enkephalins, and dynorphin A (1-17) displayed nM affinities at the mu receptors, suggesting that the mu receptors may be activated by any of these endogenous opioid peptides at physiological concentrations.

The high affinity of ß-endorphin at the mu receptors is expected, since it has long been thought that ß-endorphin is the endogenous ligand for the mu opioid receptor. Using the mu, delta, and kappa opioid receptor clones isolated from rodents, binding studies performed in parallel have demonstrated that enkephalins are also potent ligands at both the mu and the delta opioid receptors (Raynor et al. 1994). The nM affinity of dynorphin A (1-17) at the mu receptor is, however, somewhat unexpected, since dynorphin A has the highest affinity at the kappa opioid receptor (Goldstein 1987) and is thus considered a kappa agonist (Chavkin et al. 1982). However, studies have shown that while dynorphin A binds the kappa receptors with subnanomolar affinity, it does display reasonably good binding at the mu opioid receptors, with affinity values in the nM range (Goldstein 1987; Pasternak 1993). Thus, the possibility exists that the mu receptor may interact with dynorphin A under physiological conditions. Detailed studies of dynorphin A binding in the human brain are very limited. There are some reports, however, using membranes isolated from rat and bovine brain (Pasternak 1993). Using guinea pig brain membranes, it has been shown that dynorphin A (1-17) can displace radiolabeled PL-17, a mu-selective ligand, with a 5nM affinity (Kawasaki et al. 1990). In another study, dynorphin A (1-17) was found to displace radiolabeled DAMGO with 3 nM affinity (Vaughn and Taylor 1989). The authors' results further support the notion that the mu opioid receptor may serve as a natural target for dynorphin A.

CELLULAR FUNCTION OF MU OPIOID RECEPTORS

The mu opioid receptor is known to exert two types of inhibitory effects on a cell—reduction of the intracellular level of cyclic adenosine monophosphate (cAMP) and inhibition of neuronal firing. Using the cloned mu opioid receptors, the authors have begun to study the molecular mechanisms for both of these processes.

The mu opioid receptors contain seven transmembrane hydrophobic domains, a structural motif most commonly found in G-protein-coupled receptors (Chen et al. 1993a). This suggests that the mu opioid receptors may couple to heterotrimeric G-proteins to mediate intracellular signal transduction. Previous studies using cell lines that constitutively express endogenous opioid receptors also suggest that opioid receptors are

coupled to G-proteins (Carter and Medzihradsky 1993b; Childers 1991). Upon activation of a G-protein, the alpha subunit of the G-protein dissociates and displays an increase in its intrinsic GTPase activity. To test whether the cloned mu opioid receptor couples to G-proteins, GTPase activities from cell membranes expressing the mu receptor were measured in the presence and absence of mu-selective ligands. Treatment of CHO cells stably expressing the mu receptor with the mu-selective agonist DAMGO elevated the GTPase activity by 33percent. This stimulation was blocked by the opioid antagonist naloxone. In nontransfected parental CHO cells, on the other hand, GTPase activity was not affected by DAMGO treatment. Because an increase in the low affinity GTPase activity is indicative of G-protein activation, these results suggest that the mu opioid receptor is functionally coupled to the G-proteins in these mammalian cells.

Since it has been reported that activation of the mu opioid receptor exerts an inhibitory effect on adenylyl cyclase activity (Adams et al. 1991; Childers 1991; Cox 1993; Frey and Kebabian 1984; Sharma et al. 1975a), the authors next sought to examine the effect of mu opioid receptor activation on the intracellular levels of cAMP. In nontransfected COS-7 cells, treatment with the mu-selective ligands had no significant effects, while DAMGO stimulation of cells expressing the mu opioid receptor reduced the intracellular cAMP levels significantly. This effect on adenylyl cyclase activity was blocked by the mu-selective antagonist naloxonazine, indicating that the inhibitory effect was mediated through the activation of the mu opioid receptor and that the receptor was functionally coupled to the inhibition of adenylyl cyclase activity.

Studies in cell lines expressing endogenous opioid receptors demonstrated that chronic treatment with opioid agonists may modulate the coupling between opioid receptors and adenylyl cyclase (Carter and Medzihradsky 1993a; Puttfarcken et al. 1988; Sharma et al. 1975b). To test the effect of chronic morphine exposure on the intracellular levels of cAMP in CHO cells expressing the mu opioid receptor, cAMP levels were assayed by activation of the mu receptor with DAMGO before and after morphine treatment. Before chronic morphine treatment, the forskolin-stimulated cAMP level in morphine-naive cells was reduced to 70 percent relative to controls upon treatment with 1 micromlar (µM) DAMGO. After the cells were exposed to morphine for 24 hours, DAMGO-induced inhibition of cAMP levels was enhanced, resulting in a decrease of cAMP levels to 40percent relative to controls. This result suggests that morphine treatment may enhance the effectiveness of the mu receptor-mediated inhibition of adenylyl cyclase activity. When the forskolin-stimulated cAMP levels in naive cells were measured in the presence of

different concentrations of DAMGO, the intracellular cAMP levels were reduced by DAMGO in a dose-dependent fashion, with a 50percent effective concentration (EC50) value of about 30 nM. The effect of DAMGO was clearly the result of the mu receptor activation, since treatment of the cells with 1 µM DAMGO in the presence of 10 µM naloxone completely abrogated the inhibitory effect of DAMGO on cAMP accumulation. In contrast, DAMGO had no effect on the intracellular cAMP levels in non-transfected parental cells. Chronic treatment with morphine for 24hours enhanced the extent of the DAMGO-induced inhibition of adenylyl cyclase activity, but did not change the EC50 value of the DAMGO-induced inhibition. To test whether the number of receptors was changed upon chronic morphine treatment, CHO cells expressing the cloned mu opioid receptors were chronically treated with 1 µM of morphine for 24 hours. Using [3H]DAMGO and whole-cell binding to determine the number of the total surface receptors, it was observed that there was no appreciable change in the number of surface receptors, nor was there any noticeable change in the affinity for DAMGO after chronic morphine treatment. These results suggest that, unlike the changes in adenylyl cyclase activities upon morphine treatment, receptor downregulation does not occur after chronic morphine treatment in these CHO cells.

The EC50 value calculated from the dose-response curve observed with DAMGO after chronic morphine treatment was about 30 nM, compared to the 2 nM 50 percent inhibitory concentration (IC50) value derived from the competition binding studies. The difference between the functional potency and the binding affinity values may be due to the ratio of mu receptors on the cell and the G-protein or effector molecules to which they couple, but other possibilities may also exist. It should be noted that the efficacy for adenylyl cyclase inhibition by the mu opioid receptor in the CHO cells is similar to that in SK-N-SH and 7315c cells (Frey and Kebabian 1984; Yu et al. 1990). The relative efficacy of mu opioid receptors to inhibit adenylyl cyclase activity seems to be lower than that of other G-protein-coupled receptors that are linked to the inhibition of adenylyl cyclase, such as the a2-adrenergic receptor, serotonin 1A receptor, and dopamine receptors (Fraser et al. 1989; Raymond et al. 1992; Sokoloff et al. 1992). The differences in the ability of each receptor type to inhibit adenylyl cyclase suggests that different G-proteins may be involved in coupling distinct receptors to a common effector molecule. Since more than one adenylyl cyclase has been identified in the cell (Iyengar 1993), an alternative possibility may be that different subtypes of adenylyl cyclases couple differently to the various membrane receptors.

The protocol the authors used for studying the effect of chronic morphine treatment is similar to that used by many investigators (Puttfarcken and Cox 1989). First, cells are chronically exposed to a moderate concen-tration of agonist (morphine). The cells are washed to remove agonist, and then are acutely stimulated with various concentrations of agonist. Because the presence of the agonist during the chronic exposure presents a continued inhibitory input, agonist washout before acute treatment is equivalent to disinhibition, and has been compared to the withdrawal paradigm following the establishment of opioid tolerance (Sharma et al. 1975b). In the authors' cell line, both the basal and forskolin-stimulated cAMP levels were elevated after chronic morphine treatment and withdrawal. This suggests that a compensatory mechanism involving increased adenylyl cyclase activity may be responsible for the higher levels of cAMP that have been observed upon removal of mu receptor-mediated inhibition (Puttfarcken and Cox 1989; Sharma et al. 1975b). It is interesting to note that chronic morphine exposure displays a differen-tial effect on cAMP levels. Basal cAMP levels were about 60percent greater after morphine exposure than that observed in naive cells, while the forskolin-stimulated cAMP levels were more than doubled with morphine treatment. It is tantalizing to speculate that this difference between basal and forskolin-stimulated cAMP levels during chronic morphine exposure reflects a differential sensitivity to acute mu receptor activation of two populations of adenylyl cyclase molecules—those present before morphine exposure and those synthesized during morphine pretreatment as the compensatory mechanism becomes engaged. It is conceivable that these two populations of adenylyl cyclases may have different compositions with regard to cyclase subtypes, since molecular cloning has shown that multiple adenylyl cyclases exist (Iyengar 1993).

Another phenomenon of morphine treatment is that the DAMGO-induced cAMP inhibition in the morphine-treated cells is more robust, which resulted in doubling the percent inhibition of cAMP from approximately 25percent in naive cells to almost 60 percent in morphine-treated cells. These results suggest that morphine exposure "sensitized" the system, such that acute mu receptor activation was more effective in inhibiting the adenylyl cyclase activity. This is in contrast to the studies with cell lines expressing endogenous mu receptors, in which chronic morphine treatment caused a decrease in the ability of the receptor to inhibit adenylyl cyclase (Puttfarcken and Cox 1989; Werling et al. 1989). The lack of receptor desensitization in CHO cells suggests that receptor modulation of adenylyl cyclase in various cell types occurs differently. This may reflect the uniqueness in the composition of endogenous G-proteins and/or adenylyl cyclases in CHO cells versus

other cell types. Also of interest is that chronic morphine treatment of the transfected CHO cells did not cause downregulation of surface receptors. In morphine-treated cells, neither the number of cell surface receptors nor the Kd value for DAMGO was significantly different from those observed with naive cells. This is in contrast to agonist-induced downregulation in ß2-adrenergic receptors (Collins et al. 1991). The increase in cAMP levels observed with chronic morphine treatment and the sensitization of the signal transduction pathways involving adenylyl cyclase suggest that this cell line could serve as a cellular model, allowing the study of both the molecular mechanisms that link the mu opioid receptor to its effector systems and the changes associated with morphine tolerance and dependence.

Activation of opioid receptors has been known to affect membrane permeability to potassium (North 1993). Stimulation of the mu opioid receptor hyperpolarizes cellular membranes by increasing the K+ conduc-tance through an inwardly rectifying channel (North et al. 1987; Wimpey and Chavkin 1991). The recent cloning of an inwardly rectifying K+ channel (Dascal et al. 1993; Kubo et al. 1993) was shown to be expressed in the brain, and the authors were interested in testing whether the mu opioid receptor coupled to this G-protein-activated K+ channel (Chen and Yu 1994; Mestek et al. 1995). Messenger ribonucleic acids (mRNAs) encoding both proteins were generated by in vitro transcription and injected into Xenopus oocytes. Coupling of the receptor to the K+ channel was assessed by a two-electrode voltage clamp. In the oocytes injected with either the mu receptor mRNA or the K+ channel mRNA alone, no membrane current was observed with the mu-receptor agonist DAMGO. This indicated that there were no endogenous currents in oocytes activated by DAMGO, and that either the mu receptor or the K+ channel alone is not sufficient to generate DAMGO-induced currents. However, coexpression of both proteins gave rise to membrane currents upon DAMGO stimulation (Chen and Yu 1994; Mestek et al. 1995). Exposure of the oocytes to 1 µM of DAMGO produced an inward membrane current that was completely blocked by the opioid receptor antagonist naloxone. This current was carried through the inwardly rectifying K+ channel, since the current varied in amplitude with the concentration of K+ in the extracellular solution and was blocked by 100µM Ba2+, a known K+ channel blocker. The current-voltage relationship of this K+ channel was characteristic of an inward rectifier. With progressive hyperpolarization, the magnitude of current increases. However, as the membrane is depolarized, current flow decreases until there is little to none at a membrane potential of 0 millivolts (mV). Thus, the mu opioid receptor is capable of coupling to the G-protein-activated K+ channel.

Prolonged exposure to opioids is known to produce tolerance in neurons (Di Chiara and North 1992). At the cellular level, tolerance development is observed as a diminished response to opioids (Johnson and Fleming 1989). Since opioid narcotics with abuse potential such as morphine, methadone, and fentanyl interact with the mu opioid receptor with high affinities, the authors were interested in examining whether the intracellular signaling by the mu opioid receptor displays tolerance. Using the receptor-K+ channel coupling as an assay, oocytes were subjected to repeated agonist stimu-lation to determine whether functional desensitization of the mu receptor-K+ channel coupling occurred. For this purpose, a protocol of repeated agonist application was used, and "desensitization" was operationally defined as a reduction in the second response compared to the first response. The oocyte was superfused with high-potassium (HK) solution while the DAMGO-induced responses were measured. The superfusate was then switched to frog Ringer's solution and the cell was subjected to the various treatments. After treatment, DAMGO-induced responsiveness was measured again in HK solution. Comparison between the maximal response before and after the treatment thus indicates how much desensi-tization has occurred following the first stimulation with DAMGO. Repeated stimulation of the mu receptor resulted in a moderate and consistent desensitization as observed by a reduction in the second response. The second response was reduced to 80 percent relative to the first response. These results suggest that when expressed in oocytes, the coupling between the mu opioid receptor and the G-protein-activated K+ channel undergoes a process of desensitization.

Previous studies had shown that activation of PKC was capable of attenuating opioid receptor activity in neuroblastoma cells (Louie et al. 1990) as well as affecting ion conductances using acutely dissociated neurons in culture (Doerner et al. 1988). The authors were interested in observing whether stimulation of PKC affects the coupling of the mu opioid receptor to the K+ channel. Using the protocol described above, oocytes were superfused for 10 to 15 minutes after the initial stimulation with phorbol 12-myristate 13-acetate (PMA), a PKC-activating phorbol ester. Oocytes were again stimulated with DAMGO. Comparison between the peak current response to DAMGO before and after PMA treatment indicated that PMA reduced the second response by 70percent relative to the first. Thus, activation of PKC augmented the desensitization of the mu opioid receptor-activated K+ current. To control for possible non-specific effects caused by application of a phorbol ester to the cellular membrane, oocytes were treated with 4a-phorbol, a phorbol ester that does not activate PKC (Blumberg et al.

1984). Recordings of peak current amplitude show that the 4a-phorbol does not augment desensitization beyond that observed with no treatment, as membrane currents before and after 4a-phorbol treatment exhibited moderate desensitization similar to that with no treatment. This demonstrated that the inactive form of the phorbol ester does not augment desensitization. Therefore, these results indicate that augmentation of the desensitization by PMA reflects the activation of PKC.

Physiologically, PKC is activated by diacylglycerol (DAG), a second messenger of the phosphatidylinositol pathway. This signaling pathway also generates another second messenger, inositol 1,4,5-trisphosphate (IP3), which triggers an increase in intracellular calcium and results in the activation of the multifunctional Ca2+/calmodulin-dependent protein kinase (type II Ca2+/calmodulin-dependent protein kinase, CaM kinase II) (Schulman and Hanson 1993). The authors were interested in testing whether CaM kinase II could modulate the mu opioid receptor-mediated K+ current. Using the protocol described above, oocytes were injected with the activated form of CaM kinase II between the first and second stimulations with DAMGO, and the effect on the receptor-induced K+ current was evaluated. CaM kinase II clearly enhanced the desensitization, causing a twofold decrease in the second response relative to uninjected oocytes. As a negative control, the same CaM kinase II solution was placed in a boiling water bath for 5 minutes and chilled on ice before injection into the oocytes. Boiled CaM kinase II did not augment desensitization beyond that observed with uninjected controls. The authors concluded that activated CaM kinase II can therefore augment the desensitization of the mu opioid receptor-activated K+-current.

Since the mu opioid receptor can modulate the activity of adenylyl cyclase, there was also interest in determining whether cAMP, the product of adenylyl cyclase, had any effect on the mu receptor-K+ channel coupling (Chen and Yu 1994). Treatment of the oocyte with 8-chlorophenylthio-cAMP (8-CPT-cAMP), a membrane-permeable cAMP analog that can diffuse into the cell and stimulate cAMP-dependent protein kinase (PKA), completely abolished the desensitization observed in untreated oocytes, resulting in the K+ current being similar in amplitude for both the first and the second stimulation with DAMGO. To determine whether the 8-CPT-cAMP effect on preventing desensitization was mediated by PKA, the catalytic subunit of PKA was injected into the oocytes after the first DAMGO stimulation. This resulted in the same effect as 8-CPT-cAMP incubation, indicating that the blockade of desensitization was indeed mediated through PKA.

DISCUSSION

A Molecular Mechanism for Mu Opioid Receptor Function and Its Acute Desensitization

Results from the authors' studies indicate that cloned mu opioid receptor cDNAs can be used to express functional mu opioid receptors that display the expected pharmacological profile. The cellular functions affected by the activation of the mu opioid receptor include the inhibition of adenylyl cyclase activity and the activation of a K+ channel. Various protein kinases have also been shown to modulate mu receptor-mediated cellular processes. Based on these results, the authors propose the following model for mu receptor function and its acute desensitization. This model, shown schematically in figure 2, is discussed below.

A major effect of the mu opioid receptor in brain is the decrease of neuronal membrane excitability. One of the mechanisms for this effect is an increase in K+ conductance, accomplished by the opening of an inward rectifier, resulting in outward K+ currents and hyperpolarization of the cell membrane (Chavkin 1988; North 1986, 1993). With the cloning of an inwardly rectifying K+ channel that can be activated by a number of neurotransmitter receptors (Dascal et al. 1993; Kubo et al. 1993), it became possible to examine whether the mu opioid receptor could also activate this channel. It has been shown that the mu opioid receptor from both rat (Chen and Yu 1994) and human (Mestek et al. 1995) can activate this channel, causing an increase in K+ conductance. The receptor-channel coupling is clearly mediated through heterotrimeric GTP-binding proteins (G-proteins), since a nonhydrolyzable GTP analog, GTP-gS, can enhance the mu receptor-activated K+ current and pertussis toxin treat-ment can decrease it (Chen and Yu 1994). Thus, the authors' data

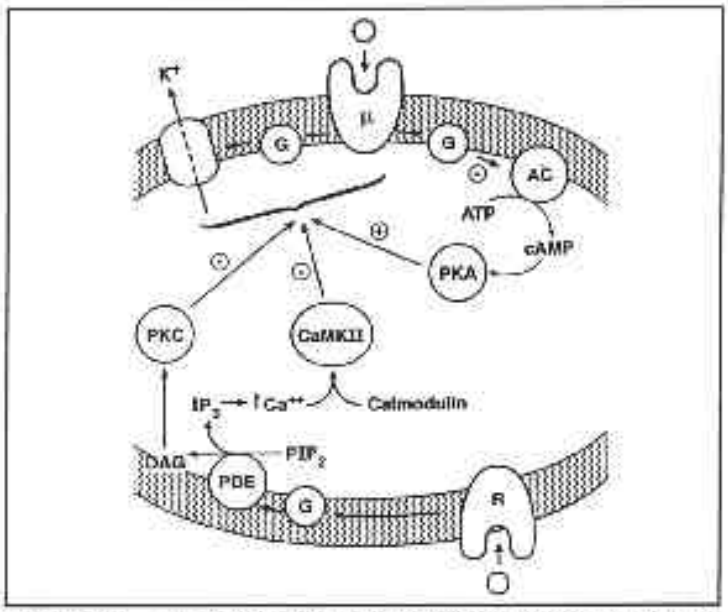

FIGURE 2. *A model depicting the molecular mechanism for mu opioid receptor function and its acute desensitization. The mu opioid receptor and another type of surface receptor in the cell membrane are shown as mu and R, respectively. Open circles represent ligands for the respective receptors. Potassium channel is shown with ionic efflux.*

KEY: G = G protein; AC = adenylyl cyclase; PKA = cAMP-dependent protein kinase; PKC = protein kinase C; CaMKII = multifunctional Ca^{2+}/calmodulin-dependent protein kinase; PDE = phosphodiesterase involved in PIP_2 hydrolysis. DAG = diacylglycerol; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = inositol 1,4,5-trisphosphate; ATP = adenosine triphosphate; cAMP = adenosine 3', 5'-cyclic monophosphate. Arrows pointing from second messenger molecules to proteins indicate a stimulatory influence. Arrows between proteins indicate either a stimulatory (+) or inhibitory (-) effect on interactions. Upward arrow adjacent to Ca^{++} indicates an increase in intracellular calcium.

SOURCE: Adapted from Mestek et al. 1995.

suggest that the mu receptor-channel coupling may be the basis for the mu receptor-mediated increase in K+ conductance.

Receptor-mediated signaling processes often display desensitization, operationally defined as a decrease in the cellular response to further agonist stimulation upon continuous or repeated exposure to agonist (Benovic et al. 1988). This may serve as a physiological mechanism to prevent overstimulation of the neuron. The mu opioid receptor is the physiological target of morphine and fentanyl, analgesics used in the clinical management of pain. Prolonged use of morphine and related opioids can lead to the development of tolerance, necessitating dosage increases to achieve the same degree of their initial physiological effect. At the cellular level, tolerance manifests itself as a desensitized responsiveness to repeated opioid applications, and it has been hypothesized that several of the intermediates in the mu receptor signaling pathway are involved (Nestler et al. 1993). In the neurons of the rat locus coeruleus, desensiti-zation was observed as a reduction in membrane hyperpolarization upon continued application of Met5-enkephalin. A decrease in K+ conductance was shown to be responsible for the observed effect (Harris and Williams 1991). In oocytes expressing the mu opioid receptor and the inwardly rectifying K+ channel, a protocol was used to evaluate desensitization by measuring the K+ currents evoked by sequential activation of the receptor with a mu-selective agonist. Comparison of the maximum K+ currents thus indicates the extent of desensitization between the first stimulus and second stimulus caused by activation of the receptor. Using this paradigm, desensitization occurred consistently, as observed by the reduction of the K+ current to 80 percent of the initial response evoked by receptor activation. Thus, desensitization of receptor-channel coupling appears to be a normal process when studied in oocytes, suggesting that such a phenomenon may exist as an adaptive process in neurons to modulate the responsiveness of the mu receptor-mediated increase in K+ conductance.

Desensitization of receptor-channel coupling may involve several mechanisms at the cellular level. For acute desensitization such as that studied here with a timescale of fewer than 30 minutes, de novo protein synthesis or receptor turnover are unlikely to account for the majority of the observed effects. Covalent modification through kinase-mediated phosphorylation, on the other hand, appears to play an essential role. The authors demonstrated that both PKC and CaM kinase II augment desensitization. Activation of PKC by the phorbol ester PMA and injection of the type II CaM kinase activated by autophosphorylation both resulted in augmentation of desensitization.

In contrast, treatment with the inactive 4a-phorbol ester or injection of boiled CaM kinase II did not augment desensitization beyond that observed in oocytes that had been untreated, suggesting that the augmentation effect is specific to the active form of these kinases. When activated individually, PKC and CaM kinase (Akasu and Tokimasa 1992; Shearman et al. 1989) have been shown to decrease K+ conductance in neurons, which supports the authors' observations in Xenopus oocytes. Furthermore, in rat locus coeruleus neurons containing mu opioid receptors, treatment with staurosporine, a nonselective kinase inhibitor that can inhibit both PKC (Tamaoki 1991) and CaM kinase II (Yanagihara et al. 1991), reduced desensitization of mu receptor-mediated hyperpolarization (Harris and Williams 1991). Processes that elevate the activity of these cellular kinases, therefore, may play an important role in regulating the extent of the mu receptor-K+ channel coupling.

Both PKC and CaM kinase II are cellular effectors of a G-protein-activated phosphodiesterase, phospholipase C (PLC). The activation of PLC causes the production of DAG and IP3, two intracellular second messenger molecules that represent a bifurcation in the signal transduction pathway (Berridge 1987). Whereas DAG activates PKC, IP3triggers Ca2+ release from intracellular stores. Since CaM kinase II is activated by physiological elevations in cytosolic Ca2+ levels (MacNicol and Schulman 1992a; Schulman and Hanson 1993), stimulation of receptors linked to PLC may cause activation of both PKC and CaM kinase (figure 2). Other neuro-transmitter receptors that belong to the family of G-protein-coupled receptors influence the steady-state levels of cAMP by either stimulating or inhibiting the activity of adenylyl cyclase, respectively, as is the case for the ß-adrenergic and opioid receptors. The widespread distribution of many G-protein-coupled receptors suggests that some may be found within similar structures of the brain. In fact, in situ hybridization has shown that messages encoding receptors that use either similar (Lester et al. 1993) or different (Weiner et al. 1990) signaling pathways do coexist within the same cell. The authors' data suggest that PLC-coupled receptors may augment the process of desensitization, which is observed as a reduction in receptor-channel coupling upon repeated stimulation of the mu opioid receptor (figure 2). Thus, it is plausible that signal transduction mechanisms may affect one another when the receptors to which they couple are present on the same neuron.

Since opioid receptor activation has been shown to affect cAMP levels, another important kinase in the regulation of opioid receptor activity is PKA. Opioid receptor activation inhibits adenylyl cyclase, resulting

in decreased levels of cAMP and a reduction in basal PKA activity. The authors previously demonstrated that an increased PKA activity, either by injection of the catalytic subunit of PKA into the cell or by exposing the cell to a membrane-permeable cAMP analog, eliminated desensitization of the mu receptor-K+ channel coupling (Chen and Yu 1994). Thus, agents that activate PKA would function antagonistically in relation to the mu opioid receptor-mediated channel activity. This presents an interesting control mechanism whereby the activity of the channel is subject to negative feedback inhibition modulated by the mu opioid receptor via a decrease in PKA activity (figure 2).

Phosphorylation by kinases is one of the most important mechanisms for the functional regulation of many cellular proteins including neuro-transmitter receptors and ion channels, with PKA and PKC being two of the most widely studied kinases (Krebs 1989; Shearman et al. 1989). Phosphorylation of ß2-adrenergic receptor by either PKA or PKC leads to its uncoupling from G-proteins, resulting in desensitization to further agonist stimulation (Lefkowitz and Caron 1988; Sibley and Lefkowitz 1985). In the case of voltage-dependent Ca2+ channels such as the endogenous oocyte Ca2+ channel, PKA- and PKC-mediated phosphory-lation is able to potentiate channel activity (Bourinet et al. 1992; Chen et al. 1993c). Cystic fibrosis transmembrane conductance regulator (CFTR), a Cl- channel associated with cystic fibrosis, is also regulated by cAMP through the PKA pathway (Anderson et al. 1991; Bear et al. 1991). The authors have attempted to investigate the role of each kinase in the mechanisms involved in acute desensitization. Figure2 depicts the individual effects of these kinases on opioid receptor-mediated K+ channel activity. Although precedents exist for their acting independently, these kinases are also subject to "cross-talk," which is the ability of one signal transduction mechanism to affect another (MacNicol and Schulman 1992b; Yamakawa et al. 1992). Taken together, the data suggest that an intricate network of modulation among receptors, G-protein effectors, and protein kinases may exist. It should be noted that although each element in the model has been demonstrated in oocytes and/or neurons, the complete scheme has not yet been confirmed with respect to the cross-modulation that may occur among multiple receptor types. With the cloning of the mu opioid receptors and the other proteins involved in cellular signaling, future efforts will undoubtedly further researchers' general understanding of the molecular mechanisms that underlie the regulation of receptor-mediated neuronal activity, with particular focus on the modulatory network involved with mu opioid receptor function.

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