μ opioid receptors mediate the analgesia induced by morphine. Prolonged use of morphine causes tolerance development and dependence. To investigate the molecular basis of tolerance and dependence, the cloned mouse μ opioid receptor with an amino-terminal epitope tag was stably expressed in human embryonic kidney (HEK) 293 cells, and the effects of prolonged opioid agonist treatment on receptor regulation were examined. In HEK 293 cells the expressed μ receptor showed high affinity, specific, saturable binding of radioligands and a pertussis toxin-sensitive inhibition of adenylyl cyclase. Pretreatment (1 h, 3 h, or overnight) of cells with 1 μM morphine or [d-Ala²MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO) resulted in no apparent receptor desensitization, as assessed by opioid inhibition of forskolin-stimulated cAMP levels. In contrast, the morphine and DAMGO pretreatments (3 h) resulted in a 3–4-fold compensatory increase in forskolin-stimulated cAMP accumulation. The opioid agonists methadone and buprenorphine are used in the treatment of addiction because of a markedly lower abuse potential. Pretreatment of μ receptor-expressing HEK 293 cells with methadone or buprenorphine abolished the ability of opioids to inhibit adenylyl cyclase. No compensatory increase in forskolin-stimulated cAMP accumulation was found with methadone or buprenorphine; these opioids blocked the compensatory effects observed with morphine and DAMGO. Taken together, these results indicate that methadone and buprenorphine interact differently with the mouse μ receptor than either morphine or DAMGO. The ability of methadone and buprenorphine to desensitize the μ receptor and block the compensatory rise in forskolin-stimulated cAMP accumulation may be an underlying mechanism by which these agents are effective in the treatment of morphine addiction.

Opioid agonists are the therapeutic choice in the management of severe chronic pain (1). Despite effective analgesic benefit, extended periods of opioid treatment can result in a range of undesirable side effects, not the least of which is the development of tolerance. Opioids such as morphine demonstrate potent and stereoselective effects that are reversed by selective antagonists, suggesting an interaction with a specific membrane receptor (1). Selective, saturable, and high affinity membrane binding sites were subsequently demonstrated by radioligand binding assays on native tissue preparations (1).

Martin et al. (2) proposed the hypothesis of multiple opioid classes and defined μ receptors as the site of morphine action. The classification of opioid receptors into μ, κ, and δ was subsequently reinforced by the development of highly selective ligands for each class (3). Recently, the genetic basis for the three opioid classes was obtained, with the molecular cloning of the δ, κ, and μ opioid receptors (for review, see Ref. 4).

Of the three opioid receptor classes, the μ receptor is thought to be the principal site of analgesic interaction, since most of the clinically relevant opioids used in pain management bind to this receptor with high affinity (5–7). Studies on the μ receptor offer potential molecular insights into the cellular basis for tolerance and dependence, serious side effects of prolonged opioid usage which may result from effects on receptor regulation (8).

Whereas morphine induces dependence, methadone is used in the treatment of opioid addiction, despite being a full agonist at the μ receptor (9). The therapeutic efficacy of methadone has been linked to a lower abuse potential, although it is not established why methadone has lower addictive properties than morphine. Buprenorphine is the other opioid agent used in the treatment of addiction and has not been found to precipitate withdrawal in animal and human studies (9, 10). Buprenorphine has been reported to be a partial agonist or mixed agonist/antagonist at the μ receptor, and these properties have been implicated as the basis for addiction therapy (10).

Despite the clinical importance of tolerance development and dependence, relatively little is known about the molecular and cellular events induced by morphine (8). Also, the cellular events that accompany the therapeutic actions of methadone and buprenorphine in treating addiction are poorly understood.

The recent cloning and heterologous expression of the μ receptor have facilitated attempts to determine the biochemical mechanisms of clinically used opioids. In this study we investigated the molecular consequences of prolonged opioid agonist exposure on the cloned murine μ receptor stably expressed in HEK 293 cells. Continuous treatment of the μ receptor with morphine or DAMGO does not appear to desensitize the receptor, but it does result in an adaptive cellular response that is manifested by an increase in forskolin-stimulated intracellular cAMP levels. In contrast, methadone and buprenorphine treatments result in a pronounced desensitization of the μ receptor while also blocking the compensatory increases in forskolin-stimulated cAMP levels observed with either morphine or DAMGO. These differential actions of opioid agonists at the μ receptor may explain the mechanism by which morphine induces dependence, whereas methadone and buprenorphine are effective therapeutic agents in the treatment of dependence.
Opioid Agonist Regulation of \( \mu \) Opioid Receptor

EXPERIMENTAL PROCEDURES

Cell Culture—HEK 293 cells were grown and maintained in minimal essential medium with Earl’s salts (Life Technologies, Inc.) containing 10% fetal bovine serum, 1,000 units ml\(^{-1}\) penicillin and streptomycin sulfate in 10% CO\(_2\) at 37°C. The mouse \( \mu \) opioid receptor gene modified with the FLAG epitope (DYKDDDDK) at the amino terminus was a gift from Dr. Mark von Zastrow, University of California at San Francisco. The modified cDNA in the expression vector pcDNA3 (Invitrogen) was stably transfected into HEK 293 cells by a modification of the calcium phosphate protocol (11). Briefly, HEK 293 cell monolayers at approximately 70% confluence in T 75-cm\(^2\) tissue culture flasks were transfected with 30 \( \mu \)g of plasmid. After an overnight incubation, the medium was removed, and the cells were treated with 5 ml of phosphate-buffered saline containing 10% glycerol for 10 min at room temperature. Cells were then washed twice with phosphate-buffered saline and incubated for 3 h at 37°C with growth medium containing 100 \( \mu \)g ml\(^{-1}\) chloroquine. Cells were then washed twice and incubated for 48 h at 37°C in growth medium. Stable transfomants were selected in growth medium containing 1 mg ml\(^{-1}\) Geneticin (Life Technologies, Inc.) and maintained in T 75-cm\(^2\) tissue culture flasks in 10% CO\(_2\) at 37°C.

Radioligand Binding Studies—Receptor binding studies were performed using membranes from stably transfected HEK 293 cells expressing the \( \mu \) FLAG cDNA. Membranes were prepared and receptor binding studies conducted as described (5) and as noted in the table and figure legends. Briefly, cell monolayers were harvested in 6 ml of buffer containing 50 mM Tris-HCl (pH 7.8) containing 1 mM EGTA, 5 mM MgCl\(_2\), 10 \( \mu \)g ml\(^{-1}\) leupeptin, 10 \( \mu \)g ml\(^{-1}\) pepstatin, 200 \( \mu \)g ml\(^{-1}\) bacitracin, and 0.5 \( \mu \)g ml\(^{-1}\) aprotinin and placed on ice. A cell pellet was prepared by centrifugation at 24,000 \( \times \) g for 7 min at 4°C and was homogenized in the same buffer using a Polytron (Brinkmann Instruments) at setting 2.5, 30 s. The cell homogenate was centrifuged for at 48,000 \( \times \) g for 20 min at 4°C, and the resulting cell pellet was homogenized and placed on ice for the binding assays. Binding assays were carried out at 25°C for 40 min in a final volume of 200 \( \mu \)l in the presence or absence of competing ligands.

For agonist pretreatment studies, a 10-fold concentrated stock of agonist was diluted into growth medium and added to individual culture flasks. The final concentration of all agonists used in regulation studies was 1 \( \mu \)M. Cell monolayers were harvested at the times indicated in the table and figure legends. Pertussis toxin treatments were carried out either in tissue culture flasks or 12-well plates overnight at 37°C with 100 ng ml\(^{-1}\) pertussis toxin (List Biological Laboratories, Campbell, CA).

cAMP Accumulation Studies—Stably transfected HEK 293 cells were subcultured in 12-well culture plates and allowed to recover for 72 h prior to experiments. For agonist pretreatment and pertussis toxin experiments, the growth medium was replaced for the times indicated in the figure legends with medium containing either ligand or pertussis toxin. After treatment, the medium was removed and replaced with 1 ml of growth medium containing 0.5 mM isobutylmethylxanthine, and the cells were incubated for 30 min at 37°C. The culture medium was then removed and replaced with fresh medium, with or without 10 \( \mu \)M forskolin and opioids, and the cells were transferred to 37°C. After 5 min, the medium was removed, 1.0 ml of 0.1 N HCl was added, and the monolayers were frozen at \(-20^\circ\)C. For determination of the cAMP content of each well, the monolayers were thawed, placed on ice, sonicated, and the intracellular cAMP levels measured by radioimmunoassay (Amersham plc, Buckinghamshire, UK). Data obtained from the dose-response curves were analyzed by nonlinear regression analysis with GraphPad Prism 2.01 (GraphPad Software, Inc., San Diego, CA).

Antibody Binding to Cell Monolayers—Antibody binding studies were performed with the selective \( \mu \) receptor agonists DAMGO, morphine, and methadone and the nonselective opioids levorphanol, etorphine, and buprenorphine. The chemical structures of these opioid ligands are shown in Fig. 1.

Bound radioactivity was determined after solubilization of the cell monolayer with 0.5 ml of 0.5 N NaOH and counted in a \( \gamma \) scintillation counter. Nonspecific radiolabeled antibody binding was determined in the presence of 10 \( \mu \)M FLAG peptide (Eastman Kodak) and accounted for 80% or less of the total binding.

Opioids Used in This Study—Cell monolayers were pretreated with the selective \( \mu \) receptor agonists DAMGO, morphine, and methadone and the nonselective opioids levorphanol, etorphine, and buprenorphine. The chemical structures of these opioid ligands are shown in Fig. 1.

RESULTS

To investigate the prolonged effects of opioid agonist treatment, we stably expressed the mouse \( \mu \) receptor in HEK 293 cells. Pharmacological characterization of the stably transfected cells showed saturable, high affinity binding for selective \( \mu \) opioid ligands, which was best defined as a single, noninteracting site (Table 1). Saturation radioligand binding with the nonselective opioid antagonist \(^{3}H\)Oheprenorphine revealed a \( \mu \) receptor density (\( B_{max} \)) of 7.7 ± 0.5 pmol mg\(^{-1}\) membrane protein with a dissociation constant (\( K_d \)) of 0.61 ±...
Buprenorphine (Fig. 2) has been shown to inhibit forskolin-stimulated cAMP accumulation in HEK 293 cells (13–15). Consistent with previous studies, the rank order of potency for opioid agonists to inhibit cAMP accumulation in HEK 293 cells couples to the G\textsubscript{i} or G\textsubscript{0}\textsubscript{0} class of G proteins. The effect of buprenorphine on the inhibition of forskolin-stimulated cAMP accumulation was blocked by pertussis toxin treatment, the effects of the agonist pretreatment impair the coupling of the µ receptor to the G\textsubscript{i} or G\textsubscript{0} class of G proteins in the HEK 293 cells. DAMGO pretreatment was found to have no effect on high affinity \[^{3}H\]DAMGO binding (102 ± 18%, n = 3). Buprenorphine and etorphine pretreatments appeared to abolish \[^{3}H\]DAMGO binding relative to control binding (99 ± 1% reduction, n = 4 for buprenorphine; 99 ± 0.3% reduction, n = 3 for etorphine); and methadone, levorphanol, and morphine pretreatments reduced radiolabeled agonist binding (reductions of 79 ± 13%, 73 ± 6% and 35 ± 8%, respectively, n = 3). The effects of buprenorphine were stereoselective, as (+) buprenorphine, the inactive stereoisomer, did not bind to the mouse µ receptor (data not shown).

To determine the effects of agonist pretreatment on µ-FLAG receptor density, two independent studies were performed. The binding of \[^{3}H\]diprenorphine, a nonselective opioid antagonist, was found to be decreased by four of the six agonist pretreatments examined, in agreement with the \[^{3}H\]DAMGO studies (Fig. 4A). Morphine and DAMGO pretreatments had negligible effects on \[^{3}H\]diprenorphine binding (reductions of 9 ± 11% and 19 ± 8%, respectively). And, whereas levorphanol reduced antagonist binding by 28 ± 4%, methadone, buprenorphine, and etorphine pretreatments resulted in marked reductions in \[^{3}H\]diprenorphine binding (61 ± 12%, 99 ± 1%, and 68 ± 8%, respectively). Since etorphine and buprenorphine have been proposed to be highly lipophilic opioids (10), the dramatic reductions in \[^{3}H\]diprenorphine binding may result from an irreversible component of the ligand binding that occurred during the 3-h pretreatment, making direct radioligand measurements inaccurate. To assess the effects of agonist pretreatment on receptor density independently of radioligand binding, an iodinated monoclonal antibody against the amino-terminal FLAG epitope was used. If the continued presence of the pretreatment agonist (e.g., buprenorphine) affected the affinity of \[^{3}H\]diprenorphine binding, then the use of the radiolabeled M2 antibody should provide an alternative means to measure the cell surface receptor density. At present, the amino terminus of the mouse µ receptor is predicted to be an extracellular site not known to be directly involved in ligand binding (3, 16, 17). Fig. 4B demonstrates that the binding of the radiolabeled M2 antibody to intact cell monolayers is unaffected by pretreatment with the agonists levorphanol (101 ± 7% of control, n = 4), buprenorphine (109 ± 7% of control, n = 8), or morphine binding (95 ± 3% of control, n = 3), whereas DAMGO resulted in small decreases in antibody binding (83 ± 5% of control, n = 4). In contrast, the 3-h pretreatment of cells with 1 µM etorphine resulted in a reduction of M2 binding to 64 ± 6% (n = 8) of the untreated control (Fig. 4B). The antibody binding studies indicate that despite the ability of levorphanol,
Opioid agonist effects on inhibition of forskolin-stimulated cAMP accumulation in HEK 293 cells. Panel A, HEK 293 cell monolayers were treated for 30 min at 37°C with growth medium containing 0.5 mM isobutylmethylxanthine. After treatment, the medium was replaced with growth medium containing agonist over the concentration range of 10^{-11} to 10^{-6} M and incubated at 37°C for 5 min. The medium was then aspirated, and 1 ml of 0.1 N HCl was added; the cells were sonicated. Intracellular cAMP levels were measured using a commercially available cAMP radioimmunoassay kit (Amerham Corp.). The inhibition of forskolin-stimulated cAMP accumulation is expressed as a percentage of the forskolin control. Forskolin-stimulated cAMP levels were typically 5–20-fold higher than basal values. The data presented are the means ± S.E. of three independent experiments, each performed in duplicate. Panel B, pertussis toxin (PTX) effects on opioid inhibition of cAMP accumulation expressing HEK 293 cells. Panel C, naloxone block of morphine inhibition of forskolin-stimulated cAMP accumulation.

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**Fig. 2. Opioid agonist inhibition of forskolin-stimulated cAMP accumulation in µ-FLAG-expressing HEK 293 cells.** Panel A, HEK 293 cell monolayers were treated for 30 min at 37°C with growth medium containing 0.5 mM isobutylmethylxanthine. After treatment, the medium was replaced with growth medium containing agonist over the concentration range of 10^{-11} to 10^{-6} M and incubated at 37°C for 5 min. The medium was then aspirated, and 1 ml of 0.1 N HCl was added; the cells were sonicated. Intracellular cAMP levels were measured using a commercially available cAMP radioimmunoassay kit (Amerham Corp.). The inhibition of forskolin-stimulated cAMP accumulation is expressed as a percentage of the forskolin control. Forskolin-stimulated cAMP levels were typically 5–20-fold higher than basal values. The data presented are the means ± S.E. of three independent experiments, each performed in duplicate. Panel B, pertussis toxin (PTX) effects on opioid inhibition of cAMP accumulation expressing HEK 293 cells. Panel C, naloxone block of morphine inhibition of forskolin-stimulated cAMP accumulation. Cell monolayers were treated overnight with 100 ng ml^{-1} of pertussis toxin (List Biologicals), and cAMP accumulation was determined the following day. The data shown are the means ± S.E. of three independent experiments, each performed in duplicate. Panel B, pertussis toxin (PTX) effects on opioid inhibition of cAMP accumulation expressing HEK 293 cells. Cell monolayers were treated as in panel A, and either morphine (1 μM) or morphine plus naloxone (1 μM) was added to the monolayers. After 5 min at 37°C the medium was aspirated, and the samples were prepared for cAMP radioimmunoassay. The results are the means ± S.E. of three separate experiments, each performed in duplicate.
sensitization was observed after pretreatment with methadone and buprenorphine, opioids used in the treatment of morphine and heroin dependence (Fig. 6, B and C). A 3-h pretreatment of cell monolayers with methadone resulted in a marked rightward shift in the EC_{50} for the methadone inhibition of forskolin-stimulated cAMP accumulation (Fig. 6B; methadone control EC_{50} = 9.6 ± 1.5 nM, n = 5; 3-h treated EC_{50} = 230 ± 3.7 nM, n = 5). The 3-h methadone pretreatment reduced the maximum inhibition of cAMP accumulation (control, 84 ± 6%; n = 7; 3-h pretreated, 55 ± 6%; n = 5). Buprenorphine pretreatment (3 h) also resulted in receptor desensitization (Fig. 6C) at buprenorphine concentrations above 10^{-5} M, with the maximal inhibition of cAMP accumulation reduced from 67 ± 3% (n = 14) for control to 2 ± 8% (n = 5) for treated cells. No effect on forskolin-stimulated cAMP accumulation was found with (+) buprenorphine (data not shown). The three opioid agonists etorphine, methadone, and buprenorphine also appeared to desensitize the effects of morphine on cAMP accumulation (Fig. 6D) by markedly reducing the ability of maximal concentrations of morphine to inhibit forskolin-stimulated cAMP accumulation. Methadone pretreatment appeared to have a pronounced effect on the observed EC_{50} for morphine inhibition, with an approximately 200-fold shift to higher agonist concentrations when compared with control (Fig. 6D; 3-h treatment EC_{50} = 467 ± 19 nM, n = 3, compared with Fig. 4A; control EC_{50} = 2.3 ± 1.5 nM). Thus, the opioids methadone and buprenorphine are able to desensitize the mouse μ receptor to morphine, with rightward shifts in the EC_{50} for inhibition and a reduction in the maximal levels of inhibition after pretreatment. The pretreatment effects of these three opioids differ markedly from the effects observed on the mouse μ receptor after pretreatment with morphine, levorphanol, or DAMGO.

Adaptive sensitization or overshoot of adenyl cyclase activity has been implicated in the cellular mechanisms of opioid tolerance (21, 22), and a recent study on the cloned rat μ receptor suggested that the physiological consequences of receptor sensitization, rather than receptor desensitization, may be critical to tolerance development (22). We examined whether a physiological adaptation phenomenon occurred after opioid agonist pretreatment of HEK 293 cells by determining the levels of forskolin-stimulated cAMP after agonist pretreatment. As shown in Fig. 7, a 3-h pretreatment with 1 μM morphine or DAMGO leads to an approximately 3.4 ± 0.3-fold increase (ANOVA, n = 17, p < 0.001) and 2.9 ± 0.2-fold (n = 12, p < 0.001) in forskolin-stimulated intracellular cAMP accumulation, respectively, for morphine and DAMGO, when compared with untreated control monolayers.
In the current study we have stably expressed an epitope-tagged mouse μ receptor (23) in HEK 293 cells and examined the pharmacological and functional consequences of prolonged agonist exposure. The mouse receptor binds the selective μ agonists morphine, DAMGO, and methadone with high affinity, as well as the nonselective opioids levorphanol, etorphine, and buprenorphine. In agreement with earlier studies on the heterologous expression of cloned opioid receptors in HEK 293 cells (13–15), the high affinity agonist binding of the expressed mouse μ receptor was sensitive to the effects of GTPγS and pertussis toxin pretreatments. The sensitivity to pertussis toxin indicates that in the HEK 293 cells, the expressed mouse μ receptor couples to a member of the G_{i} or G_{o} family of G

**DISCUSSION**

The compensatory effects of morphine and DAMGO on adenylyl cyclase were blocked by the addition of other opioid agonists. Fig. 7 shows that when cell monolayers were pretreated with morphine in combination with other opioid agonists such as levorphanol (n = 3, p < 0.001), methadone (n = 3, p < 0.001), etorphine (n = 2, p < 0.001), or buprenorphine (n = 3, p < 0.001), the forskolin-stimulated increase of adenylyl cyclase activity was reversed. In a similar manner, DAMGO pretreatment in combination with other opioid agonists also resulted in a reduction in the forskolin-stimulated levels of intracellular cAMP (Fig. 7). No statistically significant differences were observed for the effects of methadone when compared with the combinations of methadone and morphine (n = 3, p > 0.05) or methadone and DAMGO (n = 3, p > 0.05). These results demonstrate that the opioid agonists levorphanol, etorphine, methadone, and buprenorphine are capable of blocking the compensatory increases in intracellular cAMP observed after chronic treatment with morphine or DAMGO.

**FIG. 5.** Agonist pretreatment effects on opioid inhibition of forskolin-stimulated intracellular cAMP levels. Stably transfected HEK 293 cells were plated in 12-well dishes, and intracellular cAMP levels were assayed as described under “Experimental Procedures.” Forskolin-stimulated cAMP values, in the absence of opioid agonist, were defined as 100%, and the opioid percentage of inhibition of cAMP accumulation was calculated from the forskolin control value. Panel A, dose-dependent inhibition of forskolin-stimulated cAMP accumulation of control (■) or morphine-pretreated cell monolayers. The results presented represent the mean ± S.E. of at least three separate experiments, each performed in duplicate and assayed in duplicate. ▲, morphine control; ●, morphine 3-h pretreatment; ▼, overnight pretreatment; *p < 0.05. Panel B, levorphanol dose-dependent inhibition of forskolin-stimulated cAMP levels from control and 1 μM levorphanol-pretreated cell monolayers. The results shown are the mean ± S.E. of three independent experiments, each performed and assayed in duplicate. Symbols are as in Panel A. Panel C, DAMGO concentration-dependent inhibition of forskolin-stimulated cAMP levels from control and 1 μM DAMGO-pretreated cells. The results shown are the means ± S.E. of three independent experiments performed in duplicate. ■, control; ▲, 3-h pretreatment. Panel D, levorphanol or DAMGO pretreatment effects on morphine inhibition of forskolin-stimulated cAMP accumulation. HEK 293 cells were pretreated with either 1 μM levorphanol (■) or DAMGO (▲) for 3 h at 37 °C, and the concentration-dependent effects of morphine on intracellular cAMP accumulation were determined. The results are the mean ± S.E. of three independent experiments, each performed in duplicate.
FIG. 6. Etorphine, methadone, or buprenorphine desensitization of opioid agonist inhibition of forskolin-stimulated cAMP accumulation. Panel A, etorphine desensitization of etorphine effects on forskolin-stimulated cAMP accumulation. Monolayers were pretreated for 3 h with 1 μM etorphine and assayed for intracellular cAMP accumulation. The results are the mean ± S.E. of at least three independent experiments. Statistical significance (*p < 0.05) was determined by a paired Student’s t test. ■, control; ▲, 3-h pretreatment. Panel B, control or methadone pretreatment effects on methadone inhibition of forskolin-stimulated cAMP accumulation. Monolayers were pretreated with methadone for 3 h at 37 °C and then prepared for cAMP accumulation experiments as described under “Experimental Procedures.” The results shown are the means ± S.E. of at least three separate experiments, all performed in duplicate. Symbols are as in panel A. Panel C, control or buprenorphine pretreatment effects on buprenorphine inhibition of forskolin-stimulated cAMP accumulation in μ-FLAG-expressing HEK 293 cells. Cells were either untreated or treated for 3 h with 1 μM buprenorphine and assayed for intracellular cAMP accumulation, as described under “Experimental Procedures.” The results are the means ± S.E. of three or more independent experiments, each performed in duplicate. Statistical significance was determined as in panel A. Symbols are as in panel A. Panel D, etorphine, methadone, and buprenorphine effects on morphine inhibition of forskolin-stimulated cAMP accumulation. μ-FLAG-expressing HEK 293 cells were pretreated with 1 μM etorphine (▲), methadone (●), or buprenorphine (■), and the dose-dependent inhibition of intracellular cAMP by morphine was measured. Each point represents the mean ± S.E. of at least three independent experiments, performed and assayed in duplicate.
Opioid agonist pretreatment (3hr) effects on forskolin stimulated cAMP levels in μ-FLAG expressing HEK 293 cells

**FIG. 7. Effects of opioid agonist pretreatment on compensatory changes in forskolin-stimulated cAMP accumulation.** μ-FLAG-expressing HEK 293 cells were plated in 24-well plates and incubated with agonist for 3 h at 37 °C. After a 30-min incubation with 0.5 mM isobutylmethylxanthine at 37 °C, cells were then incubated with 10 μM forskolin for 5 min at 37 °C followed by radioimmunoassay for intracellular cAMP levels. Data are expressed as fold stimulation where control forskolin-stimulated cAMP accumulation is defined as 1.0. The effects of levorphanol (Lev.), etorphine (Etor.), methadone (Meth.), and buprenorphine (Bup.) were found to be significantly different (p < 0.001) from either morphine or DAMGO, as determined by ANOVA with a Neuman-Keuls post-test (GraphPad Prism 2.01). Similarly, the results of morphine or DAMGO, in combination with the other opioids, were significantly different (p < 0.001) from the effects of morphine or DAMGO alone. All of the results presented are the mean ± S.E. of three or more separate experiments, except for morphine + etorphine and DAMGO + etorphine, which are the means of two experiments.

Rat μ receptors in the COS-7 transient expression system have demonstrated that chronic μ agonist exposure results in neither receptor down-regulation nor desensitization (6). Arden et al. (13) were able to demonstrate down-regulation and phosphorylation of the rat μ receptor in stably transfected HEK 293 cells; however, it is unclear whether the down-regulation event was accompanied by receptor desensitization. Chakrabarti et al. (28) stably expressed the rat μ receptor in a murine neuroblastoma cell line and examined the effects of morphine and DAMGO pretreatment on opioid receptor binding and adenylyl cyclase inhibition. The results of this study (28) indicated that both morphine and DAMGO were able to reduce the maximum number of receptor binding sites and, following extended treatment, diminished the maximal levels of opioid-mediated inhibition of cAMP. The differences in μ receptor regulation observed in the studies of Raynor et al. (6) and Arden et al. (13) suggest that agonist regulation of the μ receptor may be a function of the surrogate cell lines or of the species isoforms of the μ receptor. None of these studies (6, 13, 28) addressed the actions of buprenorphine or methadone on μ receptor regulation.

The results presented here demonstrate that the desensitization of the μ receptor by buprenorphine is independent of down-regulation or internalization of the receptor, since cell surface labeling of the receptor with a radiolabeled monoclonal antibody revealed no changes in antibody binding after buprenorphine treatment. The loss of high affinity agonist and antagonist binding after buprenorphine pretreatment may be due to the continued presence of the opioid on the receptor, with the continuous activation of the receptor resulting in functional desensitization. Such a notion would be consistent with previous studies that have demonstrated a slow off-rate for buprenorphine binding to the μ opioid receptor (10).

However, activation of the receptor is not the sole requirement for desensitization since morphine, DAMGO, and levorphanol are more potent and effective than buprenorphine in inhibiting forskolin-stimulated cAMP accumulation. Clearly, buprenorphine must stimulate the μ receptor to induce a cascade of cellular events resulting in a desensitization of the receptor which does not occur with these other opioid agonists. This may be due to buprenorphine binding differently to the μ receptor. Preliminary mutagenesis studies suggest that buprenorphine has different requirements for binding to the μ receptor than morphine, DAMGO, or levorphanol, since point mutations at key amino acids which abolish morphine, DAMGO, or levorphanol binding do not affect buprenorphine binding (29).

In the current study etorphine induced cellular responses distinct from the other opioids examined, as a 3-h pretreatment resulted in the down-regulation and complete desensitization of the μ receptor while also lowering the compensatory increases observed for forskolin-stimulated cAMP accumulation. This unique agonist profile may be due to the extremely potent effects of etorphine and may also reflect the lipophilic nature of this opioid, as membrane binding studies point to a component of etorphine binding which is essentially irreversible (30). The in vivo actions of etorphine, as assessed in animal and human behavioral studies, also indicate that etorphine is profoundly more potent than morphine by a factor of as much as 500–10,000-fold, depending upon the species tested and behavioral paradigm employed (18, 20). It has also been noted that although etorphine has a short duration of action in man, it is capable of suppressing abstinence in morphine-dependent subjects, suggesting that etorphine may be effective in the short term curbing of opioid withdrawal (20). However, etorphine has been reported as being highly addictive in rodents and primates (19) and has been reported as having abuse potential in man (20). Studies on the ability of etorphine to induce tolerance and addiction indicate that tolerance can occur in rats and monkeys, but no evidence of tolerance has been observed in humans (19). Thus, the unique and complex biochemical actions of etorphine observed in the current study are also mirrored by the unusual effects reported for in vivo behavioral studies in a variety of species.

Methadone pretreatment also desensitized the μ receptor and prevented the adaptive sensitization of forskolin-stimulated cAMP accumulation by morphine. As observed for buprenorphine, the desensitization appeared to be independent of receptor down-regulation. Methadone, like morphine, is a full agonist at the mouse μ receptor (9). The ability of methadone to desensitize the receptor may also be due to its ability to bind differently to the μ receptor than morphine, DAMGO, or levorphanol. Consistent with this notion, recent studies have delineated distinct domains of the rat μ receptor involved in the binding of μ-selective alkaloids and peptides (17, 31).

The inability of morphine and levorphanol to desensitize the μ receptor suggests that uncoupling of the receptor from adenylyl cyclase is unrelated to the tolerance development associated with these agonists. Both agonists induce dependence in rodents (1), yet only morphine causes an adaptive sensitization of adenylyl cyclase (21, 22). These results suggest that the enhanced stimulation of forskolin-stimulated cAMP accumulation is unlikely to be the biochemical basis for morphine addiction. A recent study on adaptive sensitization in HEK 293 cells has shown that the type VI isoform of adenylyl cyclase may be
involved, suggesting that in these cells the compensatory response occurs downstream of the receptor (32).

The ability of buprenorphine and methadone to desensitize the μ receptor functionally may be critical for the therapeutic efficacy of these opioids in the treatment of addiction. Morphine and heroin dependence may be a consequence of the continued activation of the μ receptor, with subsequent long term cellular changes in the nervous system. These prolonged neurochemical alterations may result from the inability of opioids such as morphine to desensitize the μ receptor. The therapeutic benefit derived from methadone or buprenorphine treatment may reflect the ability of these agents to interfere with the effects initiated by morphine, even in its continued presence, thereby interrupting a cascade of cellular events. However, methadone and buprenorphine are not opioid antagonists and do not precipitate withdrawal, thereby making them useful in the treatment of narcotic addiction. Furthermore, buprenorphine treatment does not appear to induce dependence (10) despite having agonist properties at the μ opioid receptor. Buprenorphine and morphine have been proposed to induce their in vivo analgesic action in animals through distinct cellular mechanisms (33). The therapeutic actions and long term consequences in man may also be due to distinct cellular actions.

Our findings show that chemically distinct opioid agonists can bind to the μ receptor and produce different cellular consequences. The studies presented here reveal that the addictive agents, such as morphine, and the opioids used in the treatment of addiction, buprenorphine and methadone, can be distinguished by different effects on μ receptor regulation. These studies provide a model system for understanding the molecular and cellular basis of dependence and tolerance, thereby facilitating studies on the design and development of new opioids that are potent analgesics but devoid of addictive properties.

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