Fentanyl and its Analogs Desensitize the Cloned Mu Opioid Receptor

GEORGE BOT, ALLAN D. BLAKE, SHUIXING LI and TERRY REISINE
Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania
Accepted for publication April 3, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
Fentanyl, and its structural analogs lofentanil and sufentanil, are potent analgesics used clinically in the management of pain. However, the high analgesic potency of these compounds is limited by the development of tolerance after chronic use. To investigate whether their tolerance development may be related to mu receptor desensitization, the cloned mouse mu receptor as well as mutant forms of the receptor were stably expressed in HEK 293 cells and tested for their response to continuous opioid treatment. Fentanyl and its analogs potentely bound to the mu receptor and effectively inhibited cAMP accumulation. Three-hour pretreatment of mu receptors with fentanyl and its analogs desensitized the mu receptor by uncoupling it from adenylyl cyclase. The fentanyl analogs caused a slight internalization of the mu receptor as accessed by antibody binding to the epitope-tagged mu receptor. Truncation of the mu receptor by removal of its carboxyl terminus at Glu234 did not affect the ability of the fentanyl analogs to bind to and activate the mu receptor nor did it prevent the fentanyl analogs from desensitizing the receptor. In a previous study we showed that morphine did not desensitize the cloned mu receptor even though it is a potent and effective agonist at the mu receptor. Mutagenesis studies revealed that morphine interacts differently with the mu receptor to activate it than do the fentanyl analogs which may explain its lack of desensitization of the mu receptor. These results indicate that desensitization of the mu receptor may be a molecular basis for the development of tolerance to fentanyl and its analogs.

Opioids induce a myriad of pharmacological actions and are used extensively in the management of pain. Although three major receptors mediate the effects of opioids, most of the opioids used clinically in pain management, such as morphine, methadone, fentanyl and codeine, have high affinity for the mu opioid receptor (Raynor et al., 1994). Since the potent synthetic analgesic fentanyl was reported in the early sixties (Janssen, 1965), the 4-anilidopiperidine class of opioids, structurally distinct from the amply studied morphine analogs, have been the subject of much interest. Some of these compounds were found to have an analgesic potency far greater than that of morphine (Janssen, 1982) and are used clinically as analgesics or analgesic/anesthetics. However, the high analgesic potency of these compounds is limited by the development of tolerance, dependence and respiratory depression. Despite the host of in vivo animal and in vitro receptor binding studies (Maguire et al., 1992; Raynor et al., 1994), little is known about the functional cellular consequence of fentanyl and its derivatives. Although both the morphine (morphanin) and fentanyl (4-anilidopiperidine) class of compounds are believed to induce most of their pharmacological actions by stimulating the opioid mu receptor, studies have suggested dissimilarities between the mechanisms involved in the antinociceptive effects of compounds such as fentanyl and its analogues and morphine. For example, antisense oligodeoxyribonucleotide against the subtype G12α protein antagonized morphine- but not sufentanil-induced antinociception (Raffa et al., 1994) and the ATP-sensitive K+ channel blocker glibidone antagonized the antinociception produced by morphine but not that induced by fentanyl and levorphanol (Ocana et al., 1995). This suggests that although these compounds may be interacting with the same opioid receptor type, the mu receptor, different intracellular effector mechanisms may be induced by them in producing their effects. Hence the molecular determinants of receptor recognition may be different than for receptor activation.

Studies of opioid signal transduction has relied upon the use of tumor cell lines expressing opioid receptors or brain homogenates (Costa et al., 1992; Maguire et al., 1992; Lambert et al., 1993). These preparations, however, generally contain a heterogeneous population of opioid receptors and hence do not lend themselves to the unequivocal characterization of actions at a specific receptor subtype. Recent clon-
ing of the opioid receptors has allowed the study of the pharmacology and biochemistry of these receptors in identifying the receptor domains involved in ligand binding and intracellular effects and thus has also offered a better understanding of opioid mechanisms with the promise of safer and more effective analgesic agents (Reisine and Bell, 1993; Reisine and Pasternak, 1996).

Tolerance development has been associated with both morphine and fentanyl treatment after prolonged administration (Paronis and Holtzman, 1992). Morphine has also been shown to desensitize the coupling of the cloned mu receptor to K⁺ channels (Blake et al., 1997b), uncoupling of which has been suggested to be a molecular basis of tolerance development to morphine. Although the mechanisms underlying tolerance/dependence are unknown, it has recently been correlated with modulation of adenylyl cyclase activity via G-protein transduction systems (Nestler et al., 1993). However, differential desensitization of mu opioid receptor mediated inhibition of cAMP accumulation in selected rat brain regions after chronic morphine administration has been reported, with regions such as the nucleus accumbens and caudate putamen exhibiting no change in contrast to the thalamus which exhibited desensitization (Noble and Cox, 1996). Similarly, morphine has been reported not to uncouple the cloned mu receptor from adenylyl cyclase (Blake et al., 1997a). This suggests that adaptive responses occurring during morphine administration are not identical in all opiate-sensitive neuronal populations and that morphine may selectively desensitize some, but not all, intracellular functions of the mu receptor.

In an effort to gain cellular insight into the structure/activity relationship for the fentanyl class of opioids we have stably expressed the mouse mu opioid receptor in human HEK 293 cells. We then investigated whether fentanyl, and its structural analogs (fig. 1) can regulate the cloned mouse mu receptor. We showed that fentanyl, sufentanil and lo芬坦il uncoupled the cloned mu receptor from adenylyl cyclase that distinguished this class of opioids from morphine that has been reported not to regulate mu receptor/adenylyl cyclase coupling (Blake et al., 1997a). Desensitization by the fentanyl analogs was not dependent on the presence of the C-terminus of the mu receptor indicating that posttranslational modification of the intracellular loops of the receptor may be involved in the desensitization process. The differential ability of fentanyl and morphine to desensitize the coupling of the mu receptor from adenylyl cyclase may be due to differences in the ability of these opiates to interact with and activate the mu receptor since mutation of the conserved Asp¹¹⁴ of the mu receptor to an asparagine abolished morphine stimulation but had minimal effects on fentanyl analog activation. These findings show that morphine and fentanyl interact differently with the same receptor to cause distinct adaptive responses that may be linked to long-term functional consequences associated with their use.

Methods

Cell culture. HEK 293 cells were grown and maintained in minimal essential medium with Earle’s salts (Life Technologies, Inc., Westbury, NY) containing 16% fetal calf serum, 100 U ml⁻¹ penicillin and streptomycin sulfate in 10% CO₂ 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 mouse mu opioid receptor, the D114N mutant and the carboxyl-terminal truncated mutant cDNA in the expression vector pCDNA3 (Invitrogen, Carlsbad, CA) were stably transfected into HEK 293 cells by a modification of the calcium phosphate protocol (Chen and Okayama, 1988). Briefly, HEK 293 cell monolayers at approximately 70% confluence were transfected with 30 μg of plasmid. After an overnight incubation at 37°C, 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 48 hr at 37°C in growth medium. Stable transformants were selected in growth medium containing 1 mg ml⁻¹ Geneticin (Life Technologies, Inc., Grand Is., NY) and maintained in T 75-cm² tissue culture flasks in 10% CO₂ at 37°C.

Mutagenesis of the cloned mu opioid receptor. The mouse mu opioid receptor cDNA was mutated using the Altered Site in vitro Mutagenesis system (Promega Corp., Madison, WI). To mutate aspartic acid 114 (in the putative second transmembrane domain) to an asparagine, the mu receptor cDNA was subcloned into pALTER and a single-stranded template was produced. The 21-mer oligonucleotide GCTAGGGCTTGGCCAGAGCA containing the desired mutation was annealed to the single-stranded template. The 21-mer oligonucleotide GCTAGGGCTTGGCCAGAGCA containing the desired mutation was annealed to the single-stranded template. After annealing, T4 DNA polymerase and T4 DNA ligase were employed for elongation and ligation. The heteroduplex DNA was used to transform the repair-minus Escherichia coli strain BMH 71-18 mut S. Transformants were selected by growth on LB plates containing 125 μg/ml ampicillin. The mutation was confirmed by dideoxyDNA sequencing and the cDNA was excised and subcloned into EcoRI-EcoRV site in the expression vector pCDNA3.

Generation of the carboxyl terminal truncation mu mutant. The mu opioid receptor cDNA was mutated using a commercially available in vitro mutagenesis system (Altered Sites, Promega Corp.). To produce the carboxyl terminal truncation mutant, an
ochre termination codon (UAA) was introduced by oligonucleotide directed mutagenesis at Glu$^{341}$, which is predicted to be at the interface of the putative seventh transmembrane region and intracellular carboxyl terminal tail of the receptor. The product cDNA was subcloned into pcDNA3 and sequenced to verify the presence of the stop codon at Glu$^{341}$.

**Radioligand binding studies.** Receptor binding studies were performed using membranes from stably transfected HEK 293 cells expressing the mu WT or D114N or mu-TRUNC mutant cDNA. Membranes were prepared and receptor binding studies conducted as described (Raynor et al., 1994) 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 EDTA, 5 mM NaCl, 10 mM $\mu$M leupeptin, 10 mM $\mu$M pepstatin and 200 $\mu$g/ml bacitracin 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 (Brinkman Instruments, Westbury, NY) at setting 2.5 for 30 sec. The cell homogenate was centrifuged at 48,000 $\times g$ for 20 min at 4°C and the resulting cell pellet was resuspended by homogenization 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 with 5 nM $[^3H]$-diprenorphine as radioligand and 1 $\mu$M diprenorphine to define nonspecific binding. The binding reaction was terminated by the addition of ice-cold 50 mM Tris-HCl (pH 7.8) and rapid filtration over FP-100 Whatman (Clifton, NJ) GF/B glass-fiber filters that were pretreated with 0.5% polyethyleneimine and 0.1% bovine serum albumin. The filters were rinsed with 12 ml of ice-cold buffer, and the bound radioactivity was determined using a liquid scintillation counter.

For agonist pretreatment studies, a 10-fold concentrated stock of agonist was diluted into growth medium and added to individual culture flasks. The cell homogenate was centrifuged at 48,000 $\times g$ for 20 min at 4°C and the resulting cell pellet was resuspended by homogenization 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 with 5 nM $[^3H]$-diprenorphine as radioligand and 1 $\mu$M diprenorphine to define nonspecific binding. The binding reaction was terminated by the addition of ice-cold 50 mM Tris-HCl (pH 7.8) and rapid filtration over FP-100 Whatman (Clifton, NJ) GF/B glass-fiber filters that were pretreated with 0.5% polyethyleneimine and 0.1% bovine serum albumin. The filters were rinsed with 12 ml of ice-cold buffer, and the bound radioactivity was determined using a liquid scintillation counter.

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.

**cAMP accumulation studies.** Stably transfected HEK 293 cells were subcultured in 12-well culture plates and allowed to recover for 72 hr before the experiments. For agonist pretreatment, the growth medium was replaced containing 1 $\mu$M ligand. After 3 hr pretreatment, the medium was removed and replaced with 1 ml of growth media containing 0.5 mM IBMX and the cells were incubated for 30 min at 37°C. The medium was then replaced and refreshed with fresh medium, with or without 10 $\mu$M forskolin and opioids and the cells transferred to 37°C. After 5 min the medium was removed, 1.0 ml of 0.1 N HCl was added and the monolayers 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 (GraphPad Software, Inc., San Diego, CA).

**Radiolabeling of the M2 monoclonal antibody.** The monoclonal antibody M2 against the FLAG epitope was purchased from New England Kodak (New Haven, CT). The antibody radioliodination was performed by a chloramine T procedure previously reported (Blake et al., 1997a). Briefly, 250 $\mu$g of M2 antibody were incubated in 200 mM NaPO$_4$ buffer (pH 7.3) with 0.5 mCi of Na$^{125}$I and the reaction initiated with 100 $\mu$l of chloramine T (0.5 mg ml$^{-1}$ in NaPO$_4$ buffer). After 30 sec at room temperature, the reaction was terminated by the addition of 100 $\mu$l of sodium metabisulfite (1.25 mg ml$^{-1}$ in NaPO$_4$ buffer). The iodinated protein was separated from free $[^3H]$ by column chromatography with Sephadex G-25, aliquots from the collected fractions were counted in a LKB $\gamma$-scintillation counter and then stored at 4°C.

**Antibody binding to cell monolayers.** After agonist treatment of cell monolayers, the cells were treated with 1.5% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature and then incubated for 30 min at 37°C in growth medium containing 10% fetal calf serum. After aspiration, approximately 200,000 cpm of $^{125}$I-M2 antibody in growth medium containing 10% fetal calf serum, was added to individual wells in 24-well plates. After a 30-min incubation at 37°C, the monolayers were washed in medium, solubilized with 0.5 ml of 1 N NaOH and bound radioactivity was counted in a $\gamma$-scintillation counter. Nonspecific radiolabeled antibody binding was determined in the presence of 10 $\mu$l FLAG peptide (DYKDDD DK; Eastman Kodak) and accounted for 20% or less of the total binding.

**Results**

To investigate the agonist regulation of the cloned opioid mu receptor, the wild-type CDNA and the mutant forms of the mu receptor that contained an aspartate to asparagine substitution at amino acid 114 (D114N) and the carboxyl-mutant truncated at Glu$^{341}$ (mu-TRUNC) were stably expressed in HEK 293 cells. Pharmacological characterization of the stably transformed cells was carried out, using radioligand binding and the functional inhibition of forskolin-stimulated cAMP accumulation, as previously described (Raynor et al., 1994; Blake et al., 1997a). Saturation binding with the radioligand, $[^3H]$-diprenorphine, demonstrated a dissociation constant ($K_d$) of 1.9 $\pm$ 0.2 nM and receptor density level (B$_{max}$) of 2.3 $\pm$ 0.1 pmol mg$^{-1}$ protein for the wild-type mu receptor and 1.3 $\pm$ 0.2 nM and 4.4 $\pm$ 1.6 pmol mg$^{-1}$ protein for the mu-TRUNC mutant and 1.3 $\pm$ 0.3 nM and 3.7 $\pm$ 0.6 pmol mg$^{-1}$ protein for the D114N mutant mu receptor. No specific radioligand binding was detected in untransfected HEK 293 cells (data not shown). These results were comparable to those reported in other surrogate cell lines (Raynor et al., 1994; Costa et al., 1992; Maguire et al., 1992) and in HEK 293 cells (Blake et al., 1997a). Although we have used the same transfected cells under the same conditions as in our previous study (Blake et al., 1997a), differences in cell batches may have contributed to the 3-fold lower B$_{max}$ value for the wild-type mu receptor obtained in our study. The analysis of competitive radioligand binding data with $[^3H]$-diprenorphine showed that fentanyl and its analogs, sufentanil and lofentanil, bound potently to the mu receptor with nanomolar affinities (table 1), comparable to those previously reported (Raynor et al., 1994).

**Studies on opioid receptors expressed in HEK 293 cells** have shown that these receptors are coupled to the inhibition of adenyl cyclase and to G proteins of the $G_i$ or $G_o$ family.

### Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\mu$-WT ($K_i$, nM ± S.E.)</th>
<th>$\mu$-TRUNC ($K_i$, nM ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>1.01 ± 0.4</td>
<td>1.04 ± 0.2</td>
</tr>
<tr>
<td>Lofentanil</td>
<td>0.14 ± 0.01</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Sufentanil</td>
<td>0.45 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>17.7 ± 2.1</td>
<td>14.6 ± 2.1</td>
</tr>
<tr>
<td>Levorphanol</td>
<td>5.7 ± 1.7</td>
<td>3.5 ± 1.1</td>
</tr>
</tbody>
</table>

Binding potencies of opioid ligands for HEK 293 cell membranes expressing the mu wild-type and mu-TRUNC mutant opioid receptors stably expressed in HEK 293 cells.
the forskolin-stimulated cAMP levels were 10.2 μmol cAMP/mg cellular protein, respectively. These forskolin-stimulated levels were obtained at the 1 μM concentration and is expressed as a percentage of the forskolin control. Both results represent the mean ± S.E. of at least three separate experiments, each performed and assayed in duplicate. Statistical significance (P < .05) was determined by a paired Student’s t test.

TABLE 2
Relative potencies of opioid agonists in inhibiting forskolin stimulated intracellular cAMP production for the cloned mouse μ-opioid receptor (μ-WT) and the D114N and μ-TRUNC mutants stably expressed in HEK 293 cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC_{50} (nM)</th>
<th>Max. inhibition (%)</th>
<th>EC_{50} (nM)</th>
<th>Max. inhibition (%)</th>
<th>EC_{50} (nM)</th>
<th>Max. inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphone</td>
<td>2.4 ± 1.1</td>
<td>71.7 ± 2.8</td>
<td>213. ± 72^a</td>
<td>18.3 ± 11.4^b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>0.15 ± 0.1</td>
<td>81.3 ± 1.9</td>
<td>0.8 ± 0.1^a</td>
<td>54.3 ± 0.3^a</td>
<td>0.53 ± 0.14</td>
<td>87.3 ± 3.0^a</td>
</tr>
<tr>
<td>Lofentanil</td>
<td>0.03 ± 0.01</td>
<td>82.0 ± 1.8</td>
<td>0.3 ± 0.1^a</td>
<td>74.7 ± 2.3</td>
<td>0.08 ± 0.1</td>
<td>84.7 ± 2.1</td>
</tr>
<tr>
<td>Sufentanil</td>
<td>0.3 ± 0.1</td>
<td>810 ± 1.0</td>
<td>0.5 ± 0.1</td>
<td>76.8 ± 2.4</td>
<td>0.002 ± 0.001^b</td>
<td>81.7 ± 2.6</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>1.5 ± 0.8</td>
<td>558 ± 5.6</td>
<td>3.1 ± 1.1</td>
<td>47.7 ± 4.6</td>
<td>0.14 ± 0.6^a</td>
<td>42.7 ± 6.4</td>
</tr>
<tr>
<td>Levorphanol</td>
<td>1.0 ± 0.1</td>
<td>790 ± 2.4</td>
<td>25.3 ± 8.1^a</td>
<td>42.5 ± 6.1^a</td>
<td>0.38 ± 0.15^a</td>
<td>77.7 ± 2.6</td>
</tr>
</tbody>
</table>

Inhibition of maximal cAMP accumulation was blocked by the mu selective antagonist naloxone. Naloxone (1 μM) significantly decreased (data not shown) the maximal inhibitory effects of the mu selective agonists fentanyl, lofentanil, sufentanil and nalbuphine. Previous investigations have shown that overnight treatment with pertussis toxin also decreased the maximal inhibitory capacity of the mu selective and nonselective agonists (Blake et al., 1997a) confirming that the cloned mu receptor coupled to adenyl cyclase via G_s and/or G_m proteins in the HEK 293 cells used in our study.

**Mu receptor desensitization.** Although μ agonists can inhibit cAMP accumulation, previous studies have indicated that prolonged treatment with morphine, levorphanol and DAMGO did not desensitize the μ receptor stably expressed in HEK 293 cells (Blake et al., 1997a). However, drugs used in the treatment of opioid addiction methadone and buprenorphine, did desensitize the μ receptor after a 3-hr pretreatment (Blake et al., 1997a). To further investigate the effects of agonist regulation on the cloned μ receptor, a 3-hr pretreatment with the agonists fentanyl, lofentanil, sufentanil and nalbuphine was used. This time period was based on previous studies indicating a 3-hr pretreatment period is sufficient to allow desensitization.

TABLE 3
Agonist (10^{-6} M) pretreatment (3 hr) effects on opioid inhibition of forskolin-stimulated CAMP levels for μ-WT opioid receptor expressed in HEK 293 cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC_{50} (nM)</th>
<th>Max. inhibition (%)</th>
<th>EC_{50} (nM)</th>
<th>Max. inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>0.15 ± 0.1</td>
<td>80.3 ± 2.4</td>
<td>4.6 ± 1.1^a</td>
<td>66.7 ± 3.8^a</td>
</tr>
<tr>
<td>Lofentanil</td>
<td>0.03 ± 0.01</td>
<td>80.7 ± 1.8</td>
<td>1000</td>
<td>0.0</td>
</tr>
<tr>
<td>Sufentanil</td>
<td>0.3 ± 0.1</td>
<td>81.0 ± 1.0</td>
<td>21.5 ± 6.1^a</td>
<td>25.0 ± 5.5^a</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>1.5 ± 0.8</td>
<td>58.0 ± 5.6</td>
<td>11.1 ± 5.7^b</td>
<td>38.3 ± 3.7^a</td>
</tr>
</tbody>
</table>

Effects of agonist pretreatment on opioid inhibition of cAMP accumulation for the μ-WT opioid-receptor. HEK 293 cell monolayers were either untreated (control) or treated (pretreated) for 3 hr at 37°C with 1 μM of appropriate ligand (fentanyl, lofentanil, sufentanil or nalbuphine). After 30-min incubation with 0.5 mM IBMX at 37°C, cells were then incubated with 10 μM forskolin and 1 μM of appropriate ligand for 5 min at 37°C and then assayed for intracellular CAMP levels as described in "Methods." The pretreatment results were compared to results for cells that did not undergo pretreatment. Maximal inhibition of forskolin-stimulated CAMP accumulation was that obtained at the 1 μM concentration and is expressed as a percentage of the forskolin control. For cells pretreated for 3 hr with fentanyl, lofentanil, sufentanil and nalbuphine, the forskolin-stimulated CAMP levels were 10.2 ± 0.9, 3.5 ± 0.6, 5.4 ± 0.5 and 8.8 ± 0.7 pmol CAMP/mg cellular protein, respectively. The corresponding control (nonpretreated) cells for each group were 7.1 ± 0.7, 5.4 ± 0.6, 5.9 ± 0.3 and 5.5 ± 0.6 pmol CAMP/mg cellular protein, respectively. These forskolin-stimulated levels were typically 5- to 20-fold more than basal CAMP values for each group. Both treated- and untreated results represent the mean ± S.E. of at least three separate experiments, each performed and assayed in duplicate. Statistical significance (P < .05) was determined by a paired Student’s t test.

^a P < .05 (Student’s t test, compared to untreated).
on previous studies examining effects of other drug treatments on the opioid mu receptor (Blake et al., 1997a). Time-course studies on the regulation of mu receptor by etorphine, buprenorphine and methadone revealed that maximum regulation occurs at 3 hr. For those drugs that have been found not to regulate the mu receptor in this time course, such as morphine and DAMGO, extended overnight pretreatments were also without any effect (Blake et al., 1997a).

Pretreatment of the mu receptor with fentanyl for 3 hr desensitized the mu receptor and caused a 30-fold reduction in the potency of fentanyl to inhibit cAMP accumulation (table 3; fig. 2, a and b). Lofentanil and sufentanil also desensitized the mu receptor with lofentanil treatment abolishing the coupling of the mu receptor to adenyllyl cyclase (table 3; fig. 3, a–c). The desensitization after fentanyl pretreatment was in marked contrast to the lack of desensitization reported for the mu receptor expressed in HEK 293 cells by morphine and DAMGO reported previously (Blake et al., 1997a). This suggests that drugs of abuse of the morphine class may have different intracellular adaptive effects following interaction with the mu receptor than those of the fentanyl class. The ability of the partial agonist nalbuphine to desensitize the mu receptor (table 3) was similar to the action induced by another partial agonist buprenorphine which also desensitized the mu receptor (Blake et al., 1997a).

The functional desensitization observed after agonist pretreatment may be due to reduced cell-surface receptor density induced by the agonist. To assess the effects of agonist pretreatment on receptor expression on the cell surface, an iodinated monoclonal antibody against the amino-terminal FLAG epitope was used. Binding of 125I-M2 antibody to the extracellular epitope FLAG amino terminus of the mu receptor would reflect presence of receptor whether the binding site is occupied or not. The extent of loss of receptors from the cell surface would be reflected in the reduction in mean 125I radioactivity. At present, the amino terminus of the mouse mu receptor is predicted to be an extracellular site not known to be directly involved in ligand binding (Surratt et al., 1994). Labeling was conducted in a similar manner as described by Keith et al. (1996) for the delta-receptor and Blake et al. (1997a) for the mu receptor.

Pretreatment with fentanyl and lofentanil caused a small internalization of the mu receptor (fig. 4). The internalization did not correlate with the magnitude of mu receptor desensitization because lofentanil, which abolished coupling of the mu receptor to adenyllyl cyclase, caused no greater magnitude of internalization than fentanyl that caused only a 17% reduction in maximal accumulation of adenyllyl cyclase (table 3). However, sufentanil, which dramatically desensitized the mu receptor, did not induce receptor internalization. These results suggest that the time course for desensitization and internalization for the mu receptor are not the same and appear to be agonist dependent. Furthermore, internalization and desensitization were not dependent on the potency of the agonists because levorphanol, which is as potent and effective an agonist as the fentanyl analogs in inhibiting cAMP accumulation, caused no desensitization nor internalization (figs. 4 and 5 in Blake et al., 1997a).

**Mu-TRUNC mutant.** For a number of G protein-linked receptors, the C-terminal region, which is rich in phosphate acceptor serine and threonine residues, has been proposed to be involved in receptor desensitization (Lohse, 1993). To investigate the role of the C-terminus of the mu receptor in desensitization, the receptor was truncated by engineering a stop codon in the receptor cDNA corresponding to amino acid residue 341, at the interface of transmembrane seven and the C-terminus. The truncated mu receptor (mu-TRUNC) bound fentanyl analogs as well as levorphanol and nalbuphine with similar affinities as did the wild-type mu receptor (table 1). Furthermore, both fentanyl and lofentanil were as potent and effective in inhibiting cAMP accumulation via the mu TRUNC mutant as via the wild-type mu receptor (table 2) indicating that the C-terminus of the mu receptor was not essential for coupling to adenyllyl cyclase. However, despite the similarity in binding potency, levorphanol exhibited a
small increase whereas sufentanil exhibited a large increase in potency compared to the wild-type mu receptor, suggesting an increase in their coupling to adenylyl cyclase via the mu TRUNC mutant (table 2). All three fentanyl analogs desensitized the mu-TRUNC receptor (table 4; fig. 5, a and b), with lofentanil and sufentanil analogs exhibiting a far greater effect on efficacy and potency than fentanyl, demonstrating that the C-terminus is not essential for these compounds for receptor regulation.

Nalbuphine is traditionally considered to be a mixed opioid agonist/antagonist (Reisine and Pasternak, 1996) with a low dependence profile and lower efficacy than morphine. Morphine pretreatment failed to desensitize both the wild-type mu receptor and the mu-TRUNC receptor (data not shown). However, unlike morphine (Blake et al., 1997a), nalbuphine desensitized the mu receptor causing a 7-fold affinity reduction and a 34% reduction in maximal inhibition of cAMP accumulation (table 3; fig. 6, a and b). The desensitization was not associated with internalization of the mu receptor (fig. 4). In contrast to the findings with the fentanyl analogs, nalbuphine did not desensitize the mu-TRUNC receptor (table 4; fig. 6c), indicating that desensitization by nalbuphine did require the presence of the C-terminus. These findings indicate that fentanyl analogs are likely to desensitize the mu receptor via a different mechanism than that mediating the effects of the mixed agonist/antagonist nalbuphine.

D114N mutant. The ability of fentanyl analogs to desensitize the mu receptor and the lack of mu receptor regulation by morphine and levorphanol reported previously (Blake et
al., 1997a) may be due to different interactions that these compounds may exhibit with the mu receptor that may cause their distinct cellular adaptive responses. To test whether these drugs have different determinants for activation, a point mutation was made in the second transmembrane domain of the mu receptor to change Asp^{114} to asparagine (D114N). Previous studies have shown that this conserved amino acid was necessary for morphine to bind to the mu receptor with high affinity and to activate the receptor to inhibit cAMP accumulation (Heerding et al., 1994; Surratt et al., 1994). Similarly, we found that morphine had a greatly reduced potency and efficacy in inhibiting cAMP accumulation via the D114N mutant (table 2) than the wild-type mu receptor. Similarly, levorphanol, which as morphine, has also been found not to desensitize the mu receptor (Blake et al., 1997a), also exhibited a marked reduction in its potency and efficacy to inhibit cAMP accumulation via the D114N mutant as reflected in a rightward shift of the dose-response curve (table 2; fig. 7).

In contrast, the fentanyl analogs were effective in inhibiting cAMP accumulation via the D114N mutant (fig. 8, a and b). Similarly, the ability of nalbuphine to stimulate the mu receptor to inhibit cAMP accumulation was not dependent upon the Asp^{114} residue (fig. 8c). These findings indicate that fentanyl analogs and nalbuphine, which desensitize the mu receptor in terms of inhibition of cAMP accumulation, interact differently with the mu receptor than do opioids such as morphine and levorphanol that do not desensitize the receptor.

Discussion

In our study we have stably expressed an epitope-tagged mouse mu receptor (Kaufman et al., 1995), a mutant mu receptor with substituted asparagine for the aspartate residue at position 114 (D114N) (Heerding et al., 1994; Surratt et al., 1994) and a mutant mu receptor lacking the carboxyterminal domain in HEK 293 cells and the functional activity of fentanyl analogs via these receptors were examined. The mouse mu receptor bound the fentanyl analogs with high affinity as well as the nonselective agonist levorphanol and the partial mu agonist nalbuphine.

Although morphine and fentanyl are used extensively in the management of pain, a major limitation to their clinical effectiveness is the development of tolerance. However, opioids are not all equal in their abilities to produce tolerance or to elicit responses in animals that have been rendered tolerant. For example, in a drug discrimination study, chronic morphine injections to rats induced tolerance to the analgesic effects of both morphine and fentanyl, but chronic administration of an equieffective dose of fentanyl did not produce tolerance (Paronis and Holtzman, 1992). Similarly, patients with cancer-related pain refractory to morphine did not exhibit tolerance to fentanyl or sufentanil (Paix et al., 1995). This suggests that although both morphine and fentanyl may interact with the mu opioid receptor, they may induce distinct types of biochemical cellular adaptations.

Our results suggest that the molecular basis for tolerance development to morphine and fentanyl may be different. Although the fundamental basis of tolerance is poorly understood, it may be linked to the desensitization of the receptors and their uncoupling from cellular effector systems (Lohse, 1993). Both the cloned delta and kappa receptors have been reported to desensitize after continuous agonist exposure, with an uncoupling from adenylyl cyclase and a reduction in the inhibition of cAMP accumulation (Blake et al., 1997b). However, mu receptor coupling to the inhibition of adenylyl cyclase has been found to be resistant to desensitization as up to 24 hr of pretreatment with morphine failed to uncouple the cloned mu receptor, stably expressed in HEK 293 cells, from adenylyl cyclase (Blake et al., 1997a). In contrast, fentanyl and its analogs lofentanil and sufentanil, desensitized the mu receptor after 3 hr pretreatment, greatly reducing the ability of the receptor to couple to adenylyl cyclase.

Studies of mammalian cell lines that endogenously express opioid receptors have focused on the role of receptor desensitization and down-regulation in the development of opioid tolerance (Nestler, 1992). To a large extent, receptor desensitization has been found to occur in the absence of significant receptor down-regulation and our results indicate that this is a likely mechanism by which fentanyl induces tolerance. The desensitization could involve an uncoupling of the mu receptor from G proteins linking the receptor to effector systems and/or internalization of the mu receptor as has been reported previously for etorphine acting upon the mu receptor (Blake et al., 1997a) and upon the delta receptor (Bot et al., 1997) expressed in HEK 293 cells. Our results suggest that the former mechanism is most likely to be involved in fentanyl desensitization because fentanyl, and its analogs, only induced a small internalization of the mu receptor and the internalization induced was not correlated with the magnitude of the desensitization induced by each compound. A
TABLE 4
Agonist (10⁻⁶ M) pretreatment (3 hr) effects on opioid inhibition of forskolin-stimulated cAMP levels for µ-TRUNC mutant opioid receptor expressed in HEK 293 cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>µ-TRUNC Untreated Cells</th>
<th>µ-TRUNC Agonist Pretreated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>Max. inhibition (%)</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>0.53 ± 0.14</td>
<td>87.3 ± 0.3</td>
</tr>
<tr>
<td>Lofentanil</td>
<td>0.08 ± 0.1</td>
<td>84.7 ± 2.1</td>
</tr>
<tr>
<td>Sufentanil</td>
<td>0.002 ± 0.001</td>
<td>81.7 ± 2.6</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>14.4 ± 3.0</td>
<td>42.7 ± 6.4</td>
</tr>
</tbody>
</table>

Effects of agonist pretreatment on opioid inhibition of cAMP accumulation for the µ-TRUNC mutant receptor. HEK 293 cell monolayers were either untreated (control) or treated (pretreated) for 3 hr at 37°C with 1 µM of appropriate ligand (fentanyl, lofentanil, sufentanil or nalbuphine). After 30 min incubation with 0.5 mM IBMX at 37°C, cells were then incubated with 10 µM forskolin and 1 µM of appropriate ligand for 5 min at 37°C and then assayed for intracellular cAMP levels as described in “Methods.” Both treated and untreated results represent the mean ± S.E. of at least three separate experiments, each performed and assayed in duplicate. Statistical significance (P < .05) was determined by a paired Student’s t test.

A: Dose-dependent fentanyl inhibition of forskolin-stimulated cAMP levels from (■) control and (▲) 1 µM fentanyl-pretreated cell monolayers. Monolayers were pretreated for 3 hr with 1 µM fentanyl at 37°C and the concentration-dependent effects of fentanyl on intracellular cAMP accumulation determined. B: Dose-dependent lofentanil inhibition of forskolin-stimulated cAMP levels from (■) control and (▲) 1 µM lofentanil-pretreated cell monolayers. Monolayers were pretreated for 3 hr with 1 µM lofentanil at 37°C and the concentration dependent effects of lofentanil on intracellular cAMP accumulation determined. The results represent the mean ± S.E. from three independent experiments, each performed and assayed in duplicate.

Although we have not addressed the issue whether desensitization to fentanyl analogs results in a cross-desensitization to other agonists such as morphine or nalbuphine, we have previously shown that cross-desensitization may be agonist dependent. We have measured the effects of buprenorphine pretreatment on the ability of etorphine to stimulate the mu receptor. Buprenorphine pretreatment has been reported to desensitize the mu receptor to further buprenorphine stimulation (Blake et al., 1997a). After such pretreatment, however, etorphine was still able to stimulate the mu receptor to inhibit cAMP accumulation (A. Blake, G Bot and T. Reisine, unpublished results). As different agonists appear to be dependent on different regions of the mu receptor for binding and induction of subsequent intracellular effects, desensitization to a particular agonist may, or may not, extend to other agonists.

Studies by Arden et al. (1995) have suggested that uncoupling of mu receptors from adenyl cyclase may involve phosphorylation of the receptor by protein kinases as the mu receptor contains sites for phosphorylation in the first and third intracellular domain and in the C-terminus (Reisine and Bell, 1993). In support of this notion, it has been shown recently that both chronic morphine and heroin increased protein kinase activity in the nucleus accumbens of rats (Self et al., 1995) and immunoblotting techniques have shown a decrease in protein kinase C levels in certain brain regions of heroin addicts (Busquets et al., 1995). Furthermore, opioid-inhibited protein phosphorylation mediated through adenyl cyclase in rat brain membranes has also been reported (Fleming et al., 1992). Truncation of the mu receptor to remove the serine- and threonine-rich C-terminus did not prevent mu receptor desensitization by fentanyl analogs, indicating that if phosphorylation is involved in mu receptor desensitization, then it must involve modification of residues in the intracellular loops of the receptor. The amino acid sequences of the intracellular loops of the three cloned opioid receptors are almost identical (Reisine and Bell, 1993), suggesting that if phosphorylation of the residues in these intracellular domains is involved in mu receptor desensitization, it may also be common to the other opioid receptors, the delta and kappa receptors, which have been demonstrated to undergo desensitization (Blake et al., 1997b).

Behavioral studies in humans and animals have suggested that nalbuphine is a mixed agonist/antagonist at opioid receptor.
ceptors, used in the treatment of mild to moderate pain, and can produce mu agonist or mu antagonist effects depending on the experimental procedure (Walker and Young, 1993; Zacny et al., 1997). Our study suggests that nalbuphine may be acting as a partial agonist at the mu receptor in that its effectiveness in inhibiting cAMP accumulation via the wild-type mu receptor was less than that exhibited by the full agonists such as fentanyl and morphine (Blake et al., 1997a). However, unlike morphine (Blake et al., 1997a), nalbuphine desensitized the wild-type mu receptor without any significant receptor internalization. This ability to desensitize may have contributed to the reported ability of nalbuphine, when coadministered with morphine, to prevent development of tolerance and dependence to the antinociceptive effects of morphine in rats (Lee et al., 1997). This suggests that partial agonists with this ability may be useful in extending the clinical efficacy of the more commonly used opiates such as morphine to which tolerance readily develops.

In contrast to fentanyl desensitization that did not require the C-terminus of the mu receptor, nalbuphine induced desensitization was dependent on the C-terminus. This suggests that although fentanyl and nalbuphine were able to desensitize the wild-type mu receptor, separate domains of the receptor are involved in their desensitization process and that they have different requirements for receptor regulation. The involvement of distinct regions of the mu receptor in agonist regulation points to the possibility of distinct biochemical mechanisms being responsible for the agonist-mediated desensitization of the receptor and perhaps in the induction of tolerance.

Differences in interaction with the mu receptor may also contribute to the variations in the abilities of morphine and fentanyl to desensitize the mu receptor. Previous studies have suggested that Asp$^{114}$ in the second transmembrane domain of the mu receptor was essential for morphine binding (Surratt et al., 1994; Heerding et al., 1994). Mutation of Asp$^{114}$ residue to an asparagine reduced the ability of morphine to inhibit adenyl cyclase activity to 25% of the wild-

![Fig. 6. Nalbuphine effects on forskolin-stimulated cAMP accumulation in mu-WT and mu-TRUNC mutant receptor expressing HEK 293 cells.](image-url)
type mu receptor expressed in COS cells (Surratt et al., 1994). Consistent with the results of Surratt et al. (1994), we found that morphine and levorphanol were ineffective in inhibiting cAMP accumulation via the D114N mutant. In contrast, sufentanil and nalbuphine were just as potent and effective in inhibiting cAMP accumulation via the D114 mutant as via the wild-type mu receptors. In general those compounds, fentanyl, sufentanil, lofentanil and nalbuphine, that desensitized the mu receptor, were not dependent upon the Asp^{114} residue for activation of the mu receptor to inhibit cAMP accumulation. The necessity of Asp^{114} for morphine and levorphanol to stimulate the mu receptor and the lack of its requirement for the fentanyl analogs and nalbuphine activation, indicates that these compounds have different determinants in the mu receptor for activation. By interacting with the mu receptor differently, the fentanyl analogs and nalbuphine may activate adaptive cellular responses that result in mu receptor/adenyl cyclase uncoupling. In contrast, morphine may not stimulate these cellular pathways, even though fentanyl and morphine both bind to the same receptor and are equally effective in inhibiting adenylyl cyclase.

The ability of the mu-TRUNC mutant receptor to effectively couple to adenylyl cyclase and bind agonists with high
affinity suggests that the C-terminus of the mu receptor is not essential for associating the receptor with G proteins to inhibit cAMP accumulation and suggests that the intracellular loops of the mu receptor may be more critical for G protein coupling. These results agree with others who have also shown that the C-terminus of the mouse delta receptor (Cvejic et al., 1996) and a partial truncation of the rat mu receptor (Surratt et al., 1994) were not essential for agonist inhibition of cAMP accumulation. The agonist bound mu receptor has been proposed to couple to a subset of subclasses of G proteins namely the G\textsubscript{i}, G\textsubscript{o}, and G\textsubscript{q} sub-classes (Prather et al., 1995; Standifer et al., 1996). Interestingly, all three opioid receptors are capable of interacting with these G proteins (Prather et al., 1995; Standifer et al., 1996) and the three opioid receptors have almost identical amino acid sequences in their intracellular loops (Reisine and Bell, 1993). The ability of the mu-TRUNC receptor to functionally couple to adenylyl cyclase suggests that the three opioid receptor, mu, delta and kappa, may act via similar molecular mechanisms to couple to cellular effector systems.

Differences in the affinity of the ligand-bound receptor for G proteins has been suggested to underlie the lower activity of partial agonists (Tota and Schimerlik, 1990). The lower efficacy of nalbuphine, compared to fentanyl, might result from its inability to induce or stabilize a conformational change (Kenakin, 1995) in the receptor to a state which associates with G proteins. Evidence that partial agonists induce similar receptor conformational changes to full agonists but with a reduced magnitude has been suggested from studies by Benovic et al. (1988). Differential activation of inhibitory G-protein \alpha-subunits by nalbuphine compared to fentanyl may also exist (Gettys et al., 1994). Recently it has been reported that, after binding to their receptors, opioids exhibit different efficacy and/or potency in the activation of different classes of G proteins (Garzon et al., 1997). Evidence also indicates that a single opioid receptor type can interact with several G proteins (Prather et al., 1995) which, in turn, can couple to more than one effector (Gintzler and Xu, 1991) and these may integrate coincident signals from different G-protein subtypes (Lustig et al., 1993). Different agonists may affect distinct arrays of G proteins. Hence, multiple G protein subunits are able to influence the actions of a single opioid agonist. This may contribute to their ability to desensitize or not the mu receptor and hence to their ability to induce tolerance.

Our results suggest that the agonist morphine interacts differently with the mu receptor to activate it than do agonists such as nalbuphine and the fentanyl analogs. This ability to desensitize, or not, may play a role in the degree of tolerance to the analgesic effects induced by a particular agonist and may be useful in the development of novel opioids with limited abuse potential after continued use.

Acknowledgments

The authors thank Mr. John C. Freeman for his expert technical assistance and Mr. John Hines for performing the original mutagenesis on the mu-receptor cDNA.


Send reprint requests to: Dr. George Bot, Dept. of Pharmacology, University of Pennsylvania School of Medicine, 36th St. and Hamilton Walk, Philadelphia, PA 19104.