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Opioid Regulation of the Mouse δ-Opioid Receptor Expressed in Human Embryonic Kidney 293 Cells

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SUMMARY

Opioid analgesics are used extensively in the management of pain. Although the clinically effective opioids bind with high affinity to the μ-opioid receptor, studies have suggested that the δ-opioid agonists might represent more ideal analgesic agents, with fewer side effects. A limitation to opiate effectiveness is the development of tolerance, an event that has been linked to opioid receptor desensitization. To gain a better understanding of δ-receptor agonist regulation, the cloned mouse δ receptor was stably expressed in human embryonic kidney 293 cells, and the functional effects of agonist pretreatment were examined. With a 3-hr pretreatment protocol, the δ-selective agonists [D-Pen^2,D-Pen^5]-enkephalin, [D-Ala^2,D-Leu^5]enkephalin, and [D-Ser^2,Leu^5]enkephalin-Thr and the nonselective opioids levorphanol, etorphine, and ethylketocyclazocine were found to desensitize δ receptors. [D-Pen^2,D-Pen^5]enkephalin, [D-Ser^2,Leu^5]enkephalin-Thr, [D-Ala^2,D-Leu^5]enkephalin, and etorphine treatments also caused a pronounced internalization of the epitope-tagged δ receptor, suggesting that the desensitization and internalization may be related. In contrast, levorphanol pretreatment did not internalize the receptor but still resulted in a 400-fold reduction in potency, suggesting that prolonged treatment with levorphanol only uncoupled the δ receptor from adenylyl cyclase. In contrast to the desensitization induced by peptide-selective δ agonists, pretreatment with the δ-selective nonpeptide agonist 7-spiroindanyloxymorphone and morphine sensitized the opioid inhibition of forskolin-stimulated cAMP accumulation. This differential regulation of the δ receptor may be due to variations in the ability of agonists to bind to the receptor. This hypothesis was supported by the finding that a point mutation that converted Asp128 to Asn128 (D128N) diminished the ability of δ-selective agonists to inhibit cAMP accumulation while increasing the potency of morphine to reduce cAMP accumulation. In particular, a lack of desensitization of the δ receptor by morphine may contribute to our understanding of the molecular basis of development of morphine-induced tolerance and dependence.

Since Serturner reported the isolation of a pure substance from opium, which he named morphine in 1805, morphine and its derivatives have been extensively used in the clinical management of pain (1, 2). Considerable evidence has accumulated that analgesia can be mediated via the three major classes of opioid receptors: the μ-, δ-, and κ-opioid receptors. All of the currently used opioid analgesics bind with high affinity to the μ-opioid receptor (3), but the clinical effectiveness of these opioids is limited by serious side effects, such as the development of tolerance and physical dependence (2). A primary goal of contemporary opioid research is the discovery of opioids that would provide effective analgesia devoid of unwanted side effects (4).

Several studies have suggested that δ receptor opioid agonists might represent a more ideal analgesic than those currently available because δ receptors have been proposed to mediate analgesia (5) with a diminished opioid dependence (6, 7), making these receptors a promising target for drug design. Behavioral studies have reinforced this notion. For example, the selective blockade of δ receptors by intracerebroventricular administration of the δ-selective antagonist naltrindole inhibited the development of morphine dependence in rats without compromising the antinociceptive actions of morphine (8). In addition, the δ receptor-selective antagonist H-Tyr-Ticψ(CH_2-NH)Phe-Phe-OH suppressed the development of morphine tolerance and dependence in rats, indicating that the activation of δ receptors may be critical in the development of morphine-induced tolerance and dependence (9).

Although the development of opioid tolerance is thought to be complex (10), one potential molecular component of opioid tolerance is receptor desensitization. To gain cellular insights into the role of δ receptors in opioid tolerance, *in vitro* models of δ-opioid receptor function have been studied. Recent research has focused primarily on NG108–15 cells,

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ABBREVIATIONS: DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr; EKC, ethylketocyclazocine; HEK, human embryonic kidney; SIOM, 7-spiroindanyloxymorphone; G_i, G protein-mediating inhibition of adenylyl cyclase; G_o, G protein-mediating stimulation of adenylyl cyclase.

which endogenously express the mouse δ receptor (11, 12), or on surrogate cell lines that were transfected with the cloned δ receptor cDNA (13). Acute and chronic opioid agonist treatment of NG108–15 cells produced δ receptor desensitization (11, 12), and studies in transfected mammalian cells correlated the receptor desensitization with β-adrenergic receptor kinase activity in a cellular model for G protein-coupled receptor regulation (13).

Although the cloned δ receptor was desensitized by the δ-selective agonist DPDPE (13) and tolerance to DPDPE was demonstrated in animals (6), peptides are not extensively used as analgesics. Currently, little is known about the consequences of the more commonly used opiates, such as morphine and methadone, on δ receptor function. Radioligand binding studies with δ receptor-expressing mammalian cell lines have demonstrated that chronic morphine treatment down-regulated δ receptors in a human neuroblastoma cell line (14) but not in NG108–15 cells (11). An acute treatment of δ- receptor stably transfected HEK 293 cells with the δ peptide DADLE caused receptor internalization, whereas receptor internalization was not observed with morphine (15, 16), indicating that morphine may be less able to regulate the δ receptor than δ peptide agonists.

In the current study, we investigated the regulation of the cloned δ-opioid receptor by selective and nonselective agonists and observed that pretreatment with etorphine and the δ receptor-selective peptides DSLET, DPDPE, and DADLE potently desensitized and internalized the expressed receptor. In contrast, pretreatment of δ receptor-expressing HEK 293 cells with the δ-selective nonpeptide SIOM, methadone, and morphine did not desensitization the δ receptor. This differential agonist regulation of δ receptor function may reflect the distinct requirements of these agonists for receptor activation, a notion supported by functional studies on the D128N δ receptor mutant. Our findings suggest that clinically used opioids, such as morphine, may act at the δ receptor, an action unlikely to contribute to the development of opioid tolerance.

Experimental Procedures

Cell culture. HEK 293 cells were grown and maintained in minimal essential medium with Earl's salts (Life Technologies, Grand Island, NY) containing 10% fetal calf serum and 100 units/ml penicillin/streptomycin sulfate in 10% CO_2 at 37°. The mouse δ-opioid receptor cDNA in pcDNA3 (InVitrogen, San Diego, CA) modified with the FLAG epitope (DYKDDDDK) at the amino terminus was a generous gift from Dr. Mark von Zastrow (University of California, San Francisco). The mouse δ-opioid receptor and the D128N mutant cDNA were stably transfected into HEK 293 cells by a modification of the calcium phosphate protocol (17). Briefly, HEK 293 cell monolayers at ~70% confluence were transfected with 30 μg of plasmid. After an overnight incubation at 37°, 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° in growth medium. Stable transformants were selected in growth medium containing 0.25 mg/ml geneticin (Life Technologies) for the δ wild-type and 1.0 mg/ml for the D128N mutant, and maintained in T 75-cm^2 tissue culture flasks in 10% CO_2 at 37°.

Mutagenesis of the cloned mouse δ-opioid receptor. The mouse δ-opioid receptor cDNA was mutated using the Altered Site *In Vitro* Mutagenesis System (Promega, Madison WI). To mutate Asp128 to an asparagine, the δ receptor cDNA was subcloned into pALTER, and a single-stranded template was produced. The 21-mer oligonucleotide (GCTCTCCATTAACTACTACAA) containing the desired mutation (GAC to AAC) was annealed to the single-stranded template, elongated with T4 DNA polymerase, and transformed into *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 dideoxy-DNA sequencing, and the cDNA was excised and subcloned into the *Eco*RI/*Eco*RV site in the expression vector pcDNA3.

Radioligand binding studies. Receptor binding studies were performed using membranes from stably transfected HEK 293 cells expressing the δ-FLAG or D128N mutant cDNA. Membranes were prepared and receptor binding studies were conducted as previously described (3) 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, with 1 mM EGTA, 5 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, and 0.5 μg/ml aprotinin and placed on ice. A cell pellet was prepared by centrifugation at 24,000 × *g* for 7 min at 4° and homogenized in the same buffer using a Polytron (Brinkmann Instruments, Westbury, NY) at setting 2.5, 30 sec. The cell homogenate was centrifuged at 48,000 × *g* for 20 min at 4°, and the resulting cell pellet was homogenized and placed on ice for the binding assays. Binding assays were carried out at 25° for 40 min in a final volume of 200 μl 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 μM. 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 experiments. For agonist pretreatment and pertussis toxin experiments, the growth medium was replaced for the times indicated in the table and figure legends with medium containing either ligand or pertussis toxin. Pertussis toxin treatments were carried out overnight at 37° with 100 ng/ml pertussis (List Biochemicals, Campbell, CA). After treatment, the medium was removed and replaced with 1 ml of growth media containing 0.5 mM, and the cells were incubated for 30 min at 37°. The medium was then removed and replaced with fresh medium, with or without 10 μM forskolin and opioids, and the cells were transferred to 37°. After 5 min, the medium was removed, 1.0 ml of 0.1 N HCl was added, and the monolayers were frozen at −20°. For determination of the cAMP content of each well, the monolayers were thawed, placed on ice, and sonicated, and the intracellular cAMP levels were measured by radioimmunoassay (Amersham plc, Buckinghamshire, UK). Data obtained from the dose-response curves were analyzed by nonlinear regression analysis with Prism 2 (GraphPAD Software, San Diego, CA)

Radiolabeling of the M2 monoclonal antibody. The monoclonal antibody M2 against the FLAG epitope was purchased from Eastman Kodak (New Haven, CT). The antibody radioiodination was performed by a chloramine T procedure previously reported (18). Briefly, 250 μg of M2 antibody was incubated in 200 mM $NaPO_4$ buffer, pH 7.3, with 0.5 mCi of $Na^{125}I$, and the reaction was initiated with 100 μl of chloramine T (0.5 mg/ml in $NaPO_4$ buffer). After 30 sec at room temperature, the reaction was terminated by the addition of 100 μl of sodium metabisulfite (1.25 mg/ml in $NaPO_4$ buffer). The iodinated protein was separated from free ^{125}I by column chromatography with Sephadex G-25; aliquots from the collected fractions were counted in a LKB γ-scintillation counter and then stored at 4°.

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° in growth medium containing 10% fetal calf serum. After aspiration, ~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°, the monolayers were washed in medium and solubilized with 0.5 ml of 1 N NaOH, and bound radioactivity was counted in a γ-scintillation counter. Nonspecific radiolabeled antibody binding was determined in the presence of 10 μM FLAG peptide (DYKDDDDK; Eastman Kodak) and accounted for ≤15% of the total binding.

Results

To investigate the agonist regulation of the cloned δ-opioid receptor, the wild-type cDNA and a mutant form of the δ receptor that contained an aspartate-to-asparagine substitution at amino acid 128 (D128N) were stably expressed in HEK 293 cells. Pharmacological characterization of the of the stably transfected cells was carried out using radioligand binding and the functional inhibition of forskolin-stimulated cAMP accumulation as previously described (3, 18). Saturation binding with the δ-selective antagonist [^{3}H]naltrindole demonstrated that the wild-type receptor was expressed in HEK 293 cells at the level of 17.3 ± 5.0 pmol/mg of membrane protein (B_{max}) with a dissociation constant of (K_D) of 0.29 ± 0.06 nM (n = 3). No specific radioligand binding was detected in untransfected HEK 293 cells (data not shown). Nontransfected HEK 293 cells do not appear to endogenously express any opioid receptors as suggested by the lack of [^{3}H]U69,593, [^{3}H]DAMGO, [^{3}H]diprenorphine, [^{3}H]naloxone, and [^{3}H]bremazocine binding, as previously reported (13, 18–20). The analysis of competitive radioligand binding data with [^{3}H]naltrindole showed that the expressed wild-type δ receptor had specific, high affinity binding for δ-selective ligands, with K_i values for DADLE, DPDPE, DSLET, deltorphin (II), met-enkephalin, SIOM, 7-benylidenenaltrexone, naltriben methanesulfonate, and naltrindole of 7.6, 34, 26, 14.5, 62, 68, 6.04, 0.16, and 0.41 nM (3 experiments for each compound), respectively. These values were comparable to those reported in other surrogate cell lines (3, 20–22) and in HEK 293 cells (23). The nonselective ligands bremazocine, etorphine, diprenorphine, and naloxone displaced [^{3}H]naltrindole with K_i values of 10.6, 26, 5.7, and 486 nM, respectively. The high affinity of naltriben methanesulfonate demonstrated that the expressed δ receptor had a pharmacology consistent with the δ_2-opioid receptor subtype (3).

Studies on opioid receptors expressed in HEK 293 cells have shown that these receptors are coupled to the inhibition of adenylyl cyclase and to G proteins of the G_i or G_o family (24–26). The cloned δ receptor expressed in HEK 293 cells was functionally active and mediated agonist inhibition of forskolin-stimulated cAMP accumulation (Fig. 1). The selective δ agonists DPDPE, DADLE and DSLET and the nonpeptide-selective δ agonist SIOM (27) effectively inhibited cAMP accumulation (Table 1). These results are comparable to previously published potencies for the δ-selective and nonselective agonists acting at the δ receptor to inhibit cAMP accumulation in the mouse NG108–15 hybrid cells (11, 12, 28) and in δ receptor-transfected CHO cell line (29, 30) and HEK 293 cells (16). Nonselective opioids such as EKC, etorphine, and bremazocine also inhibited cAMP accumulation, as did the clinically used opioids morphine, methadone, and levorphanol (Table 1). Lower maximal levels of cAMP accumulation for these nonpeptide compounds have been reported by others in CHO cells (31–34) transfected with δ receptors and in NG108–15 hybrid cells (11, 28). The extent

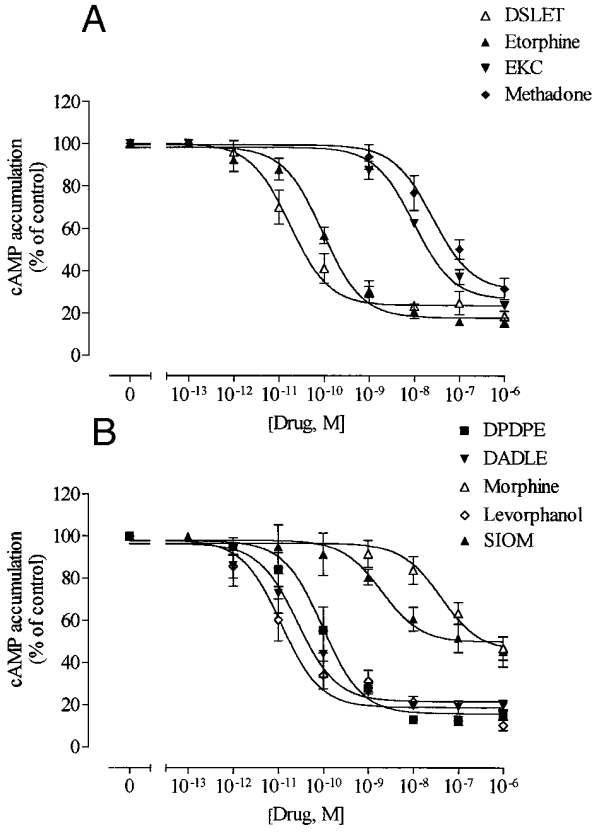

Fig. 1. Concentration-dependent inhibition of intracellular cAMP accumulation in δ-FLAG expressing HEK 293 cells by selective and nonselective δ agonists. A, △, DSLET; ▲, etorphine; ▼, EKC; ◆, methadone. (B) ■, DPDPE; ▼, DADLE; △, morphine; □, levorphanol; ▲, SIOM. Cell monolayers plated onto 12-well dishes were treated for 30 min at 37° with growth medium containing 0.5 mM isobutylmethylxanthine. After treatment, the medium was replaced with medium containing 10 μM forskolin and agonist over the concentration range of 10^{-12} to 10^{-6} M and incubated at 37° for 5 min, and cAMP accumulation was determined as described in the text. Intracellular cAMP levels were measured using a commercially available cAMP radioimmunoassay kit (Amersham). The inhibition of forskolin-stimulated cAMP accumulation is expressed as a percentage of the forskolin control. Intracellular cAMP levels of the cells incubated with forskolin alone served as controls (100%). Forskolin-stimulated cAMP levels were typically 5–20-fold higher than basal values. Basal levels were subtracted from the forskolin levels obtained. The dose-response curves were determined by computer analysis using GraphPAD Prism 2. The data presented are the mean ± standard error of three or more separate experiments, each performed in duplicate.

of maximal inhibition of morphine (maximal inhibition) (54.3 ± 4.9%, 11 experiments) and methadone (68.6 ± 5.0%, 10 experiments) compared with that of the nonselective agonists etorphine (84.4 ± 2.5%, seven experiments; Student's t test, $p < 0.01$, $p = 0.026$) and levorphanol (89.3 ± 2.0%, n = 7; $p < 0.01$, $p < 0.01$) suggests that morphine and methadone may have partial agonist activity at the δ receptor. Compared with the δ-selective peptide agonists, the maximal response elicited by SIOM at 1 μM ($p < 0.01$) suggests that it also was acting as a partial agonist (Table 1), although full agonist activity has been reported for SIOM in mouse vas deferens preparation (27).

Inhibition of maximal cAMP accumulation was blocked by the δ-selective antagonist naltrindole. Naltrindole (1 μM) significantly decreased the maximal inhibitory effects of the δ-selective agonists DPDPE and DSLET and the nonselective

TABLE 1

Relative potencies of opioid agonists in inhibiting forskolin-stimulated intracellular cAMP production for the cloned mouse δ-opioid receptor (δ-WT) and the D128N mutant and agonist (1 μM) pretreatment (3 hr) effects on opioid inhibition of forskolin-stimulated cAMP levels for the δ-WT stably expressed in HEK 293 cells

The ability of various opioid agonists to inhibit the forskolin-stimulated intracellular cAMP accumulation and the effects of agonist pretreatment on cAMP accumulation were determined. Cell monolayers were either untreated (control) or treated (pretreated) for 3 hr at 37° with 1 μM concentration of the appropriate ligand (DSLET, DPDPE, DADLE, SIOM, EKC, etorphine levorphanol, morphine, or methadone). After a 30-min incubation with 0.5 mM isobutylmethylxanthine at 37°, cells were incubated with 10 μM forskolin and 1 μM concentration of an appropriate ligand for 5 min at 37° and then assayed for intracellular cAMP levels as described in the text. The pretreatment results were compared with results for cells that did not undergo pretreatment. The EC_{50} values were determined by nonlinear regression computer analysis of the dose-response curves generated using GraphPAD Prism 2. 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. Both treated and untreated results represent the mean ± standard error of at least three separate experiments, each performed and assayed in duplicate. Statistical significance ($p < 0.05$) was determined by a paired Student's *t* test.

Ligand	δ-Opioid receptor/mutant					
	δ-WT		δ-WT agonist-pretreated cells		D128N mutant	
	EC_{50}	Maximum inhibition	EC_{50}	Maximum inhibition	EC_{50}	Maximum inhibition
	nM	*%*	*nM*	*%*	*nM*	*%*
DSLET	0.02 ± 0.01	81.5 ± 2.2	0.4 ± 0.1[a]	64.0 ± 2.1[b]	12.4 ± 7.4[a]	74.3 ± 1.5
DPDPE	0.09 ± 0.01	88.3 ± 1.0	2.0 ± 1.2[a]	73.5 ± 4.1[b]	171. ± 96[a]	40.3 ± 7.7[c]
DADLE	0.03 ± 0.01	80.3 ± 2.4	1.5 ± 0.5[b]	54.2 ± 9.6[a]	2.8 ± 0.8[b]	71.8 ± 5.2
Etorphine	0.10 ± 0.01	84.4 ± 2.5	0.7 ± 0.1[c]	78.3 ± 3.3	0.07 ± 0.01	87.0 ± 1.2
Levorphanol	0.01 ± 0.01	89.3 ± 2.0	4.4 ± 1.5[b]	96.3 ± 0.3[a]	0.12 ± 0.08	85.3 ± 2.9
SIOM	2.2 ± 1.7	54.7 ± 7.2	1.2 ± 0.2	85.0 ± 6.1[a]	69.8 ± 2.1[c]	68.0 ± 11.4
EKC	8.7 ± 1.3	76.3 ± 1.2	40.4 ± 11.2[a]	90.0 ± 0.6[c]	13.1 ± 6.7	75.3 ± 2.6
Morphine	38.0 ± 2.1	54.3 ± 4.9	12.2 ± 5.6[a]	80.2 ± 1.7[a]	0.02 ± 0.01[c]	72.3 ± 2.4[a]
Methadone	41.2 ± 8.6	68.6 ± 5.0	137 ± 212	80.0 ± 5.5	4.3 ± 1.3[a]	91.0 ± 0.6[a]

[a] $P < .05$ (Student's *t* test, compared with wild-type).
[b] $P < .01$.
[c] $P < .001$.

agonists levorphanol, etorphine, and EKC. Overnight treatment with pertussis toxin also decreased the maximal inhibitory capacity of DADLE, DPDPE, DSLET, and SIOM (Fig. 2), confirming that the cloned δ receptor couples, in part, to adenylyl cyclase via $G_{i\alpha}$ and/or $G_{o\alpha}$ in the HEK 293 cells used in the current study. The lack of full blockade by pertussis toxin suggests that the δ receptor may couple to adenylyl cyclase via non-pertussis toxin-sensitive G proteins. Recent studies have shown that the cloned δ receptor can associate with $G_{z\alpha}$ and $G_{q\alpha}$, which are insensitive to pertussis toxin (35).

Desensitization

δ-Selective agonists. Although δ agonists can inhibit cAMP accumulation, previous studies have indicated that prolonged agonist treatment can desensitize δ receptors (12, 13, 28). To further investigate the effects of prolonged agonist regulation on the cloned δ receptor, a 3-hr pretreatment with the agonists DPDPE, DSLET, and DADLE was used. The peptide δ agonists DPDPE, DSLET (Fig. 3), and DADLE desensitized the receptor and resulted in a rightward shift of the dose-response curve and a decrease in the maximal levels of cAMP inhibition (Table 1).

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

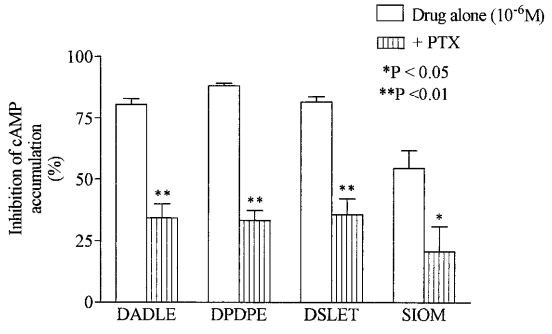

Fig. 2. Pertussis toxin effects on DADLE, DPDPE, DSLET, and SIOM inhibition of forskolin-stimulated cAMP accumulation. Cell monolayers were treated overnight with 100 ng/ml pertussis toxin. The next day, they were exposed to 10 μM forskolin and 1 μM concentration of the appropriate ligand for 5 min, and intracellular cAMP levels were assayed. The results presented are the levels of opioid inhibition relative to the forskolin-stimulated control and represent the mean ± standard error of at least three separate experiments, each performed and assayed in duplicate. Control plates in which the cells were not treated with pertussis toxin were included in each group of experiments.

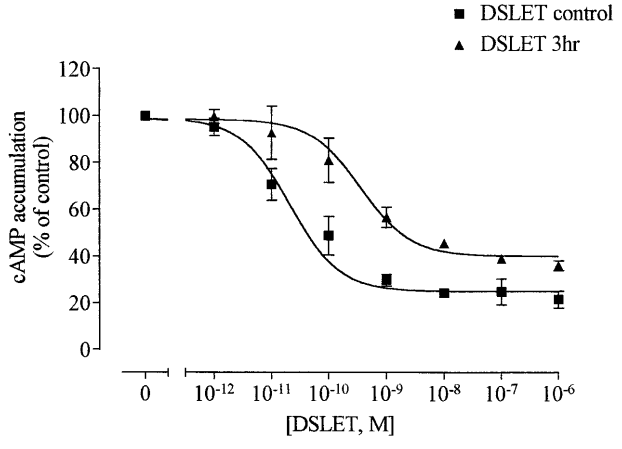

Fig. 3. DSLET desensitization of opioid agonist inhibition of forskolin-stimulated cAMP accumulation. Dose-dependent DSLET inhibition of forskolin-stimulated cAMP levels from (■) control and (▲) 1 μM DSLET-pretreated cell monolayers. Monolayers were pretreated for 3 hr with 1 μM DSLET at 37°, and the concentration-dependent effects of DSLET on intracellular cAMP accumulation were determined. The results represent the mean ± standard error from at least three independent experiments, each performed and assayed in duplicate. Statistical significance ($p < 0.05$) was determined by a paired Student's *t* test.

pretreatment on receptor expression on the cell surface, an iodinated monoclonal antibody against the amino-terminal FLAG epitope was used. Binding of ^{125}I-M2 antibody to the extracellular epitope FLAG amino terminus of the δ receptor would reflect presence of receptor regardless of whether the binding site is occupied. The extent of loss of receptors from the cell surface would be reflected in the reduction in mean ^{125}I radioactivity. At present, the amino terminus of the mouse δ receptor is predicted to be an extracellular site not known to be directly involved in ligand binding (17, 20, 33).

Pretreatment with DADLE, a membrane-impermeable ligand, caused internalization of the δ receptor (Fig. 4). Labeling was conducted in a similar manner as described by Keith *et al.* 1996 (16) for the δ receptor and in a similar manner as described by Blake *et al.* 1997 (18) for the μ receptor. As shown in Fig. 4A, DADLE pretreatment caused a 79% loss of cell surface labeling. Internalization was time dependent, with half the receptor population internalized ~30 min after treatment (Fig. 4B). Similar results were reported by Keith *et al.* (16). The results suggest that the desensitization induced by DADLE pretreatment may be due, in part, to internalization of the receptor. DPDPE also internalized the δ receptor with a similar time course as DADLE, whereas DSLET caused a slightly slower internalization (Fig. 4). The different time course of DSLET compared with that of DADLE and DPDPE cannot be due to variation in potency or efficacy to activate the receptor because these three peptides exhibited similar EC_{50} values and maximal inhibitory capacities (Table 1).

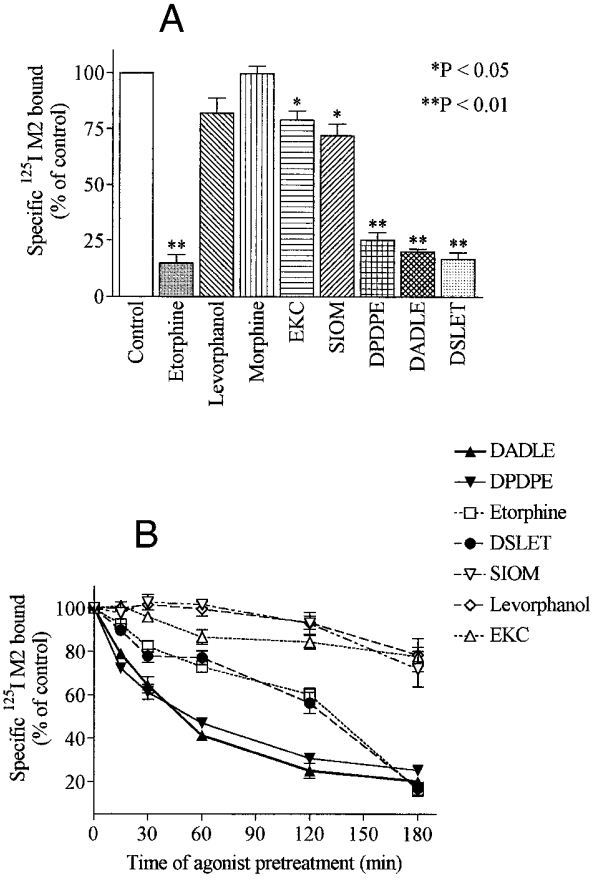

Fig. 4. Agonist pretreatment effects on ^{125}I-M2 monoclonal antibody binding to membranes prepared from HEK 293 cells stably expressing the δ-FLAG cDNA. A, Time course kinetics of receptor internalization. B, Cumulative effects at 3 hr. HEK 293 cells were plated onto 24-well plates and pretreated with the appropriate agonist for the times indicated at 37°. After agonist treatment, the cell monolayers were processed as described in the text. Total ^{125}I-M2 binding was in the range of 9646 ± 507 cpm for untreated cells; nonspecific binding, determined in the presence of 10 μM FLAG peptide, was 10–15% of the total bound counts. The results are presented as percent of untreated control monolayers and are the mean ± standard error of at least three experiments. Statistical significance was determined by paired Student's *t* test with significance defined as $p < 0.05$.

In contrast to the peptide δ-selective agonists, the newly developed nonpeptide, δ-selective agonist SIOM did not desensitize the δ receptor after a 3-hr pretreatment (Fig. 5). In fact, SIOM pretreatment caused a sensitization of the δ receptor in that after pretreatment, SIOM increased the maximal inhibition of cAMP accumulation compared with control [85.0 ± 6.1 (three experiments) versus 54.7 ± 7.2 (three experiments), $p = 0.033$]. SIOM caused a small internalization of the δ receptor that was apparent only after 3 hr of pretreatment (Fig. 4).

Nonselective agonists. A number of opioid agonists have been reported to exhibit high affinity at δ receptors but are nonselective because they also interact potently with other opioid receptors. To determine whether they regulate the δ receptor in a similar manner as the selective agonists, a series of potent opioids were tested for their ability to desensitize or internalize the δ receptor. Pretreatment with levorphanol, etorphine, and EKC caused a significant desensitization of the δ receptor, resulting in increased EC_{50} values (Tables 1). The maximal inhibitory capacities of levorphanol and EKC were increased after pretreatment, whereas that of etorphine was unaffected (Table 1). The desensitization exhibited by etorphine was agonist specific in that it did not cross-desensitize the effects of morphine; instead, it shifted the EC_{50} value of morphine to a lower concentration (EC_{50}: control, 38.0 ± 2.1, 11 experiments; treated, 0.9 ± 0.2, three experiments; $p < 0.001$, Student's *t* test to control) (Table 1).

Etorphine caused 85% of δ receptors to internalize (Fig. 4) with a similar time course as that induced by DSLET. These results are similar to those reported by Keith *et al.* 1996 (16). Despite the large internalization induced by etorphine, the maximal capacity of etorphine to inhibit cAMP accumulation was not attenuated in pretreated cells (Table 1), suggesting

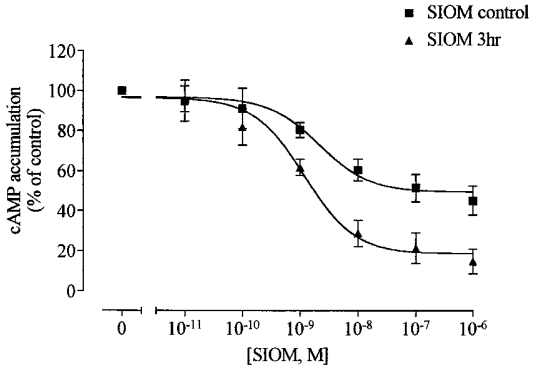

Fig. 5. Dose-dependent SIOM inhibition of forskolin-stimulated cAMP levels from (■) control and (▲) 1 μM SIOM-pretreated cell monolayers. Monolayers were pretreated for 3 hr with 1 μM SIOM at 37°, and the concentration-dependent effects of SIOM on intracellular cAMP accumulation were determined. The results represent the mean ± standard error from three independent experiments, each performed and assayed in duplicate.

that a significant proportion of cloned δ receptors expressed in HEK 293 cells are "spare receptors."

Levorphanol pretreatment did not cause a significant internalization of δ receptors (Fig. 4), but it did cause a 400-fold reduction in agonist potency (Table 1). This suggests that levorphanol pretreatment may primarily uncouple δ receptors from intracellular effector systems such as adenylyl cyclase rather than induce receptor internalization. Because levorphanol was as potent and efficacious in inhibiting cAMP accumulation as were DSLET and DPDPE (Fig. 1), different modes of cellular regulation induced by these agonists may be related to variations in how they bind to or activate the cloned δ receptor. This differential binding may activate different classes of G proteins and hence different intracellular effector systems, as have been suggested by others (35).

In contrast to the peptide-selective δ agonists and some nonselective alkaloids, the clinically used opiates morphine and methadone did not desensitize the δ receptor (Fig. 6, A and B). Morphine pretreatment caused an increase in maximal inhibitory capacity (maximal inhibition: control, 54.3 ± 4.9%, 11 experiments; pretreated, 80.2 ± 1.7%, three experiments, $p = 0.020$ Student's t test to control) and a leftward shift in the dose-response curve for morphine (EC_{50}: control, 38.0 ± 2.1%, 11 experiments; treated, 12.2 ± 5.6%, three experiments, $p < 0.001$, Student's t test to control) suggesting that morphine may have sensitized the δ receptor. Morphine pretreatment did not internalize the δ receptor (Fig. 4). Similar results have been reported by Keith *et al.* (16). Methadone pretreatment failed to affect significantly the response of the δ receptor in HEK cells to either methadone or morphine (data not shown).

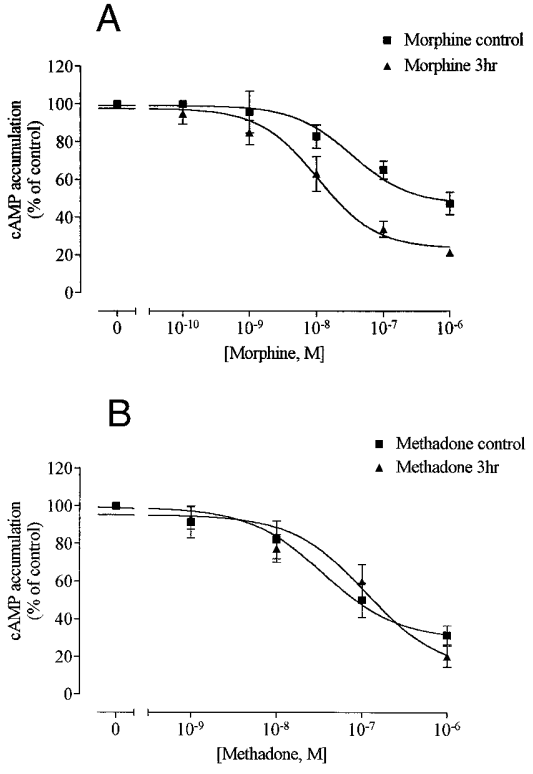

Fig. 6. Effects of morphine and methadone on forskolin-stimulated cAMP accumulation. A, Dose-dependent morphine inhibition of forskolin-stimulated cAMP levels from control (■) and 1 μM morphine-pretreated (▼) cell monolayers. Monolayers were pretreated for 3 hr with 1 μM morphine at 37°, and the concentration-dependent effects of morphine on intracellular cAMP accumulation were determined. B, Dose-dependent methadone inhibition of forskolin-stimulated cAMP levels from control (■) and 1 μM methadone-pretreated (▲) cell monolayers. Monolayers were pretreated for 3 hr with 1 μM methadone at 37°, and the concentration-dependent effects of methadone on intracellular cAMP accumulation were determined. The results represent the mean ± standard error from three independent experiments, each performed and assayed in duplicate.

D128N mutant. The differential regulation of the δ receptor by various agonists could be due to differences in the molecular interaction of these agonists with the δ receptor. In a previous study (21), the charged aspartate at residue 128 (Asp128) was reported to be critical for the binding of peptide δ agonists. Mutation of Asp128 to asparagine resulted in a receptor with greatly reduced affinity for DADLE and DPDPE as well as selective nonpeptide agonists such as BW 373U86 [(±)-4-[(α-*R**)-α-[(2*S**,5*R**)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide]. These findings showed that the Asp128 of the δ receptor has an important role in selective ligand recognition.

To further investigate whether differences in agonist regulation of the δ receptor are due to variations in agonist binding, a series of opiates were tested for binding and function to the same mutant receptor as described by Befort *et al.* (21). The mutant δ receptor, with asparagine at residue 128 (D128N), was stably expressed in HEK 293 cells. Saturation binding analysis with the opioid antagonist [^{3}H]naltrindole revealed that the B_{max} and K_D values for binding to the D128N mutant were 236.4 ± 30.3 fmol/mg of protein (three experiments) and 1.45 ± 0.17 nM, respectively. These results indicate that the mutant δ receptor was expressed at a lower density than the wild-type. Consistent with the results of Befort *et al.* (21) showing that the D128N mutant had lower affinity for δ-selective agonists, DADLE, DPDPE, SIOM, and DSLET were less potent in inhibiting cAMP accumulation in D128N mutant δ receptor-expressing HEK 293 cells than in cells expressing the wild-type δ receptor (Table 1, Fig. 7). Except for DPDPE, all of the selective agonists had similar maximal inhibitory effects on cAMP accumulation via the D128N and wild-type δ receptors but exhibited decreased potency, suggesting that Asp128 is critical for the affinity of the receptor for these agonists.

In contrast, the nonselective opioids etorphine, levorphanol, and EKC had similar potencies and efficacy to inhibit cAMP accumulation in HEK 293 cells expressing the wild-type and D128N mutant δ receptors (Table 1). This suggests the lower expression of the D128N mutant is not the cause of the reduced potency of the δ-selective ligands at the receptor and Asp128 is not critical for these nonselective agonists to activate the δ receptor. These findings indicate that levorphanol has different requirements for activating the δ receptor than the peptide-selective agonists, which may explain the ability of levorphanol to desensitize the δ receptor without causing internalization, in contrast to the effects of the selective peptide agonists, which caused internalization and desensitization.

In contrast to the δ-selective peptides, the clinically used opiates morphine and methadone were more potent and effective in inhibiting cAMP accumulation in cells with the D128N mutant than the wild-type δ receptor (Table 1, Fig. 8). The enhanced functional responses were mirrored by a higher receptor binding affinity of the D128N mutant for the

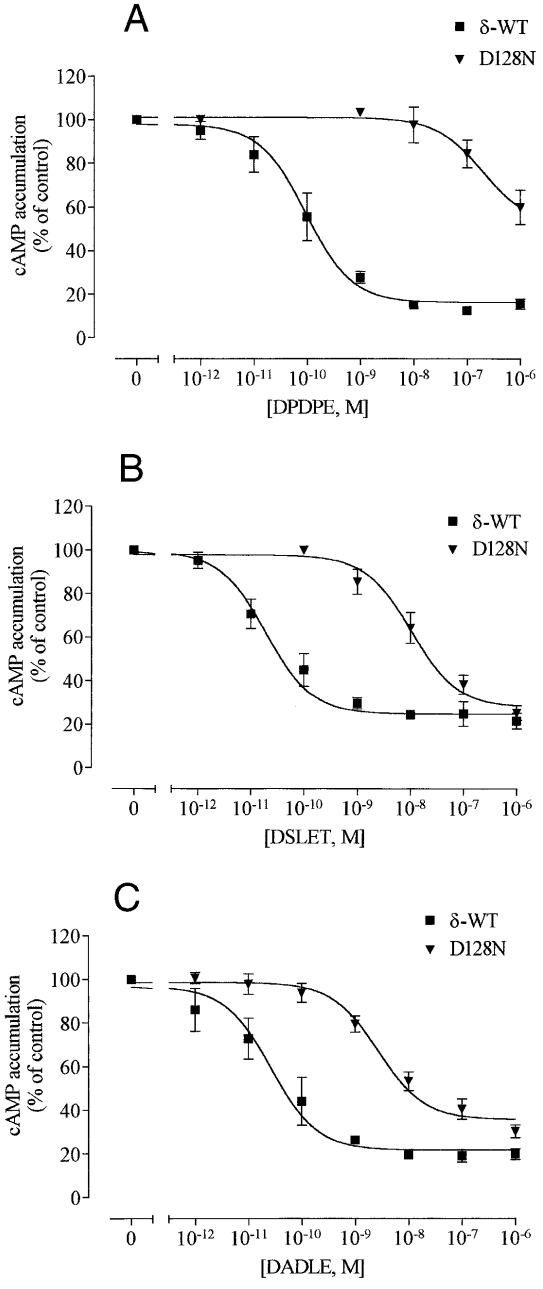

Fig. 7. Effects of selective δ agonists (A) DPDPE, (B) DSLET, and (C) DADLE on inhibition of forskolin-stimulated cAMP accumulation for the (■) δ wild-type and (▼) D128N mutant opioid receptor. Stably transfected HEK 293 cells were plated onto 12-well plates, and intracellular cAMP accumulation was assayed as described in the text. The dose-response curves, over the concentration range of 10^{-12} to 10^{-6} M, were determined by computer analysis using GraphPAD Prism 2. The data presented are the mean ± standard error of three or more separate experiments, each performed in duplicate.

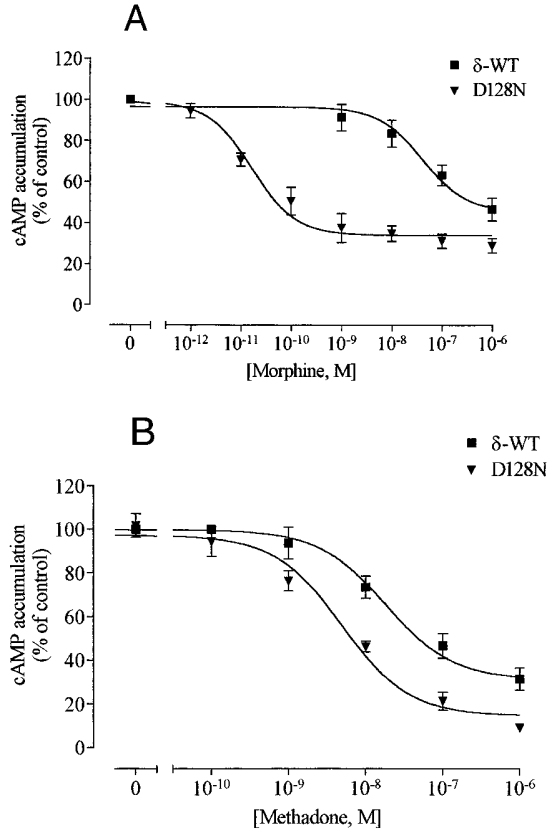

Fig. 8. Effects of morphine and methadone on inhibition of forskolin-stimulated cAMP accumulation for the (■) δ wild-type and the (▼) D128N mutant opioid receptor. A, Dose-dependent morphine inhibition of forskolin-stimulated cAMP levels. B, Dose-dependent methadone inhibition of forskolin-stimulated cAMP levels. Cell monolayers plated onto 12-well dishes were treated for 30 min at 37° with growth medium containing 0.5 mM isobutylmethylxanthine. After treatment, the medium was replaced with medium containing 10 μM forskolin and agonist over the concentration range of 10^{-12} to 10^{-6} M, incubated at 37° for 5 min, and then assayed for intracellular cAMP levels. The inhibition of forskolin-stimulated cAMP accumulation is expressed as a percentage of the forskolin control. Intracellular cAMP levels of the cells incubated with forskolin alone served as controls (100%). Forskolin-stimulated cAMP levels were typically 5–20-fold higher than basal values. Basal levels were subtracted from the forskolin levels obtained. The dose-response curves were determined by computer analysis using GraphPAD Prism 2. The data presented are the mean ± standard error of three or more separate experiments, each performed in duplicate.

agonists than the wild-type receptor [K_i (nM), wild-type versus D128N mutant, morphine >1000 versus 2.3; methadone >1000 versus 0.22]. These results indicate that Asp128 in the δ receptor is critical for the functional activity of δ-selective peptide agonists but not for morphine, methadone, EKC, and etorphine, demonstrating that these opioids interact differently with the cloned δ receptor than with selective peptide δ agonists.

Discussion

In the current study, the cloned mouse δ-opioid receptor (32, 34) and a mutant with substituted asparagine-for-aspartate residue at position 128 (D128N) (21) were stably expressed in HEK 293 cells, and the functional activity with selective and nonselective agonists was examined. A significant finding of the current study was the differential agonist regulation of the mouse δ-opioid receptor function in HEK 293 cells. A range of δ-selective peptide (DPDPE, DADLE, and DSLET) and nonpeptide, SIOM, and nonselective opioid agonists (levorphanol, EKC, and morphine, methadone) inhibited forskolin-stimulated cAMP accumulation. This functional activity was pertussis toxin sensitive, suggesting involvement of G_i/G_o family of G proteins. The coupling of δ receptors to the G_i or G_o class of proteins is consistent with immunoprecipitation studies on other surrogate cell lines

that have been used to stably express the cloned opioid receptors (35). However, involvement of non-pertussis toxin-sensitive G proteins was also suggested by the lack of full blockade by pertussis toxin. This supports reports of coupling of pertussis toxin-resistant G proteins with δ receptors (35).

Pretreatment of cell monolayers with the peptide δ agonists DPDPE, DSLET, and DADLE, desensitized the cloned δ receptor. They also internalized the receptor, consistent with the findings of Keith *et al.* (16), suggesting that desensitization to these agonists may involve both an uncoupling from adenylyl cyclase and an internalization of the receptor.

The nonpeptide δ-selective agonist SIOM was recently developed (27) in an endeavor to improve the stability and bioavailability of δ-selective agonists, which, till recently, have been based on structural analogs of traditional opioid peptides with limited success. This nonpeptide agonist may be useful in elucidating the pharmacological function of δ-opioid receptors *in vivo* (see introductory paragraphs). In the current study, we observed that SIOM acted as a partial agonist compared with δ-selective peptide agonists and did not desensitize the cloned mouse δ receptor. This was in contrast to the peptide δ-selective agonists and to the nonselective agonists etorphine and levorphanol. This partial agonist characteristic, combined with its persistent, nondesensitizing effect, may offer an advantage of this compound over the more commonly used nonpeptide δ-selective agonists in that it may have a different affect on intracellular effector systems, which have been implicated in opioid tolerance and dependence development, and hence have more prolonged therapeutic actions. Limited studies have reported that SIOM was 7 times more potent in antinociceptive tests in mice than DPDPE (27), possibly because of its more prolonged activation of the δ receptor.

In addition to SIOM, the clinically used opiates, morphine and methadone also did not desensitize the δ receptor. In fact, a slight sensitization of morphine inhibition was observed. This sensitization did not extend to methadone, a drug used for the treatment of opiate addiction, suggesting that the actions of morphine and methadone at the δ receptor may be different. This difference could perhaps contribute to the ability of methadone to substitute for morphine in morphine-tolerant rats and humans (36, 37). Although morphine and methadone are generally accepted as μ-preferring agonists, they also interact with δ-opioid receptors (38, 39). The significant difference between the extent of maximal inhibition by levorphanol and etorphine and that of morphine and methadone ($p < 0.05$) suggests that morphine and methadone may have partial agonist activity at the δ receptor. This property of morphine as a partial agonist at the δ-opioid receptor has also been identified in δ-opioid receptor-expressing NG108–15 cells (11) and in stably transfected CHO cells (30, 32). The partial agonist property of these compounds may explain their inability in our study to desensitize δ receptor.

Chronic exposure to opiates has been shown to elicit adaptations in some of the same intracellular pathways, such as the cAMP pathway, that mediate the acute actions of the drugs. Moreover, some adaptations, such as an up-regulation of the cAMP pathway, have been related to tolerance and dependence phenomena that have been demonstrated at the level of individual neurons (10, 40). Opioid treatment has also been reported to desensitize the opioid receptors on phosphorylation by G protein receptor kinases (e.g., β-adrenergic receptor kinase; Ref. 13), and β-adrenergic receptor kinase 1 up-regulation has been reported after chronic morphine treatment (10). However, the resistance of the δ receptor to morphine desensitization suggests that morphine could cause long term activation of the δ receptor. Hence, some of the effects of morphine may be partially mediated by the δ receptor, especially after chronic administration. Several studies have demonstrated that repeated administration of opiates produces a progressive sensitization to the locomotor-activating and -reinforcing effects of these opiates (40). Perhaps chronic morphine treatment sensitizes the δ receptor to further morphine treatment but may not affect the action of other agonists, such as methadone, that are used to treat morphine tolerance and addiction. Furthermore, because the δ receptor has been implicated in mediating a number of diverse functions, morphine, methadone, or a compound with similar partial activity could serve as a prolonged activator of the δ receptor system required for functional continuity.

Mutagenesis studies on the δ receptor have revealed a conserved aspartate at residue 128 as being involved in ligand binding of peptide agonists. We found that the activation of the D128N mutant by agonists was varied and type dependent. Consistent with previous reports, we found that peptide agonists were less potent in activating the D128N mutant in comparison to the wild-type δ receptor. However, morphine and methadone were more potent in binding to and stimulating the D128N mutant than the wild-type receptor, and etorphine, levorphanol, and EKC were equipotent. Hence, the D128N was not essential for these compounds to inhibit cAMP accumulation via the δ receptor. This indicates that the former agonists have different requirements for activation of the δ receptor than δ-selective agonists DADLE, DSLET, DPDPE, and SIOM, which were much less potent in inhibiting cAMP accumulation via the D128N mutant than the wild-type receptor. Although the Asp128 moiety is essential for δ-selective agonists to bind to and/or activate the δ receptor, this residue is unlikely to be part of the ligand binding pocket because the surrounding amino acid sequences are similar among the μ-, δ-, and κ-opioid receptors and δ-selective agonists do not bind to μ or κ receptors. The residue may, however, contribute to the binding affinity of δ agonists by contributing to the stabilization of the spatial conformation of the binding pocket. It may also affect the rates of association/disassociation by providing the negative counterion for the positively charged nitrogen found in many opioid ligands and take part in ligand/receptor electrostatic interaction. Furthermore, the residue may be critical for the partial agonist activity of SIOM observed at this receptor because SIOM acted as a full agonist at the D128N mutant. The distinct manner by which these agonists activate the receptor and induce changes in intracellular effector systems may be linked to their different abilities to desensitize the δ receptor.

The mechanism of δ receptor desensitization induced by peptides has been reported to involve receptor phosphorylation and an uncoupling of the receptor from G proteins via a β-adrenergic receptor kinase (13). It also may involve internalization of the receptor, particularly in response to the δ agonist DADLE (15). The potent and selective agonists such as DSLET, DPDPE, and DADLE desensitized and internalized the δ receptor. Hence for these agonists, the two phe-

nomena desensitization and internalization could not be distinguished and may be interrelated. On the other hand, levorphanol caused a 400-fold reduction in agonist potency to inhibit cAMP accumulation but did not internalize the receptor, indicating that desensitization is not dependent on receptor internalization. Furthermore, etorphine treatment caused a dramatic 85% reduction in cell surface receptor but only a 7-fold reduction in agonist potency, indicating that there is a significant spare receptor population and that the phenomena of desensitization and internalization may be via interrelated but distinct processes. A similar proposal has been made to explain μ receptor regulation (18).

If desensitization and internalization involve different intracellular adaptive processes, then etorphine may predominantly induce one pathway, levorphanol may predominantly induce another, and the selective peptides may predominantly induce both pathways. Because our mutagenesis results indicate that etorphine and levorphanol do not depend on Asp128 for binding to and activation of the δ receptor, whereas peptides do, these agonists may bind to the receptor and induce distinct conformational changes with subsequent differential effect on intracellular effector systems to produce these different adaptive responses.

The results we present revealed that the addictive agent morphine and the opioid used in treatment of addiction, methadone, exhibited partial agonist activity at the δ receptor and failed to desensitize it. This characteristic was also demonstrated with the nonpeptide δ agonist SIOM, whereas the more potent δ-selective and -nonselective agonists desensitized it. This lack of desensitization of the δ receptor by a partial agonist such as SIOM may contribute to our understanding of the molecular and cellular basis of tolerance and dependence and hence facilitate the development of new opioids with a longer duration of action and that are devoid of addictive properties.

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