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Interaction of Co-Expressed μ- and δ-Opioid Receptors in Transfected Rat Pituitary GH_3 Cells

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ABSTRACT

μ- and δ-Opioid agonists interact in a synergistic manner to produce analgesia in several animal models. Additionally, receptor binding studies using membranes derived from brain tissue indicate that interactions between μ- and δ-opioid receptors might be responsible for the observation of multiple opioid receptor subtypes. To examine potential interactions between μ- and δ-opioid receptors, we examined receptor binding and functional characteristics of μ-, δ-, or both μ- and δ-opioid receptors stably transfected in rat pituitary GH_3 cells (GH_3MOR, GH_3DOR, and GH_3MORDOR, respectively). Saturation and competition binding experiments revealed that coexpression of μ- and δ-opioid receptors resulted in the appearance of multiple affinity states for μ- but not δ-opioid receptors. Additionally, coadministration of selective μ- and δ-opioid agonists in GH_3MORDOR cells resulted in a synergistic competition with [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE) for δ-opioid receptors. Finally, when equally effective concentrations of [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) and two different δ-opioid agonists (DPDPE or 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino-[2,3,3-*g*]-isoquinoline; TAN67) were coadministered in GH_3MORDOR cells, a synergistic inhibition of adenylyl cyclase activity was observed. These results strongly suggest that cotransfection of μ- and δ-opioid receptors alters the binding and functional characteristics of the receptors. Therefore, we propose that the simultaneous exposure of GH_3MORDOR cells to selective μ- and δ-opioid agonists produces an interaction between receptors resulting in enhanced receptor binding. This effect is translated into an augmented ability of these agonists to inhibit adenylyl cyclase activity. Similar interactions occurring in neurons that express both μ- and δ-opioid receptors could explain observations of multiple opioid receptor subtypes in receptor binding studies and the synergistic interaction of μ- and δ-opioids in analgesic assays.

Three opioid receptor subtypes (μ, δ, and κ) mediate the physiological responses to opioid drugs (Mansour et al., 1994). Analgesia produced by opioids is believed to be mediated primarily by μ-opioid receptor stimulation (Reisine and Pasternak, 1996); however, δ-opioid receptor agonists can also elicit an antinociceptive response (Quock et al., 1999). Behavioral studies suggest that μ- and δ-opioid receptors can also interact to modulate the ultimate antinociceptive response. For example, when subantinociceptive doses of the δ-opioid agonist DPDPE are coadministered with intracerebroventricular morphine (μ-opioid agonist), the antinociception produced by morphine is potentiated (Qi et al., 1990). However, in this same study, when other δ-opioid agonists are coadministered, such as [Met]enkephalin, the antinociception produced by morphine is attenuated. In another study, intrathecal coadministration of the μ-opioid agonists morphine, DAMGO, or PL017 with δ-opioid agonists DPDPE or [D-Ala^2,D-Leu^5]-enkephalin resulted in a synergism of μ-/δ-opioid antinociception (Malmberg and Yaksh, 1992). Such interaction between μ- and δ-opioid receptors has led to the idea of combined therapy for pain management to both decrease the dose of administered agents and avoid undesired side effects (Solomon and Gebhart, 1994; Cahill et al., 1996).

Several mechanisms could be responsible for the observed μ-/δ-opioid interaction that occurs at the behavioral level. Rossi et al. (1994) demonstrated that simultaneous activation of μ- and δ-opioid receptors from different brain regions at different sites within the nociceptive pathway resulted in a synergistic enhancement of analgesia. Activation of the receptors in the same brain region resulted in additive effects only. This suggests that the μ-/δ-opioid interaction occurs between opioid receptors located in different pathways involved in the pain response instead of a direct receptor-receptor interaction (Rossi et al., 1994).

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ABBREVIATIONS: DPDPE, [D-Pen^{2,5}]enkephalin; DAMGO, [D-Ala^2,N-MePhe^4,Gly-ol^5]enkephalin; DMEM, Dulbecco's modified Eagle's medium; TAN67, 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino-[2,3,3-*g*]-isoquinoline; MOR, μ-opioid receptor; DOR, δ-opioid receptor; MORDOR, μ- and δ-opioid receptor.

Alternatively, other lines of evidence indicate that μ- and δ-opioid receptors contained in the same cell or neuron may interact to alter receptor binding characteristics and modulate the ultimate response. For example, from receptor binding studies using rat brain membranes, Rothman et al. (1988, 1989) demonstrated that the μ-opioid alkylating agent β-funaltrexamine inhibited a proportion of the binding of δ-opioid receptor ligands. Therefore, it was proposed that δ-opioid receptors can be divided into two classes: those that are associated with μ-opioid receptors (i.e., $\delta_{complexed}$ or δ_{cx}) and those that are not (i.e., $\delta_{noncomplexed}$ or δ_{ncx}). The μ-/δ-opioid receptor complex has different binding characteristics than either the μ- or δ-opioid receptor alone. Other binding and behavioral studies have led to the proposal of two subtypes of μ- (μ_1 and μ_2) and δ- (δ_1 and δ_2) opioid receptors. The μ_1-opioid receptor demonstrates high affinity for enkephalins and opiate alkaloids and is antagonized by naloxonazine, whereas the μ_2-opioid receptor is assumed to be the traditional μ-opioid receptor (Wolozin and Pasternak, 1981). The δ-opioid receptor subtypes are classified based on selective agonist-induced analgesia and blockade of this response by the corresponding antagonist. Thus, DPDPE acts at the δ_1-opioid subtype and is antagonized by [D-Ala^2,Leu^5,Cys^6]enkephalin, but not naltrindole 5′-isothiocyanate, whereas [D-Ala^2]deltorphin II and [D-Ser^2,Leu^5,Thr^6]enkephalin act at the δ_2-opioid subtype and are antagonized by naltrindole 5′-isothiocyanate, but not [D-Ala^2,Leu^5,Cys^6]enkephalin (Jiang et al., 1991). The idea of two different subtypes of the δ-opioid receptor is also supported by the ability of 7-benzylidenenaltrexone to selectively antagonize the antinociceptive actions of DPDPE and [D-Ser^2,Leu^5,Thr^6]enkephalin in vivo (Portoghese et al., 1992).

Interestingly, despite the evidence for multiple μ- and δ-opioid receptor subtypes, only a single gene has been found to encode for either μ- or δ-opioid receptors and knockout of these genes eliminates the response to all μ- or δ-opioid ligands in transgenic mice, respectively [for review, see Kieffer (1999)]. It has recently been demonstrated that coexpression of two different opioid receptor subtypes, δ- and κ-, in human embryonic kidney 293 or COS-7 cells results in the physical association of these receptors to form a heterodimer with unique binding and functional properties (Jordan and Devi, 1999). While our study was in progress, it was also demonstrated that μ- and δ-opioid receptors coexpressed in COS-7 or CHO cells form hetero-oligomers that not only bind ligands differently but also couple to additional G-proteins (George et al., 2000). It is possible that an interaction between μ- and δ-opioid receptors existing in the same cell or neuron might be responsible for the observation of multiple opioid receptor subtypes and also contribute to the synergism of μ-/δ-opioid analgesia observed in behavioral studies. Therefore, the purpose of the present study was to first determine the effect of simultaneous expression of μ- and δ-opioid receptors on the binding of selective opioid ligands. Next, we examined whether cotransfection altered the ability of these receptors to functionally couple to adenylyl cyclase. Finally, we investigated the consequence of coadministration of μ- and δ-opioid ligands on both receptor binding and regulation of adenylyl cyclase activity.

Experimental Procedures

Materials. Penicillin/streptomycin (10,000 IU/ml and 10,000 μg/ml), geneticin (G418), and Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g glucose, L-glutamine, and pyruvate were purchased from Cellgro (Herndon, VA). Hygromycin-B was supplied by Calbiochem (San Diego, CA) and fetal calf serum was from Summit Biotechnologies (Fort Collins, CO). DPDPE and DAMGO were obtained from Peninsula Laboratories (Belmont, CA). TAN67 was purchased from Tocris Cookson, Inc. (Ballwin, MO). [^{3}H]DAMGO was obtained from Multiple Peptide Systems (San Diego, CA) and Amersham Pharmacia Biotech (Piscataway, NJ) with specific activities of 51 Ci/mmol and 68 Ci/mmol, respectively. [^{3}H]DPDPE was obtained from Multiple Peptide Systems and PerkinElmer Life Sciences (Boston, MA) with specific activities of 48 Ci/mmol and 45 Ci/mmol, respectively. [8-^{3}H]Adenine (26 Ci/mmol) and [α-^{32}P]ATP (30 Ci/mmol) were purchased from Amersham Pharmacia Biotech. [α-^{32}P]cAMP was prepared by enzymatic conversion of [α-^{32}P]ATP using GH_3MOR membranes. All other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture. GH_3 cells (CCL 82.1) were stably transfected with μ, δ, and both μ- and δ-opioid receptors as described previously (Piros et al., 1995, 1996; Prather et al., 2000) and are denoted as GH_3MOR, GH_3DOR, and GH_3MORDOR, respectively. Transfected cells were maintained in a DMEM-based media supplemented with $NaHCO_3$ (3.7 g/L), 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml geneticin. The addition of 200 μg/ml Hygromycin-B to the culture media was used for cells transfected with both μ- and δ-opioid receptors. Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were harvested each week with a 10 mM phosphate-buffered saline containing EDTA (1 mM), pH 7.4. Cells harvested for binding or adenylyl cyclase assays were then reseeded either at 30% of their original density into 175-cm^3 flasks (binding assays) or at a density of 8×10^6 cells per 17-mm (24-well) culture plate (adenylyl cyclase assays). Cells collected for membrane preparation were centrifuged (1000 rpm, 4°C, 10 min.) and pellets stored at −80°C until used.

Membrane Preparation and Receptor Binding. GH_3 membranes containing the transfected opioid receptor(s) of interest were prepared for binding assays as follows. Harvested cell pellets were re-suspended in 0.32 M sucrose and 0.010 M HEPES, pH 7.4, and homogenized with 10 strokes of a glass Dounce Homogenizer (Wheaton, Philadelphia, PA). The cell homogenates were then centrifuged at 1000*g* for 10 min at 4°C. The supernatant was collected and the resultant pellet was resuspended in one half the original volume of homogenization buffer. The homogenization procedure was repeated twice more and the pooled supernatants were then subjected to a 70-min centrifugation at 100,000*g* at 4°C. The resulting pellet of partially purified membrane was re-suspended in 50 mM Tris, pH 7.4, and the protein concentration determined by the method of Lowry et al. (1951). All opioid receptor binding assays were performed using 200 μg of membrane protein.

Saturation and competition binding assays were performed in 50 mM Tris, pH 7.4, with 5 mM $MgCl_2$, at room temperature using a 90-min incubation period, as described previously (Prather et al., 2000). For saturation binding studies, 0.05 to 30 nM [^{3}H]DAMGO (μ-opioid receptor ligand), 0.05 to 20 nM [^{3}H]DPDPE (δ_1-opioid receptor ligand) or 0.02 to 20 nM [^{3}H]deltorphin II (δ_2-opioid receptor ligand) were used. Nonspecific binding was determined in the presence of nonradioactive DAMGO, DPDPE, or deltorphin II (1 μM), respectively. In competition binding experiments, the ability of increasing concentrations (0.03 nM–30 μM) of selective opioid receptor ligands to displace the binding of [^{3}H]DAMGO or [^{3}H]DPDPE (1 nM) was assessed. Binding reactions were terminated by filtration with a Brandel 24-sample Standard Format Harvester (Gaithersburg, MD), and the amount of radioactivity on the filters was determined using a Packard Tri-Carb 2100TR liquid scintillation counter (Meriden, CT).

Measurement of cAMP Levels. The effect of opioids on the conversion of [^{3}H]ATP to [^{3}H]cAMP by adenylyl cyclase was determined as described previously (Prather et al., 2000). Briefly, cells were seeded into 24-well culture plates and cultured for 24 h (~90% confluency). On the day of the assay, media was removed and replaced with an incubation mixture (37°C) of DMEM containing 0.9% NaCl, 500 μM 3-isobutyl-1-methylxanthine and 1.25 μCi/well [^{3}H]adenine for 2 h. After incubation, the mixture was removed and each plate was floated in an ice-water bath for 5 min. During this time, an assay mixture of ice-cold Krebs-Ringer-HEPES buffer containing 500 μM 3-isobutyl-1-methylxanthine, 10 μM forskolin, and the appropriate concentration of the opioid ligand of interest was added. Plates were then floated in a water bath at 37°C for 15 min. The reaction was terminated by the addition of 50 μl of 2.2N HCl. An internal standard of [α-^{32}P]-cAMP was added to each well and radioactive cAMP was separated using alumina column chromatography (Alvarez and Daniels, 1992). Scintillation fluid (10 ml) was added to each sample before counting in a Packard Tri-Carb 2100TR liquid scintillation counter (Meriden, CT).

Isobolographic Analysis. Isobolographic analysis was used to evaluate interactions of coadministered agonists in binding and adenylyl cyclase assays (Tallarida et al., 1989). Briefly, the IC_{50} or K_i value for a single agonist (i.e., DPDPE or DAMGO) was determined from adenylyl cyclase or competition binding assays, respectively. The assays were then repeated with the agonists coadministered at a constant dose ratio based on an equieffective dose. Equieffective dose ratios were based on the IC_{50} or K_i values for each agonist in adenylyl cyclase and competition binding assays, respectively. (The K_i value was used to determine the dose ratio for competition binding studies rather than the IC_{50} value because the K_i value was less subject to experimental variability.) For example, if the IC_{50} value of agonist A was 20 nM and the IC_{50} value of agonist B was 5 nM, then the equieffective dose ratio of A to B was 4:1. Therefore, at each concentration of the dose-effect curve, the concentration of drug A was always 4 times the concentration of drug B. The IC_{50} value for each agonist in the presence of the other coadministered agonist was then determined by analyzing two separate curves in which the same Y values (i.e., percentage effect) were plotted against two different X values (i.e., concentrations for each agonist).

The isobolograph was constructed by plotting the experimentally determined IC_{50} values for agonists A and B administered alone on the X and Y axes, respectively. The diagonal, linear regression line connecting these values represents the theoretical line of additivity. On this additivity line lies the theoretical IC_{50} value. The X/Y coordinates of the theoretical IC_{50} value are calculated for each agonist when coadministered in equieffective concentrations and represent the IC_{50} values if the interaction were purely additive. The experimental IC_{50} values of each agonist when coadministered then provided the X/Y coordinates for the observed IC_{50} value to be graphed on the isobolograph. If the combination of agonists resulted in only an additive interaction, the observed IC_{50} value was on the line of additivity. An observed IC_{50} value significantly below the line of additivity indicated a synergistic (or greater than additive) interaction between agonists. An observed IC_{50} significantly above the additivity line suggested a less than additive interaction between agonists.

The effective dose ratios for the adenylyl cyclase assays were 500:1 for DAMGO and DPDPE, and for DAMGO and TAN67, respectively. In competition binding studies with [^{3}H]DPDPE, the dose ratio was 1:150 for DPDPE and DAMGO, respectively. The effective dose ratio for competition binding using [^{3}H]DAMGO was 1:170 for DAMGO and DPDPE, respectively.

Data Analysis and Statistics. For saturation binding experiments, determination of receptor affinity (K_d) and receptor density (B_{max}) was performed using the nonlinear regression analysis of GraphPad Prism v2.0b for Macintosh (GraphPad Software, San Diego, CA). GraphPad Prism was also used to determine IC_{50} values from the competition binding experiments. IC_{50} values were converted to K_i values using the Cheng-Prusoff (1973) equation. Isobolographic analysis, by the method outlined by Tallarida et al. (1989), was used to statistically evaluate interactions of coadministered agonists in binding and adenylyl cyclase assays. In some cases, differences between means were determined by the nonpaired Student's t test. Data are expressed as mean ± S.E.M. or mean (95% confidence interval), as indicated. Unless otherwise stated, data are represented by three separate experiments, done in duplicate or triplicate.

Results

Saturation Binding of [^{3}H]DPDPE, [^{3}H]Deltorphin-II and [^{3}H]DAMGO to GH_3DOR, GH_3MOR and GH_3MORDOR Membranes. Saturation binding of the selective δ-opioid agonist [^{3}H]DPDPE revealed the presence of a single, high-affinity site in both GH_3DOR or GH_3MORDOR membranes (Fig. 1A; Table 1). The K_d value of [^{3}H]DPDPE was similar between cell lines (K_d = 1.86 versus 2.36 nM, respectively) and the Scatchard plot in Fig. 1A clearly identifies a 3-fold difference in receptor density between

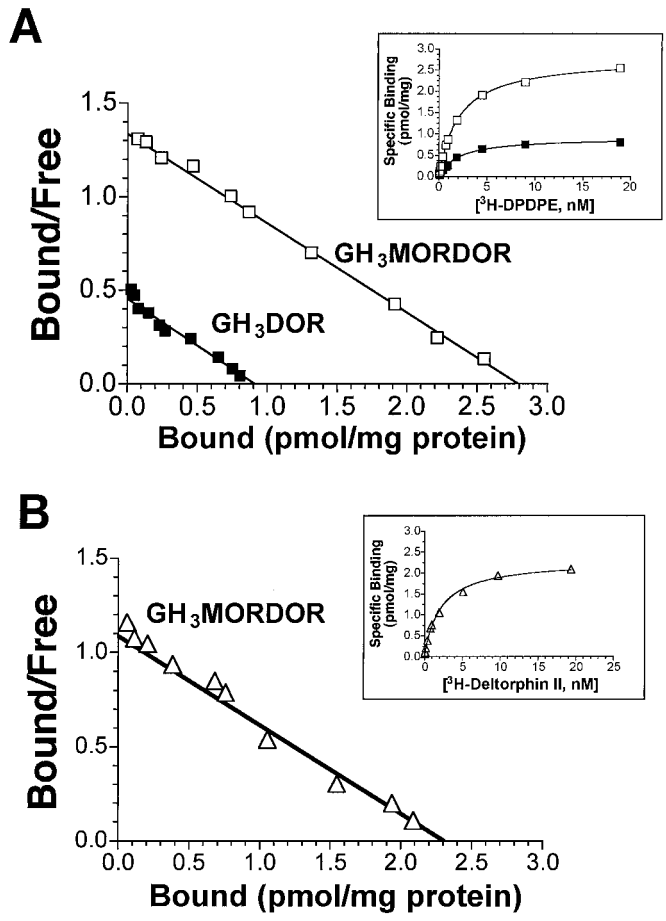

Fig. 1. Scatchard analysis of saturation binding of the δ_1-opioid agonist [^{3}H]DPDPE and the δ_2-opioid agonist [^{3}H]deltorphin-II to GH_3DOR and GH_3MORDOR membranes. A, Comparison of [^{3}H]DPDPE saturation binding (0.05–20 nM) in GH_3DOR (■) and GH_3MORDOR (□) membranes. Binding identifies a single, high affinity site with similar affinities between clones, but differing receptor densities. *B*, Scatchard analysis of [^{3}H]deltorphin-II saturation binding (0.02–20 nM) in GH_3MORDOR (△) membranes. Binding identifies a single, high-affinity site. *Insets*, corresponding saturation curves. Nonspecific binding determined by 1 μM DPDPE or deltorphin II. K_d and B_{max} values are reported in Table 1. Data are representative figures of three or four experiments performed in duplicate.

GH_3DOR and GH_3MORDOR cells (B_{max} = 0.85 and 3.09 pmol/mg protein, respectively). This difference in receptor density is attributed to the high expression vector used to stably transfect the δ-opioid receptor into GH_3 cells already expressing the μ-opioid receptor. DPDPE and deltorphin-II have been suggested to have selective activity at the δ_1- and δ_2-subtypes of opioid receptors, respectively (Jiang et al., 1991). Because the cloned δ-opioid receptor demonstrates properties most closely associated with the δ_2 subtype of opioid receptors (Raynor et al., 1994), we next performed saturation binding with the δ_2-selective agonist [^{3}H]deltorphin-II and compared it with that of the δ_1-selective agonist DPDPE in GH_3MORDOR cells (Fig. 1B; Table 1). Nonlinear analysis of the binding curve demonstrated that similar to [^{3}H]DPDPE, [^{3}H]deltorphin-II bound to a single site with high affinity (K_d = 2.1 nM). The density of binding sites identified by [^{3}H]deltorphin-II (B_{max} = 2.3 pmol/mg protein) was slightly lower than that determined for [^{3}H]DPDPE (B_{max} = 3.09 pmol/mg protein). Although this decrease was statistically significant ($p < 0.05$), it most likely indicated differences between membrane preparations used in the binding assays. Additionally, similar variability between B_{max} values determined by saturation binding using different opioid agonists as radioligands in transfected cells has been observed previously (Brown et al., 1997).

Saturation binding of the μ-opioid selective agonist [^{3}H]DAMGO to GH_3MOR membranes also revealed the presence of a single, high-affinity site with a K_d value of 0.58 nM (Fig. 2A; Table 1). In contrast, the best-fit of the saturation curve for the binding of [^{3}H]DAMGO to GH_3MORDOR membranes was a two-site model indicating the presence of a high- and low-affinity site for DAMGO (Fig. 2B; Table 1). The K_d of DAMGO for the high affinity site in GH_3MORDOR cells (0.46 nM) was similar to that observed for the single site in GH_3MOR cells (0.58 nM), but was 56-fold greater than that for the low affinity site (25.9 nM). DAMGO recognized approximately 26 and 74% of the μ-receptor population in the high and low affinity states, respectively. In addition, the total receptor density of the combined two sites in GH_3MORDOR cells was significantly less than the receptor density determined for GH_3MOR cells (B_{max} = 0.14 and 0.39 versus 1.02 pmol/mg of protein, respectively). This is surprising because GH_3MOR cells were the parent cell line used to produce GH_3MORDOR cells by transfection with δ-opioid receptors; thus, it was predicted that the μ-opioid receptor population would remain constant.

The results of saturation binding using GH_3MOR and GH_3DOR membranes are similar to that reported previously (Piros et al., 1995; Prather et al., 2000). However, results from the present study differ in the case of GH_3MORDOR cells. In an earlier study, we reported that [^{3}H]DAMGO bound in GH_3MORDOR membranes to a single, high-affinity site (K_d = 0.58 nM) (Piros et al., 1996), compared with two sites in the present study. We feel that this difference may be explained by two potential factors. First, in our earlier report we used crude membrane homogenates for receptor binding, whereas in the present study, a more pure membrane preparation was obtained by centrifugation of homogenates minus nuclei at 100,000*g* (see *Experimental Procedures*). Second, and more importantly, in our initial report we performed saturation binding with concentrations of [^{3}H]DAMGO up to a maximal concentration of only 10 nM. Because the estimated K_d value of the low affinity site is 25.9 nM, 10 nM

TABLE 1

Saturation binding of [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]deltorphin-II to μ- and δ-opioid receptors expressed singly or co-expressed in GH_3 cells.

Saturation binding was used to determine receptor affinity (K_d) and receptor density (B_{max}) in the presence of increasing concentrations of [^{3}H]DAMGO (μ-opioid receptors), [^{3}H]DPDPE (δ_1-opioid receptors), or[^{3}H]deltorphin-II (δ_2-opioid receptors). (High) and (low) represent data for high- and low-affinity sites, respectively. Data are presented as mean ± S.E.M. from three or four experiments performed in duplicate or triplicate.

Cell Line	K_d	B_{max}
	nM	*pmol/mg*
μ-Opioid Receptor		
[^{3}H]DAMGO		
GH_3MOR	0.58 ± 0.013	1.02 ± 0.052
GH_3MORDOR (high)	0.46 ± 0.080	0.14 ± 0.045
GH_3MORDOR (low)	25.9 ± 8.80	0.39 ± 0.040
δ-Opioid Receptor		
[^{3}H]DPDPE		
GH_3DOR	1.86 ± 0.16	0.85 ± 0.042
GH_3MORDOR	2.36 ± 0.29	3.09 ± 0.35
[^{3}H]Deltorphin-II		
GH^{3}MORDOR	2.10 ± 0.29	2.30 ± 0.35*

*$p<0.05$, non-paired Student's *t* test, significantly different from B_{max} in GH_3MORDOR.

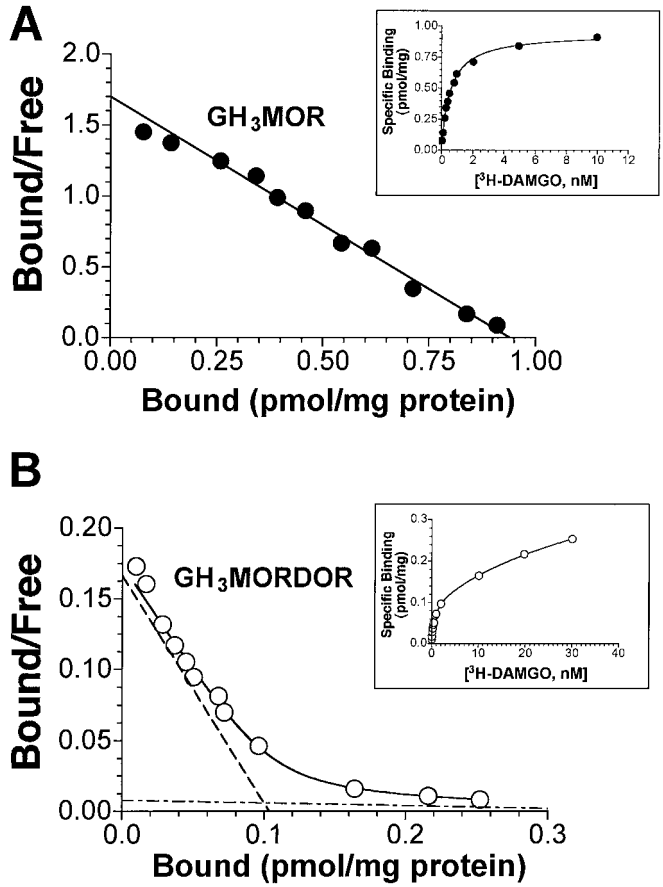

Fig. 2. Scatchard analysis of saturation binding of the μ-opioid agonist [^{3}H]DAMGO to GH_3MOR and GH_3MORDOR membranes. A, Scatchard analysis of [^{3}H]DAMGO saturation binding (0.05–10 nM) in GH_3MOR (●) membranes. Binding identifies a single, high-affinity site. B, Scatchard analysis for [^{3}H]DAMGO saturation binding (0.05–30 nM) in GH_3MORDOR (○) membranes. Binding identifies two affinity sites. *Insets*, corresponding saturation curves. Nonspecific binding determined by 1 μM DAMGO. K_d and B_{max} values are reported in Table 1. Data are representative figures of four experiments performed in triplicate.

[^{3}H]DAMGO would be predicted to occupy only approximately 25% of μ-opioid receptors in the low affinity state. Therefore, in the present study, we employed concentrations of [^{3}H]DAMGO up to 30 nM, which allowed us to fit more points on the saturation curve and more accurately predict the presence of the lower affinity site.

Competition Binding with [^{3}H]DPDPE Using GH_3DOR and GH_3MORDOR Membranes. To compare the binding profile of selective δ- and μ-opioid ligands for δ-opioid receptors expressed in GH_3DOR and GH_3MORDOR membranes, competitive binding studies using [^{3}H]DPDPE were performed. A concentration of 1 nM [^{3}H]DPDPE was chosen so as to occupy ~50% of the δ-opioid receptors. Nonlinear regression determined the IC_{50} values of the competing ligands, and a measure of affinity (K_i) was subsequently derived from the IC_{50} value using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). DPDPE dose-dependently reduced the amount of [^{3}H]DPDPE binding in both GH_3DOR and GH_3MORDOR membranes with similar IC_{50} values of 2.54 and 3.3 nM, respectively (Fig. 3A; Table 2). The μ-selective opioid agonist DAMGO also dose dependently decreased [^{3}H]DPDPE binding, but much higher concentrations were required to produce half-maximal inhibition (i.e., 667 and 588 nM, respectively) (Fig. 3B; Table 2). As expected, conversion of these values to a measure of affinity (K_i) indicate that the δ-opioid agonist DPDPE binds with much higher affinity to expressed δ-opioid receptors in both GH_3DOR and GH_3MORDOR cells than the μ-opioid agonist DAMGO. Additionally, the K_i and K_d values for DPDPE determined by competition binding and saturation binding, respectively, are very similar (compare Tables 1 and 2).

To test for a potential interaction between the binding of DPDPE and DAMGO to the expressed δ-opioid receptors in GH_3MORDOR membranes, the ability of coadministered DAMGO and DPDPE in equally effective concentrations to compete against 1 nM [^{3}H]DPDPE was examined and these results were analyzed isobolographically. The observed IC_{50} values for each coadministered, unlabeled ligand (DAMGO or DPDPE) were calculated individually and in combination. Theoretically, if DPDPE and DAMGO interact in an additive fashion to compete with [^{3}H]DPDPE, their coadministration will result in a shift to the left in the competition curve such that the observed IC_{50} value will not differ significantly from the theoretical IC_{50} value predicted from the isobolograph (Tallarida et al., 1989). In contrast to this prediction, when coadministered DPDPE and DAMGO (dose ratio 1:150, respectively) competed against 1 nM [^{3}H]DPDPE, the observed IC_{50} value was significantly less ($p < 0.05$) than the theoretical IC_{50} value (Fig. 3C; Table 3). This indicated that the coadministered ligands synergistically displaced [^{3}H]DPDPE from the δ-opioid receptor in GH_3MORDOR membranes.

Competition Binding with [^{3}H]DAMGO Using GH_3MOR and GH_3 MORDOR Membranes. The same experimental design was used to compare the binding profile of selective δ- and μ-opioid ligands for μ-opioid receptors expressed in GH_3MOR and GH_3MORDOR membranes by performing competitive binding studies using [^{3}H]DAMGO. DAMGO dose-dependently reduced the amount of 1 nM [^{3}H]DAMGO binding in GH_3MOR membranes with an IC_{50} value of 1.62 nM (Fig. 4A; Table 2). As predicted, the δ-selective agonist DPDPE also dose dependently decreased [^{3}H]DAMGO binding in GH_3MOR membranes, but a signif-

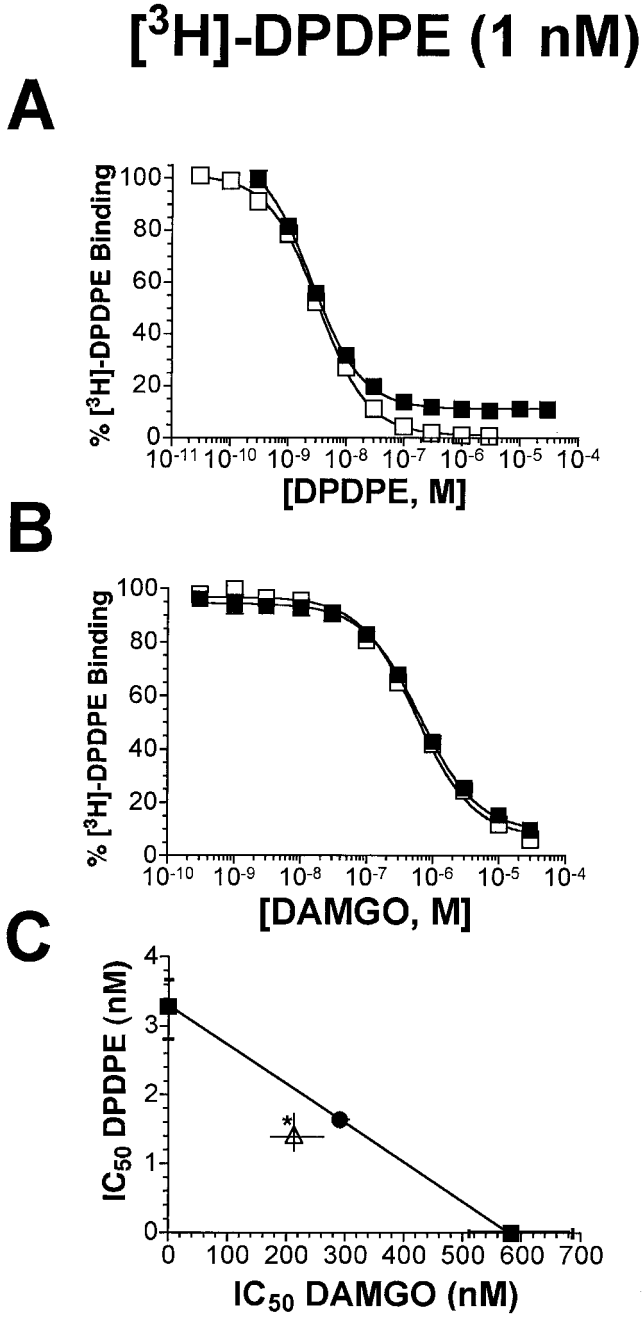

Fig. 3. [^{3}H]DPDPE competition binding experiments in GH_3DOR and GH_3MORDOR. A, competition binding of 1 nM the δ-opioid agonist [^{3}H]DPDPE in GH_3DOR (■) and GH_3MORDOR (□) membranes with DPDPE (0.3 nM–30 μM). B, competition binding of 1 nM [^{3}H]DPDPE in GH_3DOR (■) and GH_3MORDOR (□) membranes with the μ-opioid agonist DAMGO (0.03 nM–30 μM). The IC_{50} and K_i values were determined as stated under *Experimental Procedures* and are reported in Table 2. C, isobolographic plot of the competition for 1 nM [^{3}H]DPDPE by coadministered DPDPE (0.45 nM–45 μM) and DAMGO (0.003–300 nM) in GH_3MORDOR membranes. Analysis shows a synergistic interaction. Refer to *Experimental Procedures* for a more complete description of this technique. Briefly, IC_{50} values of DAMGO or DPDPE alone are plotted on the X and Y axes, respectively, and the line connecting them represents the theoretical line of additivity. The IC_{50} values of each ligand when coadministered provide the X/Y coordinates for the observed IC_{50}, represented by △. The theoretical IC_{50} value (●) is based on the coadministration of equieffective concentrations and an additive interaction. Lines through the observed and theoretical IC_{50} data points represent 95% confidence intervals. All IC_{50} values for the isobolograph analyses are reported in Table 3. Graphs represent three separate experiments performed in duplicate or triplicate. *$P < 0.05$, significant synergistic interaction.

icantly greater concentration (509 nM) was required to produce half-maximal inhibition (Fig. 4B; Table 2). The concentration-effect curve for the competition of [^{3}H]DAMGO by both DAMGO and DPDPE was best fit by a single site model with a Hill slope of ~1.

In contrast to the results obtained in membranes prepared from GH_3MOR cells, the competition curve of DPDPE for [^{3}H]DAMGO binding was clearly best fit by a two-site model with a Hill slope significantly less than 1 in GH_3MORDOR membranes (Fig. 4B; Table 2). The K_i values of DPDPE for these apparent high- and low-affinity sites were 0.95 and 242 nM, respectively. This observation provides additional support for the two affinity states for DAMGO identified previously by saturation binding in GH_3MORDOR membranes (Fig. 2B). Interestingly, competition binding between [^{3}H]DAMGO and DAMGO revealed only a single affinity site in GH_3MORDOR membranes (Fig. 4A). A probable explanation for this apparent discrepancy involves differences between the affinity ratios of DPDPE and DAMGO for the low- and high-affinity binding sites. Competition binding demonstrated that DPDPE had a low/high affinity ratio of 255-fold (242 versus 0.95 nM). However, saturation binding showed that this ratio was only 56-fold (25.9 versus 0.46 nM) for DAMGO. Therefore, it is likely that competition binding easily detected a 255-fold difference in binding affinity of DPDPE for these two sites but was not sensitive enough to identify a smaller 56-fold difference in binding affinity of DAMGO.

TABLE 2

Competition binding of [^{3}H]DAMGO and [^{3}H]DPDPE to μ- and δ-opioid receptors expressed singly or co-expressed in GH_3 cells.

Competition binding was performed in the presence of either 1 nM [^{3}]DAMGO or [^{3}H]DPDPE and the determination of K_i and IC_{50} values in GH_3MOR, GH_3DOR, and GH_3MORDOR membranes was determined. (High) and (low) represents data for high- and low-affinity sites, respectively. Data are presented as mean ± S.E.M. from three experiments performed in triplicate.

Cell Line	Ligand	K_i	IC_{50}
		nM	
μ-Opioid Receptor [^{3}H]DAMGO (1 nM)			
GH_3MOR	DAMGO	0.61 ± 0.050	1.62 ± 0.13
	DPDPE	181 ± 3.04	509 ± 8.6
GH_3MORDOR	DAMGO	0.50 ± 0.069	1.63 ± 0.13
	DPDPE (high)	0.95 ± 0.47	3.22 ± 0.92
	DPDPE (low)	242 ± 48.7	820 ± 165
δ-Opioid Receptor [^{3}H]DPDPE (1 nM)			
GH_3DOR	DAMGO	404 ± 12.0	667 ± 19.8
	DPDPE	1.55 ± 0.07	2.54 ± 0.12
GH_3MORDOR	DAMGO	393 ± 32.4	588 ± 43.2
	DPDPE	2.30 ± 0.20	3.30 ± 0.28

Similar to our studies of the δ-opioid receptor, we next designed experiments to test for a potential interaction between the binding of DAMGO and DPDPE to the expressed μ-opioid receptors in GH_3MORDOR membranes (data not shown). Competition binding was performed with 1 nM [^{3}H]DAMGO and increasing concentrations of coadministered DAMGO and DPDPE (effective dose ratio, 1:170, respectively). Interestingly, coadministration of DAMGO and DPDPE resulted in a two-site competition curve for both ligands. For this reason, an isobolograph could not be used to test statistically for a potential interaction. However, examination of the competition curves for DAMGO competing against [^{3}H]DAMGO, given alone versus in the presence of DPDPE, suggests an alteration in the binding characteristics of coexpressed μ- and δ-opioid receptors, as evidenced by the change of the competition curve from a one-site to a two-site model. Importantly, coadministered DAMGO and DPDPE competed against 1 nM [^{3}H]DAMGO in an additive fashion from the μ-opioid receptors in GH_3MOR membranes and the competition curve was best fit by a one-site model (data not shown).

Opioid Receptor-Induced Inhibition of Adenylyl Cyclase in GH_3 Cells. Opioid receptors are known to inhibit adenylyl cyclase activity through coupling to the G-protein α-subunits, $G_i/G_{o\alpha}$. The ability of selective δ- and μ-opioid agonists to inhibit adenylyl cyclase activity, and thus decrease cAMP production, was compared between GH_3DOR, GH_3MOR and GH_3MORDOR cells. In GH_3DOR and GH_3MORDOR cells, increasing concentrations of DPDPE resulted in a concentration-dependent decrease in intracellular cAMP production (Fig. 5A). There was no significant difference in the potency of DPDPE to inhibit adenylyl cyclase activity between GH_3DOR and GH_3MORDOR cells (IC_{50} = 2.2 versus 1.1 nM, respectively). However, the maximal inhibition of intracellular cAMP levels (i.e., efficacy) varied between clones. Specifically, DPDPE inhibited adenylyl cyclase activity by 85% in GH_3MORDOR cells, compared with only 55% in GH_3DOR cells. This difference in efficacy of DPDPE is attributed to the greater receptor density in GH_3MORDOR compared with GH_3DOR cells (B_{max} = 0.85 versus 3.09 pmol/mg of protein, respectively; Table 1). In GH_3MOR cells, the concentration of DAMGO required to half maximally inhibit adenylyl cyclase activity (i.e., IC_{50}) was 21.1 nM (Fig. 5B). Quite remarkably, there was a >30-fold increase in the IC_{50} value of DAMGO to inhibit adenylyl cyclase in GH_3MORDOR cells (671 nM) (Fig. 5B). These results indicate that although coexpression of δ- and μ-opioid receptors does not change the potency of DPDPE, a δ-opioid agonist, to inhibit adenylyl cyclase activity, it greatly in-

TABLE 3

Competition binding—IC_{50} values for isobolographic analysis in GH_3MORDOR membranes.

Values were determined from competitive binding experiments in GH_3MORDOR membranes examining competition between 1 nM of the δ-opioid agonist [^{3}H]DPDPE and increasing concentrations of DPDPE or the μ-opioid agonist DAMGO, alone, or in combination. Refer to *Experimental Procedures* for a more complete description of this technique. The theoretical IC_{50} values were derived from the line of additivity obtained from the isobolograph (Fig. 3C). Data are presented as mean (95% confidence intervals) from three experiments performed in duplicate.

	IC_{50} (95% Confidence Interval)			
	Ligand Alone	Ligand in Combination	Theoretical IC_{50}	Interaction
		nM		
DAMGO	582 (509–667	214 (174–262)	291 (278–305)	Synergestic*
DPDPE	3.29 (2.92–3.70)	1.43 (1.16–1.75)	1.64 (1.57–1.72)	

*$p < 0.05$

creases the amount of DAMGO, a μ-opioid agonist, required to regulate the same intracellular effector.

To determine whether μ- and δ-opioid receptors interacted to regulate adenylyl cyclase activity in GH_3MORDOR cells, we examined the ability DAMGO and DPDPE administered alone or coadministered in an effective dose ratio of 500:1 to reduce intracellular cAMP levels (Fig. 6A; Table 4). Isobolographic analysis revealed that coadministration of these two agonists in GH_3MORDOR cells resulted in a synergistic inhibition of adenylyl cyclase activity. These experiments were repeated using the nonpeptide, δ-selective agonist TAN67, and isobolographic analysis showed that DAMGO and TAN67 (effective dose ratio of 500:1), also interacted synergistically to inhibit adenylyl cyclase (Fig. 6B; Table 4).

Discussion

Saturation binding in GH_3MOR membranes using the μ-selective opioid receptor agonist [^{3}H]DAMGO indicated the presence of a single high affinity site. Remarkably, in GH_3 cells transfected with both μ- and δ-opioid receptors, [^{3}H]DAMGO bound to two sites, one with high (0.46 nM) and one with lower (25.9 nM) affinity. There are several possibilities that could explain the nature of the lower affinity binding sites for DAMGO in GH_3MORDOR cells. First, DAMGO might simply bind to over-expressed δ-opioid receptors with lower affinity. However, this seems unlikely because the affinity of DAMGO for δ-opioid receptors as determined by competition binding in GH_3DOR and GH_3MORDOR were 404 and 393 nM, respectively. In both cases, these affinities for the δ-receptor are much lower than the affinity (25.9 nM) for the DAMGO binding site determined by saturation binding. Second, it is also possible that if over-expressed δ-opioid receptors had a high affinity for a limited concentration of the G-proteins in GH_3MORDOR cells, this could decrease the

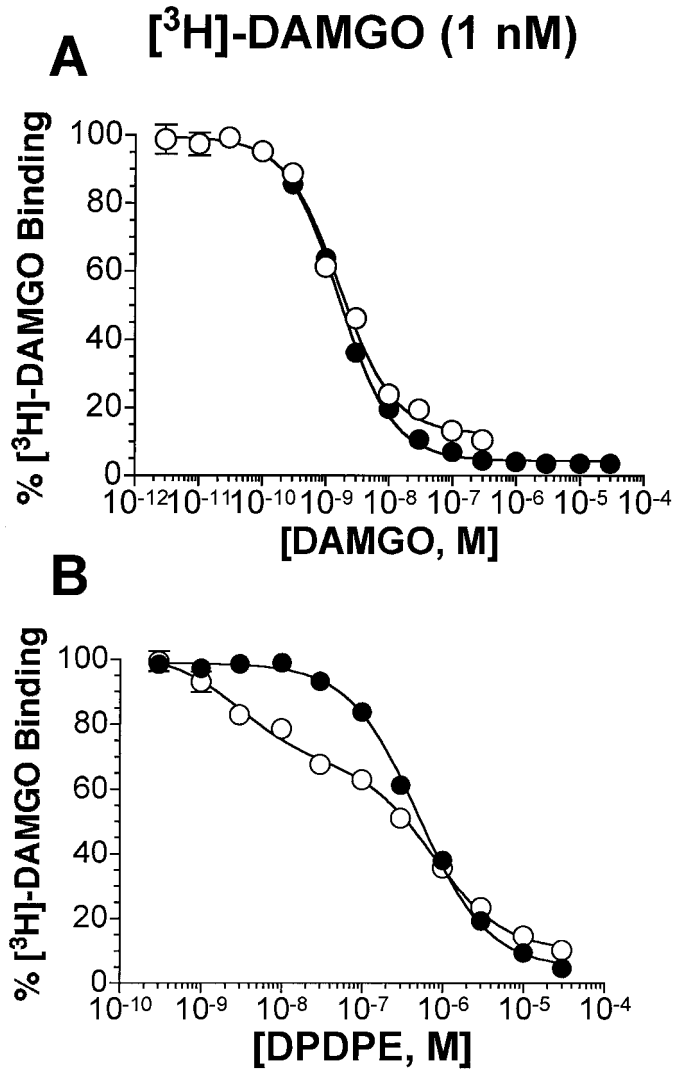

Fig. 4. [^{3}H]DAMGO competition binding experiments in GH_3MOR and GH_3MORDOR membranes. Competition binding of 1 nM the μ-opioid agonist [^{3}H]DAMGO in GH_3MOR (●) and GH_3MORDOR (○) membranes with DAMGO (0.03 nM-30 μM; A), and the δ-opioid agonist DPDPE (0.3 nM–30 μM; B). The IC_{50} and K_i values were determined as stated under *Experimental Procedures* and are reported in Table 2. Graphs represent three experiments performed in triplicate.

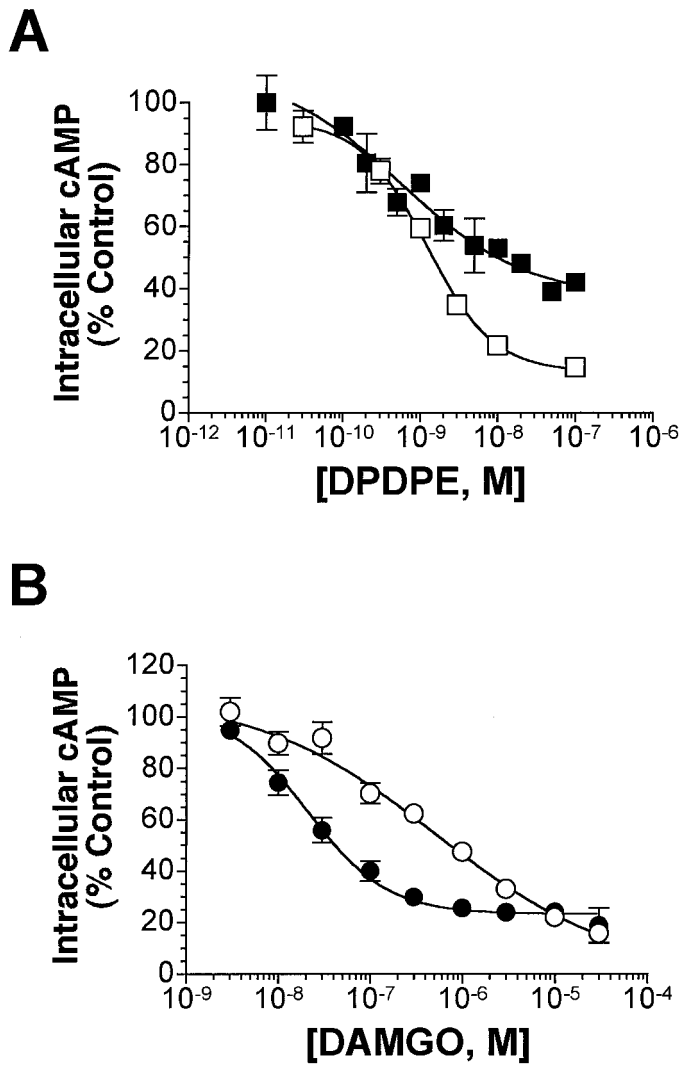

Fig. 5. Inhibition of adenylyl cyclase activity by δ- and μ-opioid receptors in GH_3DOR, GH_3MOR, and GH_3MORDOR cells. The ability of δ- and μ-opioid agonists to reduce 10 μM forskolin-stimulated cAMP accumulation was assessed in transfected GH_3 cells. Data are presented as the percentage of control cAMP levels (i.e., no opioid). A, δ-opioid receptor-induced inhibition of adenylyl cyclase activity by DPDPE in GH_3DOR (■) and GH_3MORDOR (□) cells (IC_{50} = 2.2 and 1.1 nM, respectively). B, μ-opioid receptor-induced inhibition of adenylyl cyclase activity by DAMGO in GH_3MOR (●) and GH_3MORDOR (○) cells (IC_{50} = 21.1 and 671 nM, respectively). Data points represent the mean ± S.E.M. for five experiments performed in triplicate.

accessibility of G-proteins to a portion of μ-opioid receptors required to establish a high-affinity state of the receptor. For example, in rat hepatocytes, high-affinity binding of epinephrine to α_1-adrenergic receptors was greatly reduced by vasopressin, which sequestered G-proteins needed to produce the high affinity conformation of α_1-adrenergic receptors (Dasso and Taylor, 1992). Additionally, CB1 cannabinoid receptors can sequester G-proteins, making them unavailable to provide coupling of α_2-adrenergic or somatostatin receptors to voltage-gated Ca^{2+} channels (Vasquez and Lewis, 1999). A third possibility is that in GH_3MORDOR cells, μ- and δ-opioid receptors might physically interact to form a new "hybrid" receptor for which DAMGO has a reduced affinity. Support for this possibility comes from recent literature reporting that when μ- and δ-opioid receptors were coexpressed in COS-7 cells they formed hetero-oligomers for which the affinity of DAMGO was reduced by 10-fold relative to μ-opioid receptors expressed alone (George et al., 2000). Experiments in our laboratory are ongoing to further characterize the nature of this low-affinity binding site for DAMGO in GH_3MORDOR cells.

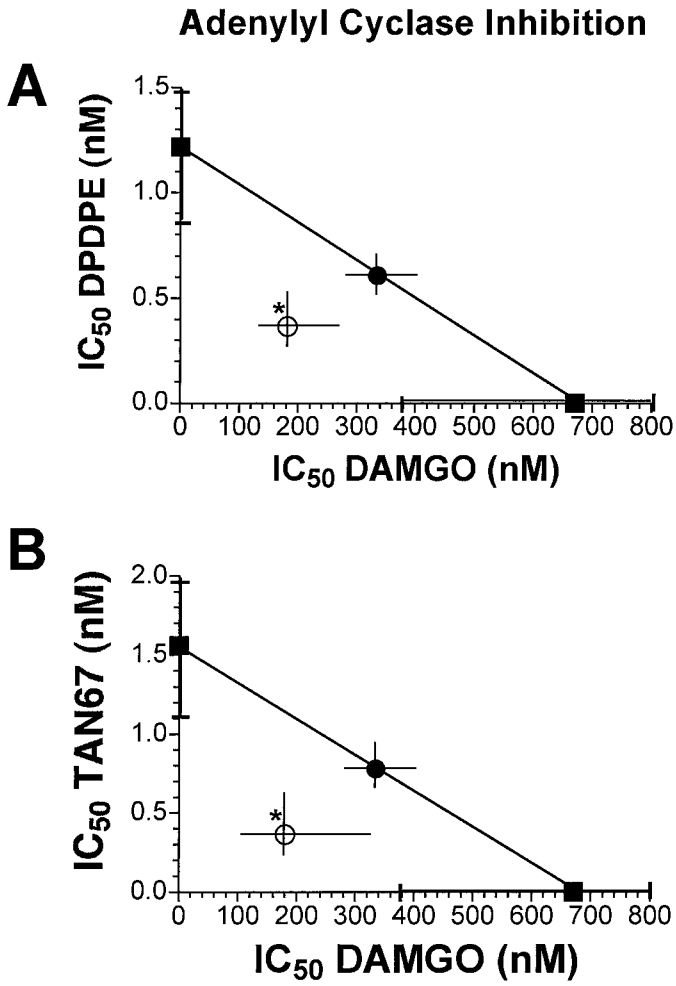

Fig. 6. Isobolographic analysis of the effect of coadministration of the μ-opioid agonist DAMGO with δ-opioid agonists DPDPE or TAN67 on the inhibition of adenylyl cyclase activity in GH_3MORDOR cells. Graphs are constructed as described in Fig. 3C. Refer to *Experimental Procedures* for a more complete description of technique. A, coadministration of equieffective concentrations of the μ-opioid agonist DAMGO (10 nM–30 μM) and the selective δ-opioid peptide agonist DPDPE (0.02 nM–60 nM) show a significant, synergistic inhibition of adenylyl cyclase activity. B, coadministration of DAMGO (10 nM–30 μM) and TAN67 (0.02 nM–60 nM), a selective δ-opioid nonpeptide agonist, also produce synergistic inhibition of adenylyl cyclase activity. Data represent five experiments performed in triplicate. *$P < 0.05$, significant synergistic interaction.

In addition to the presence of multiple affinity sites for DAMGO in GH_3MORDOR cells, the total μ-opioid receptor density of the combined affinity sites in the cotransfected clone was also significantly less than the receptor density determined for the parent GH_3MOR cell line. There are several potential mechanisms that might explain this intriguing observation. First, cotransfection with δ-opioid receptors might decrease either the transcription and/or the translation of μ-opioid receptors. Second, even if production of the μ-opioid gene product is not altered, coexpression with δ-opioid receptors might interfere with the trafficking of μ-opioid receptors to the membrane. For example, chronic stimulation of μ-opioid receptors in primary neuronal cultures results in an increased targeting of δ-opioid receptors to the plasma membrane (Morinville et al., 2000). Finally, in addition to the two affinity states identified by saturation binding, there may be an additional population of μ-opioid receptors in GH_3MORDOR cells that is not even recognized by [^{3}H]DAMGO. These sites might result from physical association between coexpressed μ- and δ-opioid receptors producing a significantly altered receptor conformation unable to bind selective ligands. Future studies are planned and must be evaluated before any definitive conclusions can be made concerning the mechanism of the reported results. These studies will include employing radiolabeled antagonists to examine receptor binding. Unlike agonists, the affinity of antagonists for G-protein coupled receptors is not sensitive to regulation by sodium, guanine nucleotides, or coupling to G-proteins.

Altered binding characteristics of μ-opioid receptors in GH_3MORDOR cells was further supported by results from

TABLE 4

Adenylyl cyclase inhibition—IC_{50} values for isobolographic analysis in GH_3MORDOR cells.

The ability of the μ-opioid agonist DAMGO (10 nM–30 μM) and the δ-opioid agonists DPDPE or TAN67 (0.02 nM–60 nM) administered alone or in equally effective concentrations to reduce intracellular cAMP levels was assessed in GH_3MORDOR cells. Refer to *Experimental Procedures* for a more complete description of this technique. The theoretical IC_{50} values were derived from the line of additivity obtained from the isobolograph (Fig. 6). Data are presented as mean (95% confidence intervals) from five experiments performed in triplicate.

	IC_{50} (95% Confidence Interval)			
	Ligand Alone	Ligand in Combination	Theoretical IC_{50}	Interaction
		nM		
DAMGO	671 (375–1199)	183 (127–263)	335 (279–402)	Synergistic*
DPDPE	1.22 (0.872–1.70)	0.366 (0.254–0.525)	0.608 (0.506–0.731)	
DAMGO	671 (375–1199)	181 (105–313)	335 (280–402)	Synergistic*
TAN67	1.56 (1.14–2.13)	0.363 (0.210–0.627)	0.780 (0.652–0.934)	

*$p < 0.05$

competition binding studies. It was observed that the δ-selective opioid receptor agonist DPDPE recognized both a high- (0.95 nM) and low- (242 nM) affinity site in GH_3MORDOR but not GH_3MOR membranes when competing against 1 nM [^{3}H]DAMGO. In contrast to the μ-opioid receptors, coexpression of μ- and δ-opioid receptors in GH_3 cells did not alter the binding characteristics of the δ-opioid receptor. Collectively, results from both saturation and competition binding studies indicate that coexpression of μ- and δ-opioid receptors results in the formation of multiple affinity states of μ- but not δ-opioid receptors.

Not only was the binding of μ-selective opioid ligands altered upon coexpression of δ-opioid receptors, but the amount of DAMGO required to half-maximally inhibit the intracellular effector adenylyl cyclase was also increased by more than 30-fold in GH_3MORDOR cells compared with GH_3MOR cells. This reduction in potency of DAMGO in the cotransfected clone might reflect the fact that 74% of the μ-opioid receptor sites recognized by DAMGO exist in a state for which DAMGO has a 56-fold lower affinity relative to the high affinity sites (Table 1). Additionally, the K_d value for DAMGO in GH_3MOR cells is 0.58 nM (Table 1), whereas the IC_{50} for inhibition of adenylyl cyclase activity is reported to be 21.1 nM (Fig. 5B). The K_d/IC_{50} ratio for DAMGO is significantly less than one (i.e., 0.026). This indicates that μ-opioid receptors in GH_3MOR cells have little or no receptor reserve for the inhibition of adenylyl cyclase activity. Therefore, slight changes in the μ-opioid receptor signal transduction pathway produced by cotransfection with δ-opioid receptors (i.e., sequestration of G-proteins) might be predicted to result in dramatic alterations in μ-opioid receptor function. In contrast to DAMGO, the potency of the δ-opioid agonist DPDPE to reduce intracellular cAMP levels was not significantly different between GH_3DOR and GH_3MORDOR cells. Therefore, similar to our results with receptor binding, this suggests that coexpression of μ- and δ-opioid receptors alters the ability of μ- but not δ-opioid receptors to regulate adenylyl cyclase activity.

Our evidence that cotransfection effects primarily μ- but not δ-opioid receptor binding and function may be influenced by the greater δ-opioid receptor density in GH_3MORDOR cells or limitations in the sensitivity of our assays. For example, there is a 5:1 ratio of δ- to μ-opioid receptors in GH_3MORDOR cells. If, for example, all the μ-opioid receptors interacted with δ-opioid receptors, then approximately 80% of δ-opioid receptors would not be involved in such interactions. Consequently, our assays may be limited in their ability to detect differences between the relatively small population of δ-opioid receptors interacting with μ-opioid receptors and the larger population of noninteracting δ-opioid receptors. However, based on the recent study indicating that cotransfection of μ- and δ-opioid receptors results in the formation of hetero-oligomers (George et al., 2000), it is also possible that one μ-opioid receptor might interact with several δ-opioid receptors.

Interestingly, several studies examining saturation binding of [^{3}H]DAMGO to membranes containing both endogenous μ- and δ-opioid receptors, including whole brain (Chan et al., 1997), spinal cord (Hoskins et al., 1998), and neuroblastoma cell lines (Standifer et al., 1994; Palazzi et al., 1996; Yabaluri and Medzihradsky, 1997), have not revealed multiple affinity sites for μ-opioid receptors. All of these tissues and cell lines contain similar ratios of opioid receptors (i.e., 1:1) or even slightly higher densities of μ-opioid receptors relative to δ-opioid receptors (i.e., 2:1). In contrast, in the present study, our cotransfected cells express five times more δ- than μ-opioid receptors. Therefore, interactions between opioid receptors might depend on the ratio of expressed receptors present in a given cell or tissue. We are presently generating GH_3 cells expressing different levels of δ-opioid receptors cotransfected with a single density of μ-opioid receptors to investigate this hypothesis.

To quantify the potential interactions between μ- and δ-opioid receptors, isobolographic analysis was employed to examine the effect of coadministration of μ- and δ-opioid agonists on competition receptor binding and the inhibition of adenylyl cyclase activity in GH_3MORDOR cells. Coexpressed receptors interacted synergistically in assays that measured the levels of receptor binding and in assays measuring effector regulation. First, when equally effective concentrations of DAMGO and DPDPE were coadministered, a synergistic competition for the binding of 1 nM [^{3}H]DPDPE to δ-opioid receptors was observed. Second, coadministration of DAMGO with the δ-selective agonists, DPDPE or TAN67, produced a synergistic inhibition of adenylyl cyclase. Therefore, we propose that the simultaneous exposure of GH_3MORDOR cells to selective μ- and δ-opioid agonists produces an interaction between receptors resulting in enhanced receptor binding. This effect is translated into an augmented ability of these agonists to inhibit adenylyl cyclase activity.

It is unclear why coadministration of DPDPE with DAMGO enhances the ability of DAMGO to compete with low affinity for δ-opioid receptors. Perhaps DPDPE binding alters the conformation of interacting δ-opioid receptors in a manner that improves recognition by DAMGO. Other studies have shown that interaction between μ- and δ-opioid receptors alters receptor binding and function. For example, Jordan and Devi (1999) demonstrated that κ/δ-opioid heterodimers display synergistic binding and regulation of intracellular effectors in response to selective opioid ligands. In a second study, SK-N-BE neuroblastoma cells that express a similar ratio of endogenous μ- and δ-opioid receptors, showed DAMGO produced a rightward shift in the DPDPE dose-response curve for adenylyl cyclase activity inhibition. DPDPE also increased the amount of DAMGO required to produce half-maximal reduction in cAMP levels (Palazzi et al., 1996). A final example reports that activation of μ- and δ-opioid receptors produced a synergistic release of adenosine from spinal cord synaptosomes (Cahill et al., 1996). Thus, it is conceivable that coexpression results in interactions between the μ- and δ-opioid receptors, resulting in altered binding characteristics of the δ-opioid receptor.

In conclusion, the present study demonstrated that when μ- and δ-opioid receptors are coexpressed in GH_3 cells, selective μ-opioid ligands exhibit altered receptor binding and altered regulation of the intracellular effector adenylyl cyclase. In contrast, no changes were observed in the actions of selective δ-opioid ligands. However, when μ- and δ-opioid ligands were coadministered, a synergistic interaction between the coexpressed receptors at the levels of both binding and effector regulation occurred. Therefore, we propose that the simultaneous exposure of GH_3MORDOR cells to selective μ- and δ-opioid agonists produces an interaction between receptors resulting in enhancement of receptor binding that

is translated into an augmentation of their ability to inhibit adenylyl cyclase activity. It is tempting to speculate that similar interactions occur in neurons that express both μ- and δ-opioid receptors (Ji et al., 1995), which explains observations of multiple opioid receptor subtypes in receptor binding studies and the synergistic interaction of μ- and δ-opioids in analgesic assays. However, further studies are needed and future research will be directed to address this question.

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