ABSTRACT

A C6 glioma cell line stably transfected with the rat delta opioid receptor (C6δ) was used to characterize receptor binding and G protein activation by both peptide and nonpeptide delta opioid ligands. The ligand binding affinities for [3H]naltrindole and [3H]nalorphine were similar to those observed in monkey brain membranes. The nonpeptide agonists, BW373U86 and SN80, as well as peptide agonist [D-Ser2,L-Arg5]enkephalin-Thr maximally stimulated [35S]GTPγS binding by 640, 654 and 576%, respectively, over basal. The peptide agonists DPDPE and deltorphin II, both stimulated [35S]GTPγS binding by 375%. Etorphine, diprenorphine, oxymorphindole and 7-spiroindanyloxymorphone were also partial agonists in this assay, although they were less efficacious than deltorphin II. Stimulation of [35S]GTPγS binding by agonists was blocked completely by pertussis toxin pretreatment. Both delta-1 and delta-2 selective antagonists 7-benzylidenenaltrexone and a benzofuran analog of naltrexone displayed high affinity for the cloned receptor (0.04 and 0.08 nM) and antagonized the stimulation of [35S]GTPγS binding by BW373U86 and DPDPE with similar potencies. Other evidence suggesting the lack of receptor subtypes includes the finding that stimulation of [35S]GTPγS binding by receptor subtype selective ligands DPDPE and deltorphin II was not additive. BW373U86, SN80 and DPDPE maximally inhibited forskolin-stimulated adenyl cyclase. These cells highly express a homogenous population of delta opioid receptor that couple to inhibitory Gαi/G protein. Ligand affinity for the delta opioid receptor correlates with ligand EC50 values for stimulation of [35S]GTPγS binding.

Activation of the delta opioid receptor is suggested to play a role in multiple behavioral and physiological effects ranging from analgesia and mood-driven behaviors to olfaction and gastrointestinal motility (for review, see Dhawan et al., 1996). Delta opioid receptors are members of the seven-transmembrane G protein-coupled receptor superfamily (for review, see Reisine and Bell, 1993). Delta opioids, acting at the delta opioid receptor, have mediated the inhibition of forskolin-stimulated adenyl cyclase (Evens et al., 1992), an increase in the production of inositol phosphates (Tsu et al., 1995) as well as modulation of ion channel opening (Taussig et al., 1992; Ikeda, 1996) in a pertussis toxin-sensitive manner.

The delta opioid receptor transduces its signal through inhibitory G proteins, Gαi2, Gαi3, and Gαo in tumor cell lines (Taussig et al., 1992; Prather et al., 1994a) and in vivo (Heeschler et al., 1987; Sanchez-Blazquez and Garzon, 1993). The G proteins Gαo, Gαi1,2 and Gαi3 have also been found to mediate inhibition of adenyl cyclase by delta opioids (McKenzie and Milligan, 1990; Carter and Medzihradsky, 1993). There have also been reports of coupling of the delta opioid receptor transduces its signal through inhibitory G proteins, Gαi2, Gαi3, and Gαo in tumor cell lines (Taussig et al., 1992; Prather et al., 1994a) and in vivo (Heeschler et al., 1987; Sanchez-Blazquez and Garzon, 1993). The G proteins Gαo, Gαi1,2 and Gαi3 have also been found to mediate inhibition of adenyl cyclase by delta opioids (McKenzie and Milligan, 1990; Carter and Medzihradsky, 1993). There have also been reports of coupling of the delta opioid receptor

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3 The International Union of Pharmacology (IUPHAR) subcommittee on Opioid Receptors (Dhawan et al., 1996) recommended the name of OP receptor for the delta opioid receptor (OP for opioids, and the chronological order of the first formal demonstration of the existence of the receptors).

ABBREVIATIONS: BW373U86, (−)-4-[(α-R)-α-(22S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl-N,N-diethylbenzamide, methyl ether of (+)-BW373U86 (SN80); DSLET, [D-Ser2,L-Leu5]enkephalin-Thr; DPDPE, [D-Pen2,D-Pen5]enkephalin; pCl-PPDPE, [D-Pen2,pCl-Phe4,D-Pen5]enkephalin; DAMGO, Tyr-β-Ala-Gly-MePhe-Gly-ol; BNTX, 7-benzylidenenaltrexone; NTB, naltrexin, benzofuran derivative of naltrexone; SIOM, 7-spiroindinooxymorphine; ICI 174864, N,N-diallyl-Tyr-Ala-Phe-Leu-OH (Alb, a-aminoisobutyric acid); U69,593, 5α,7α,8β(-)-N-methyl-N(7-Cr-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzene acetamide; G protein, GTP binding protein; Gα, pertussis toxin-sensitive G protein that mediates adenylyl cyclase inhibition; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GTPγS, guanosine-5′-O-(3-thio)triphosphate; PTX, pertussis toxin; CTX, cholera toxin; A2 buffer, 128 mM NaCl, 2.4 mM KCl, 1.3 mM CaCl2, 2.0 mM NaHCO3, 3.0 mM MgSO4, 10 mM Na2HPO4, 10 mM glucose, 8 mM theophylline, pH 7.4; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; SINTX, 7-spiroindanylnaltrexone.
opioid receptor to the stimulatory G protein (Shen and Crain, 1990; Gintzler and Xu, 1991). In addition, intrathecal administration in mice of antisense oligodeoxynucleotides directed against \( G_{\alpha}\) blocked DPDPE-induced spinal analgesia (Standafer et al., 1996). Thus, we were interested in determining if the \( \delta \) opioid receptor expressed in C6 cells would interact with the stimulatory G protein.

Although definitive proof of \( \delta \) opioid receptor subtypes has yet to be provided, behavioral and biochemical data suggest the presence of two receptor subtypes (Sofuoglu et al., 1991; Jiang et al., 1991; Buzas et al., 1994; for review see Traynor and Elliott, 1993). Study of a homogeneous \( \delta \) receptor population was mostly limited to the NG108–15 neuroblastoma cell line until the cloning of the \( \delta \) opioid receptor (Evans et al., 1992; Kieffer et al., 1992). The pharmacological properties of the three cloned opioid receptors have also been investigated in COS monkey fibroblast cells and the CHO cell line (Raynor et al., 1994). They proposed that the cloned \( \delta \) opioid receptor has a pharmacological profile similar to that of the “\( \delta \)-2” opioid receptor subtype. In addition to examining the pharmacology in non-neural cell lines, the ligand binding was determined with use of conditions to maximize agonist interactions. We were interested in examining the pharmacological profile of the cloned rat \( \delta \) opioid receptor expressed in C6 glioma cells.

Although there is a fair amount of data describing ligand affinities at the \( \delta \) opioid receptor, there is less information regarding ligand efficacy. Drugs with similar receptor binding affinities are not necessarily equally efficacious in evoking behavioral or biochemical responses. Efficacy is of importance for potential analgesic drugs; maximally efficacious ligands may relieve more severe pain than weaker agonists. In a continuation of our interest in studying the opioid receptor in its native milieu, we examined ligand pharmacology and efficacy at the cloned rat \( \delta \) opioid receptor stably expressed in a rat C6 glioma cell line under somewhat more physiological conditions (100 mM sodium chloride and 50 \( \mu \)M GDP). Recent results from our laboratory (Yabaluri and Medzihradszky, 1997) support a role for extracellular sodium in regulating not only the ligand interactions with the receptor, but also signal transduction through the \( \mu \) opioid receptor. We estimated the efficacy of coupling to the G protein by measuring the agonist-stimulated binding of the nonhydrolyzable GTP analog, \([\text{\textsuperscript{35}S}] \text{GTP} \gamma \text{S}\), which has been an effective method of assessing \( \mu \) opioid efficacy in SH-SY5Y neuroblastoma cell membranes (Traynor and Nahorski, 1995). C6 glioma cells transfected with the cloned rat \( \mu \) opioid receptor (Emmerson et al., 1996), as well as in COS cells transiently expressing the mouse \( \delta \) opioid receptor (Befort et al., 1996). Under conditions of physiological extracellular sodium concentration and in the presence of GDP, we determined the efficiencies for stimulation of \([\text{\textsuperscript{35}S}] \text{GTP} \gamma \text{S}\) binding and the ligand affinities for the \( \delta \) opioid receptor, and found no evidence for the cloned receptor favoring \( \delta \)-1- or \( \delta \)-2-selective ligands. Given the robust signal, we were also able to determine efficacies for relatively weak agonists at the \( \delta \) opioid receptor. Given the essential role of receptor-G protein coupling in \( \delta \) opioid receptor signal transduction, the efficacies of opioid ligands at the \( \delta \) opioid receptor to activate G proteins in \textit{vitro} may be an important indicator of their in \textit{vivo} efficacy.

### Materials and Methods

**Materials.** \([\text{\textsuperscript{35}S}] \text{GTP} \gamma \text{S}\) (1300 Ci/mmol), \([\text{\textsuperscript{3}H}] \text{naltrindole}\) (32 Ci/mmol) and \([\text{\textsuperscript{3}H}] \text{pCIP-DPDP}\) (41 Ci/mmol) were purchased from DuPont NEN (Boston, MA). The cAMP assay kit was purchased from Diagnostic Products (Los Angeles, CA). BW373U86 was obtained from Burroughs Wellcome Co. (Research Triangle Park, NC). DPDP and pCIP-DPDP were generous gifts from H. Mosberg (University of Michigan). Naltrindole, NTB, BNTX, ICI 174864, SINTX, SIOM, SNC50, DSLET, deltorphin II, etorphine, U69,593, oxymorphone and diprenorphine were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI). Fetal bovine serum and Geneticin were purchased from Gibco Life Sciences (Gaithersburg, MD). Pertussis toxin and cholera toxin were purchased from List Biochemicals (Campbell, CA). DAMGO, DMEM, Trizma and other biochemicals were purchased from Sigma Chemical (St. Louis, MO).

**Cell culture.** The cDNA encoding the rat \( \delta \) opioid receptor was cloned by Meng et al. (1995), and the coding region is identical with the sequence reported by Fuku da et al. (1993). A pCMV-neo expression vector, courtesy of Dr. Mike Uhler (University of Michigan) (Huggenvik et al., 1991), was used to express the receptors in C6 glioma cells. Twenty micrograms of plasmid DNA were transfected into a 100-mm dish of cells by the method of Chen and Okayama (1987). Two days after transfection, cells were maintained in tissue culture medium (DMEM and 10% fetal bovine serum) with 1 mg/ml Geneticin for 14 days. After this selection period, individual colonies were removed and plated in 24-well plates, maintaining antibiotic selection pressure. Individual colonies were screened for opioid receptor binding, and a single clone (C6513) was used for this study.

Cells were grown to confluence under 5% \( \text{CO}_2 \) in DMEM containing 10% fetal bovine serum and either with 1 mg/ml Geneticin (for subculture) or without Geneticin (for harvest). The cells were typically subcultured at a ratio of 1:20 to 1:30 with partial replacement of the media on the day before subculturing or harvesting at day 5 or 6. Pertussis or cholera toxin treatment was carried out by addition of pertussis toxin (100 ng/ml) or cholera toxin (1000 ng/ml) at the time of media refreshment 24 hr before harvesting.

**Membrane preparation.** Plasma membranes were prepared by lysis of cells in isotonie sucrose (Emmerson et al., 1996). Cells were washed two times with ice-cold phosphate-buffered saline (0.9% NaCl, 0.61 mM NaHPO\(_4\), pH 7.4). Cells were detached from flasks by incubation in lifting buffer (5.6 mM glucose, 5 mM KCl, 5 mM HEPES, 137 mM NaCl, 1 mM ethyleneglycol-bis (2-aminoethyl ether)-\( \text{N}, \text{N}', \text{N}' \)-tetraacetic acid, pH 7.4) at 37°C and pelleted by centrifugation at 200 \( \times \) 3 min for 3 min. The cell pellet was resuspended in 10 volumes of ice-cold 0.32 M sucrose, 1 mM Tris HCl (pH 7.4) with a Teflon-glass dounce mounted to a Tri-R Stir-R motor at 1000 rpm. The suspension was then centrifuged for 10 min at 1000 \( \times \) g at 4°C, and the supernatant was removed and kept on ice. The resuspension and centrifugation was repeated with the remaining pellet an additional three times, saving the supernatant from each spin in tubes kept on ice, to further break up the membranes and increase the yield. The combined supernatants were then centrifuged at 15,000 \( \times \) g for 20 min at 4°C. After the centrifugation, the upper pellet was removed from the lower pellet by gently washing with ice-cold 0.32 M sucrose. The upper pellet was resuspended in 50 mM Tris HCl buffer (pH 7.4) and centrifuged 20 min at 20,000 \( \times \) g and 4°C. The final pellet was resuspended in 50 mM Tris buffer and frozen at –80°C in 0.5-ml aliquots (0.6–1.0 mg/ml).

Because of the great loss of cellular material during the sucrose lysis procedure, a crude membrane preparation was used for toxin-treated cells, with some loss in receptor density. Treated cells were collected as described above and resuspended in 10 volumes of hypotonic phosphate buffer (0.61 mM Na\(_2\)HPO\(_4\), 0.38 mM KH\(_2\)PO\(_4\), 0.2 mM MgSO\(_4\), pH 7.4) by glass-glass dounce and centrifuging for 20 min at 20,000 \( \times \) g at 4°C. The pellet was then resuspended in 50 mM Tris buffer, and aliquots of 0.3 to 0.6 mg/ml were frozen at –80°C.
Protein determination. Protein concentration was determined by the method of Lowry et al. (1951) with a bovine serum albumin standard. Samples were dissolved with 1 N NaOH for 30 min at room temperature before protein determination.

Receptor binding assay. Ligand binding was carried out as described previously (Fischel and Medzihradszky, 1981). The assay medium for determination of [3H]pCl-DPDPE binding contained membrane protein (8.3 μg, 41 fmol receptor) diluted in Tris-Mg buffer (50 mM Tris HCl, 5 mM MgCl₂, pH 7.4), 50 μM water- or unlabeled ligand (1 μM pCl-DPDPE final concentration for maximum specific displacement) and [3H]naltrindole (final concentration of 0.04 nM for the competition experiment or 0.01–1.4 nM for saturation curve) in 50 mM Tris HCl, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 50 μM GDP in a total volume of 2 ml. After the membranes were preincubated for 15 min at 25°C in the assay buffer, the binding was initiated by addition of unlabeled and radiolabeled ligands. After incubation for 90 min at 25°C to reach equilibrium, the samples were quickly filtered through glass fiber filters (Schleicher and Schuell no. 32, Keene, NH) mounted in a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). Each filter was removed and placed in a 5-ml polypropylene scintillation vial with 0.4 ml ethanol and 4 ml scintillation cocktail and subjected to liquid scintillation counting.

For the determination of Kᵢ values (0.04 nM [3H]naltrindole), seven concentrations of competing ligand in duplicate were included in the binding assay. Kᵢ values were calculated from the EC₅₀ for inhibition of the specific binding of 0.04 nM [3H]naltrindole in [35S]GTP-S binding assay buffer obtained from two to three experiments and analyzed by the one-site competition curve fit with GraphPad Prism (San Diego, CA).

[35S]GTP-S binding assay. Agonist stimulation of [35S]GTP-S binding was measured as described by Tian et al. (1994). Membranes (5–7 μg/tube) were mixed with ligand and preincubated for 10 min at 25°C. The experiment was initiated by the addition of assay buffer to yield a final concentration in 100 μl of 50 mM Tris HCl, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (added fresh), 50 μM GDP, 50 pM [35S]GTP-S (pH 7.4). Tubes were incubated for 30 min at 25°C and the reaction was terminated by diluting the sample with 2 ml of ice-cold 50 mM Tris HCl buffer containing 5 mM MgCl₂ and 100 mM NaCl and rapidly filtering the tube contents through glass fiber filters (Schleicher and Schuell no. 32). The filters were then washed an additional three times with 2 ml of buffer. Filters were placed in vials containing 400 μl ethanol and 4 ml Econo-Safe scintillation cocktail for liquid scintillation counting. Basal activity was defined as the difference between the [35S]GTP-S binding in the absence or presence of 50 μM unlabeled GTP-S. To determine the percent of increase in [35S]GTP-S binding over basal, the basal binding was subtracted from each point, and each value was divided by the basal value and then multiplied by 100%. The experiment was performed three to four times in duplicate.

Whole-cell adenylyl cyclase assay. Cells grown to confluence were washed twice with phosphate-buffered saline (as above) and lifted off the surface by incubation with lifting buffer as described above. The cell suspension was then centrifuged for 3 min at 200 × g and the pellet was resuspended in A2 buffer (128 mM NaCl, 2.4 mM KCl, 1.3 mM CaCl₂, 2.0 mM NaHCO₃, 3.0 mM MgSO₄, 10 mM Na₂HPO₄, 10 mM glucose, 8 mM theophylline, pH 7.4). After 10 min of preincubation at 37°C, inhibition of adenylyl cyclase activity was initiated by the addition of 50 μl of cells (5–10 μg protein) to 50 μl of A2 buffer with forskolin (final concentration, 10 μM) and opioid. The assay was terminated after 15 min (37°C) by the addition of 50 μl of ice-cold 0.15 M HCl. The samples were heated at 80°C for 3 to 4 min, and then frozen at −80°C overnight. After thawing, the samples were neutralized with 0.5 M Tris and the CAMP content was determined with a radioligand binding assay kit from Diagnostic Products (Los Angeles, CA). The experiment was performed four to five times in duplicate.

Data analysis. [35S]GTP·S binding and adenylyl cyclase data from three to five experiments were combined and fitted to a sigmoidal curve with a variable slope with use of GraphPad Prism, and the radioligand binding displacement curves were best fit to one-site competitive curves. Kᵢ values were calculated as IC₅₀/(1 + [L]/Kₐ) (Cheng and Prusoff, 1973) with 0.027 nM for the naltrindole Kₐ value. Saturation binding data for [3H]pCl-DPDPE and [3H]naltrindole were fit to a one-site binding hyperbola. Efficacy was calculated as the fraction of the maximum inhibition of adenylyl cyclase by BW373U86. Unpaired, two-tailed t tests comparing control with drug addition (fig. 4) or toxin treatment (fig. 5) were performed with the GraphPad Prism.

Results

Radioligand binding to the delta opioid receptor in C60 membranes. Equilibrium binding of antagonist [3H]naltrindole revealed a single population of saturable binding sites on membranes prepared from C6 glioma cells stably expressing the rat delta opioid receptor (fig. 1). Membrane preparations from these cells routinely expressed 4 to 6 pmol receptor/mg membrane protein. The binding affinity (0.03 nM) was similar to that found for [3H]naltrindole binding in monkey brain membrane preparations (0.04 nM, Emerson et al., 1994). Saturation binding of [3H]pCl-DPDPE in 50 mM Tris, pH 7.4, containing 5 mM MgCl₂ (Tris-Mg buffer), conditions which typically favor high-affinity binding, was also best described by a single saturable binding site. The Kₛ was 1.5 nM, which is similar to the Kₛ of 1.2 nM found for [3H]pCl-DPDPE in monkey cortex membranes (Emerson et al., 1994). The B₅₀ was 3.3 pmol/mg protein. (data not shown.) The binding affinity was determined with a radioligand binding assay kit from Diagnostic Products (Los Angeles, CA). The experiment was performed four to five times in duplicate.

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review). However, the subnanomolar binding of [3H]pCl-DPDPE in Tris-Mg buffer (table 1).

$K_i$ values for several agonists and antagonists were determined by displacement of 0.04 nM [3H]naltrindole (table 1). The binding assay buffer contained 100 mM NaCl and 50 μM GDP (as did the buffer for the stimulation of [35S]GTPγS binding). Antagonists naltrindole, NTB, BNTX and SINTX had subnanomolar $K_i$ values whereas the $K_i$ values for ICI 174,864, SIOM, oxymorphindole and diprenorphine were in the nanomolar range. Both $mu$ and $kappa$ selective ligands, DAMGO and U69,593, respectively, displayed very low affinity for the $delta$ receptor in the C6 glioma membrane preparation. However, the nonselective agonist, etorphine had a $K_i$ value of 40 nM. The higher affinity of the C6$delta$ receptor for DLETS than for DPDPE suggests that this receptor is of the delta-2 subtype (see Traynor and Elliot (1993); Zaki et al. (1996) for review). However, the subnanomolar $K_i$ for BNTX, a putative delta-1 selective ligand, and the high nanomolar $K_i$ for deltorphin II, a delta-2 selective ligand, suggest that the expressed receptor is a delta-1 subtype. In addition, some of the binding affinities measured here did not correlate with the binding affinities found in monkey membranes for the delta receptor (data not shown). The ratio of ligand $K_i$ values determined in the two different assay buffers did not correlate with the maximal stimulation of [35S]GTPγS binding (fig. 2a, table 1). The maximum increase in [35S]GTPγS binding of 0.10 pmol/mg was similar to the increase observed by $mu$ agonists in C6$mu$ cells, despite the higher level of receptor expression in the C6$mu$ cells (Emmerson et al., 1996). The peptide $delta$ opioid agonists BW373U86 and SNC80 stimulated basal [35S]GTPγS binding of 640 ± 20% (EC50, 1.3 nM) and 650 ± 20% (EC50, 57 nM), respectively, over basal [35S]GTPγS binding (fig. 2a, table 1). The maximum increase in [35S]GTPγS binding of 0.10 pmol/mg was similar to the increase observed by $mu$ agonists in C6$mu$ cells, despite the higher level of receptor expression in the C6$mu$ cells (Emmerson et al., 1996). The peptide $delta$ opioid agonists DLETS and pCl-DPDPE were also highly efficacious with a maximum stimulation of [35S]GTPγS binding of 576 ± 20% (EC50, 72 nM) and 510 ± 20% (EC50, 69 nM).

The putative $delta$-1 agonist DPDPE and putative delta-2 agonist deltorphin II were less efficacious in stimulation of [35S]GTPγS binding with maximum stimulations of 380 ± 20% (EC50, 332 nM) and 380 ± 10% (EC50, 98 nM), respectively (fig. 2b and table 1). DPDPE and deltorphin II also were partial agonists compared with SNC80 in 293S cells (see Traynor and Elliot (1993); Zaki et al. (1996)). The EC50 values for inhibition of the specific binding of 1 nM [3H]naltrindole in [35S]GTPγS binding assay buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (added fresh), 50 μM GDP) obtained from two to three experiments and analyzed by the one-site competition curve fit with Graph Pad Prism as described under “Materials and Methods.” The affinity for the $delta$ receptor in the C6 membrane preparation (Emmerson et al., 1996).

### TABLE 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding Affinity, K (95% CI) (nM)</th>
<th>[35S]GTPγS Binding</th>
<th>Adenylyl Cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Na⁺</td>
<td>−Na⁺</td>
<td>EC50 (95% CI) (nM)</td>
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<tr>
<td>BW373U86</td>
<td>0.45 (0.38–0.54)</td>
<td>0.85 (0.69–1.0)</td>
<td>1.3 (0.98–1.8)</td>
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<tr>
<td>SNC80</td>
<td>60 (45–80)</td>
<td>2.1 (1.7–2.5)</td>
<td>57 (43–74)</td>
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<tr>
<td>DLETS</td>
<td>93 (80–109)</td>
<td>1.9 (1.5–2.3)</td>
<td>72 (46–113)</td>
</tr>
<tr>
<td>pCl-DPDPE</td>
<td>135 (105–174)</td>
<td>–</td>
<td>69 (48–99)</td>
</tr>
<tr>
<td>DPDPE</td>
<td>487 (447–531)</td>
<td>8.0 (6.6–9.8)</td>
<td>332 (210–510)</td>
</tr>
<tr>
<td>Deltorphin II</td>
<td>81 (65–99)</td>
<td>4.9 (3.7–5.6)</td>
<td>8 (69–139)</td>
</tr>
<tr>
<td>Etophene</td>
<td>40 (36–44)</td>
<td>–</td>
<td>52 (27–96)</td>
</tr>
<tr>
<td>SIOM</td>
<td>8.4 (7.3–6.9)</td>
<td>–</td>
<td>12.5 (4.3–27)</td>
</tr>
<tr>
<td>Oxymorphindole</td>
<td>6.5 (6.0–7.1)</td>
<td>0.88 (0.80–0.96)</td>
<td>3.6 (1.2–11)</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1.6 (1.5–1.8)</td>
<td>–</td>
<td>1.5 (0.66–3.4)</td>
</tr>
<tr>
<td>DAMGO</td>
<td>&gt;10,000 (–)</td>
<td>–</td>
<td>&gt;5,000 (–)</td>
</tr>
<tr>
<td>U69,593</td>
<td>&gt;10,000 (–)</td>
<td>–</td>
<td>&gt;10,000 (–)</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>0.035 (0.032–0.038)</td>
<td>0.31 (0.24–0.39)</td>
<td>–</td>
</tr>
<tr>
<td>NTB</td>
<td>0.037 (0.034–0.042)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BNTX</td>
<td>0.079 (0.069–0.086)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SINTX</td>
<td>0.082 (0.069–0.094)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ICI 174,864</td>
<td>37 (29–47)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* $K_i$ values were calculated from the EC50 for inhibition of the specific binding of 0.04 nM [3H]naltrindole in [35S]GTPγS binding assay buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (added fresh), 50 μM GDP) obtained from two to three experiments and analyzed by the one-site competition curve fit with Graph Pad Prism as described under “Materials and Methods.” The 95% confidence intervals (95% CI), calculated with Graph Pad Prism, are given in parentheses.

* $K_i$ values were calculated from the EC50 for inhibition of the specific binding of 1 nM [3H]Cl-DPDPE in 50 mM Tris, pH 7.4, 5 mM MgCl2 obtained from two to three experiments and analyzed by the one-site competition curve fit with Graph Pad Prism as described under “Materials and Methods.” The affinity of [3H]Cl-DPDPE for the delta opioid receptor in Tris-Mg buffer was 1.5 nM. The 95% confidence intervals, calculated with Graph Pad Prism, are given in parentheses. The ratio of $K_i$ values in the presence and absence of sodium and GDP did not correlate with the efficacies to stimulate [35S]GTPγS binding (data not shown).

* The EC50 values for stimulation of [35S]GTPγS binding or inhibition of adenylyl cyclase activity were obtained by fitting the data from three to four experiments to dose-response curves with Graph Pad Prism as described under “Materials and Methods.” The 95% confidence intervals are given in parentheses.

* The efficacies were calculated as the maximum stimulation of [35S]GTPγS binding by the ligand/the maximum response by BW373U86.

* $-$, this parameter was not measured or could not be calculated for this ligand.
expressing the human delta opioid receptor (Payza et al., 1996).

Etorphine, a nonselective opioid agonist, had an even lower maximum stimulation value of 230 ± 20% (EC_50 52 nM). The kappa specific agonist, U69,593, and DAMGO, a selective mu opioid agonist, had EC_50 values greater than 5 μM. Maximal binding stimulation was not reached with 100 μM ligand (fig. 2b and table 1).

Oxymorphindole and SIOM are two structurally similar oxymorphone derivatives. Oxymorphindole, a partial agonist at the delta opioid receptor (Portoghese et al., 1988), had a low efficacy for stimulation of [35S]GTPγS binding with a maximum of only 73 ± 5% over basal, but unlike the non-delta selective agonists, it had a relatively low EC_50 of 3.6 nM. SIOM (Portoghese et al., 1993), a putative delta-1 selective agonist, is a potent partial agonist in the guinea pig ileum and a full agonist in the mouse vas deferens. SIOM also appeared to be a partial agonist in C6δ membranes, stimulating [35S]GTPγS binding by 120 ± 10% (EC_50 12.5 nM). Although both oxymorphindole and SIOM exhibited similar high-affinity binding for the C6δ receptor, they were both weak partial agonists. The lack of discrimination between the two ligands also agreed with the lack of receptor subtype displayed by the C6δ receptor.

In an acute sensitization behavioral assay, diprenorphine was a pure antagonist at the mu opioid receptor (White-Gbadebo and Holtzman, 1994). Although it exhibited no agonist actions in the mouse vas deferens preparation (Miller et al., 1986), diprenorphine exhibited weak partial agonist characteristics at the delta opioid receptor in that it was minimally, yet significantly efficacious at stimulation of [35S]GTPγS binding (51 ± 5%) with an EC_50 of 1.5 nM. Thus, despite high-affinity binding and nanomolar EC_50 values for stimulation of [35S]GTPγS binding, diprenorphine, oxymorphindole and SIOM functioned as partial agonists in this assay. Both partial and full agonists displayed a linear in-

Fig. 2. Stimulation of GTPγS binding. Plasma membranes were preincubated with opioids for 10 min at 25°C. [35S]GTPγS and the GTPγS binding assay buffer were added, and the samples were incubated for an additional 30 min at 25°C. The reactions were terminated by rapid filtration as described under “Materials and Methods.” The data are expressed as stimulation relative to GTPγS binding in the absence of added ligand (0.1–0.2 pmol/mg membrane protein). Shown are the mean and standard error for three to four experiments, each carried out in duplicate. The efficacy values are listed in table 1.
crease in stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding with increasing receptor occupancy ($(L)/K_i + [L]$) where $[L]$ is ligand concentration and $K_i$ is binding affinity), which indicates that no “spare receptors” are present under these assay conditions (data not shown).

Costa et al. (1990) found that the antagonists naloxone and ICI 174,864 functioned as reverse antagonists in a GTPase assay from NG108–15 cell membranes. In addition, ICI 174,864 inhibited basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 30% in a stable Rat 1 fibroblast clone expressing the mouse delta opioid receptor (Mullaney et al., 1996). We found no significant inhibition of basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by ICI 174,864 ($-7 \pm 10\%$) or naloxone ($0.6 \pm 12\%$). The effect of these antagonists on basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding was also measured in the presence of KCl instead of NaCl (Costa et al., 1990) because KCl was shown to magnify the effect of inverse agonists in the stimulation of GTPase activity in NG108–15 cells. We found no significant change in the effect of the antagonists on basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding (data not shown).

**Evaluation of the delta opioid receptor subtype.** To ensure that the difference in efficacies between the presumably full delta opioid agonists were not caused by the presence of both delta-1 and delta-2 receptors, and to assess the delta receptor subtype in this preparation, we antagonized $[^{3}\text{H}]\text{naltrindole}$ binding as well as the stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding with selective and nonselective antagonists. If there were more than one subtype present, we would expect an antagonist selective for one subtype to be less potent in antagonizing the effect of a different receptor subtype. The antagonists naltrindole (both delta-1 and delta-2), NTB (delta-2 selective) and BNTX (delta-1 selective) displayed similar relative potencies for inhibition of $[^{3}\text{H}]\text{naltrindole}$ binding (table 1, fig. 3c). However, the relative potencies of the selective antagonists, NTB and BNTX, did not change whether they were antagonizing a delta-1 selective agonist or a nonselective (delta-1 and delta-2) agonist for delta opioid receptor subtypes. Both selective and nonselective antagonists were able to completely inhibit stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by maximally efficacious concentrations of BW373U86 (delta-1 and delta-2) or DPDPE (delta-1 selective) (fig. 3, a and b). The $K_B$ values for inhibition of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding stimulation by 10 nM BW373U86 were $-8.53$, $-8.56$ and $-8.14$ for naltrindole, NTB and BNTX, respectively. The $K_B$ values for inhibition of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding stimulation by 3 $\mu M$ DPDPE were similar at $-8.74$, $-8.76$ and $-8.54$ for naltrindole, NTB and BNTX, respectively. In contrast, BNTX possessed a 100-fold greater affinity for $[^{3}\text{H}]\text{DPDPE}$ binding sites (delta-1) relative to those of $[^{3}\text{H}]\text{DSLET}$ (delta-2) in guinea pig brain membranes (Portoghese et al., 1992).

**Verification of partial agonism.** To verify partial agonist characteristics of oxymorphindole, deltorphin II and DPDPE in this assay, the following experiments were performed. We measured the effect of 10 $\mu M$ oxymorphindole on the stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by 10 nM BW373U86 or 3 $\mu M$ DPDPE, concentrations which induced nearly maximum stimulation. The presence of 10 $\mu M$ oxymorphindole reduced the stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by BW373U86 and DPDPE to $9 \pm 1\%$ and $17 \pm 5\%$ that of full agonist alone, respectively (fig. 4). Deltorphin II and DPDPE, which appear to be partial agonists compared to BW373U86, also antagonized the stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by BW373U86 to the level stimulation observed with 10 $\mu M$ deltorphin II or 10 $\mu M$ DPDPE alone (fig. 4). Antagonist ICI 174,864 (10 $\mu M$) was able to completely inhibit stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by DPDPE (fig. 4), but not BW373U86 (not shown). However, 90 $\mu M$ ICI 174,864 did completely inhibit stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by BW373U86 (fig. 4). The decreased inhibition of BW373U86 by ICI 174,864 was possibly caused by a slower ligand dissociation rate observed with BW373U86 (Childers et al., 1993). Furthermore, addition of both 10 $\mu M$ deltorphin II and 3 $\mu M$ DPDPE did not significantly increase the stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding.
binding compared with 3 μM DPDPE alone (fig. 4). If DPDPE and deltorphin II acted through separate receptor types in this system, we would expect their effects to be at least partially additive. However, there was no additive effect on stimulation by the deltorphin II and DPDPE, providing further evidence for partial agonist actions by these ligands, as well as for a single delta opioid receptor subtype present in these cells.

Treatment of C6δ cells with pertussis toxin and cholera toxin. To determine whether the stimulation of [35S]GTPγS binding in the membranes is caused by coupling of the delta opioid receptor to the pertussis toxin-sensitive G proteins, G," or whether there is some stimulation of the cholera toxin-sensitive stimulatory G protein, Gs, cells were pretreated with pertussis or cholera toxin. Pertussis toxin pretreatment completely inhibited the stimulation of [35S]GTPγS binding by 1 μM BW373U86, 10 μM DSLET and 10 μM DPDPE (fig. 5), which indicates that the stimulation of [35S]GTPγS binding by both full and partial agonists is mediated by pertussis toxin-sensitive G proteins. Treatment with cholera toxin reduced the basal and stimulated [35S]GTPγS binding with BW373U86 and DSLET (fig. 5). After cholera toxin pretreatment, the apparent percent stimulation (expressed as percent of basal binding) actually increased because the basal [35S]GTPγS binding decreased. Although there is basal [35S]GTPγS binding to Gs, pertussis toxin pretreatment completely eliminated agonist-stimulated [35S]GTPγS binding, which indicates that opioid receptor activates only pertussis toxin inhibitory (Gt/Gi) G proteins.

Inhibition of adenylyl cyclase activity in whole cells. To determine whether the greater stimulation of [35S]GTPγS binding by BW373U86 and SNC80 in membranes resulted in greater inhibition of adenylyl cyclase activity in intact cells than by DPDPE, cAMP accumulation in whole cells in the presence of BW373U86, SNC80 or DPDPE was measured. BW373U86 inhibited forskolin-stimulated adenylyl cyclase activity by 79 ± 9% with an EC50 of 0.9 nM (fig. 6 and table 1). As with [35S]GTPγS binding, SNC80 inhibited adenylyl cyclase activity to nearly the same extent (71 ± 5%), but was less potent (EC50, 15 nM). Surprisingly, DPDPE similarly inhibited adenylyl cyclase activity by 72 ± 6% with an EC50 of 80 nM (fig. 6). Therefore, the greater stimulation of [35S]GTPγS binding by BW373U86 than with DPDPE did not result in greater inhibition adenylyl cyclase activity.

Discussion

The purpose of this study was to characterize the efficacy of G protein coupling of the cloned delta opioid receptor stably expressed in a C6 glioma cell line. The C6δ cells highly express a homogeneous population of delta opioid receptor and thus provide an excellent means to assess efficacy of ligands at the delta opioid receptor. The magnitude of receptor-stimulated [35S]GTPγS binding in the C6δ membrane preparation enables one to evaluate efficacy at the delta opioid receptor for opioid ligands including very weak partial agonists. We found that both peptide and nonpeptide ligands were maximally efficacious in the stimulation of [35S]GTPγS binding by 72 ± 6% with an EC50 of 80 nM (fig. 6). Previously, our laboratory has shown that C6 cells provide a suitable environment for the mu opioid receptor, which was similarly transfected into the C6 cells at a high receptor density (Emmerson et al., 1996). Recently, the stimulation of [35S]GTPγS binding by opioid agonist was also characterized for the mouse delta receptor transiently expressed in COS cells (Be- foret et al., 1996).

Ligand binding affinities for naltrindole and pCl-DPDE measured in the membranes from C6 cells transfected with the cloned delta opioid receptor were similar to those found in monkey membranes. For example, the Kd of [3H]pCl-DPDE in the absence of sodium was 1.5 nM in membranes from C6δ and 1.2 nM in monkey brain membranes (Emmerson et al., 1994). However, the binding affinities of other ligands measured here did not correlate with the binding affinity found in monkey membranes for the delta receptor, which indicates that the cloned delta receptor differs pharmacologically from those characterized in the monkey membrane preparation, despite their characterization in a cell line of glial origin. In
addition, little correlation was found between the literature $K_i$ values for delta receptor ligands measured in the membranes from CHO cells transfected with the mouse delta opioid receptor and those measured in other membrane preparations (Raynor et al., 1994). The differences in receptor pharmacology may be caused by the heterogeneous population of delta receptor subtypes in other systems, different post-translational modifications or unknown proteins associated with the receptor.

Raynor et al. (1994) proposed that the cloned mouse delta receptor is of the delta-2 subtype. Although we saw a similar trend in the binding data regarding receptor subtype (i.e., deltorphin II and NTB having higher affinities than DPDPE and BNTX), the efficacies of deltorphin II and DPDPE to stimulate $[^{35}\text{S}]\text{GTP} \gamma S$ binding were identical. Because of these equal efficacies and the greater efficacy of ligands that bind to both subtypes, i.e., BW373U86 and SNC80 (Bilsky et al., 1995), we were concerned about the possibility of the presence of both delta receptor subtypes, perhaps because of a posttranslational modification. However, naltrindole, NTB and BNTX antagonized stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding by BW373U86 or DPDPE with equal potencies. Furthermore, the intrinsic activities of DPDPE and deltorphin II were not additive, which indicates that their action was through the same receptor. Based on these data, we are unable to conclude that the cloned delta receptor is of the delta-2 or delta-1 subtype. Although substantial evidence from rat and mouse both in vivo (Mattia et al., 1991; Sofuoglu et al., 1991) and in vitro (Buzas et al., 1994) studies indicates the existence of delta opioid receptor subtypes, this clone does not exhibit the characteristics of one subtype, which suggests that perhaps neuroanatomical and subcellular localization of the receptor may contribute to the observation of receptor subtypes (Zaki et al., 1996).

Although the $[^{35}\text{S}]\text{GTP} \gamma S$ binding data in membranes from the C66 cells suggest that the receptor-G protein interaction was functional, the data do not discriminate which population of G proteins were interacting with the delta opioid receptor. There is evidence that the delta opioid receptor may couple to $G_s$ (Shen and Crain, 1990; Gintzler and Xu, 1991; Standifer et al., 1996). Cruciani et al. (1993) found that opioid receptors couple to both cholera toxin- and pertussis toxin-sensitive G proteins in F-11 (neuroblastoma-dorsal root ganglion neuron) hybrid cells. Specificity of G protein coupling did not appear to depend on the level of delta opioid receptor expression. Prather et al. (1994b) showed that overexpression of the delta opioid receptor in neuroblastoma and neuroblastoma × glioma cell lines does not alter the population of G proteins that couples to the delta opioid receptor. In addition, Befort et al. (1996) demonstrated that transiently expressed mouse delta opioid receptor (25 pmol receptor/mg protein) showed similar agonist efficacy and maximal stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding compared with the receptor stably expressed in CHO cells (4 pmol receptor/mg protein). In C6 cells transfected with high levels of the mu opioid receptor, the receptor retained its specificity for inhibitory G proteins (Emmerson et al., 1996); however, cells expressing high levels of alpha-2 adrenergic receptor did not (Eason et al., 1992). In this study, pertussis toxin treatment completely abolished agonist stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding, which demonstrated that the delta opioid receptor is only coupled to inhibitory (G$i$/G$s$) G proteins.

Despite the high levels of receptor expression, we found no evidence of spare receptors. As receptor occupancy increased, stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding also increased with a linear correlation where maximal receptor occupation was necessary for maximal stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding. Given the differential sensitivity of ligands to sodium and guanine nucleotides (Childers et al., 1993; Emmerson et al., 1994), $K_i$ values were determined by displacement of $[^{3}H]$naltrindole under conditions identical with the $[^{35}\text{S}]\text{GTP} \gamma S$ binding assay. The $K_i$ values were similar to the $EC_{50}$ values for stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding. Thus, receptor occupancy $(|L|)/(K_i + |L|)$ where $|L|$ is ligand concentration and $K_i$ is binding affinity) correlated closely with stimulation of $[^{35}\text{S}]\text{GTP} \gamma S$ binding. In addition, there appears to be a relationship between the potency of agonist to inhibit adenyl cyclase and the potency for activation of G proteins.

Although we observed a wide range of responses for $[^{35}\text{S}]\text{GTP} \gamma S$ binding stimulation by agonists, the differences did not carry over to inhibition of adenylyl cyclase activity. We found no significant difference between the maximum inhibition of adenylyl cyclase by BW373U86, SNC80 or DPDPE. Knapp et al. (1995) also found similar maximum inhibition of adenylyl cyclase by SNC80 and pCI-DPDPE in CHO cells stably transfected with the human delta opioid
receptor. SNC80 and DPDPE were also fully efficacious in bioassays in mouse vas deferens and guinea pig ileum preparations (Bilsky et al., 1995). In addition, the relative efficacies of several peptide ligands, including deltorphin II and DPDPE, were indistinguishable in the mouse vas deferens bioassay (Kramer et al., 1993). The full agonist properties of DPDPE could possibly be caused by an excess number of G proteins relative to adenylyl cyclase, thus an apparent partial agonist for stimulation of [35S]GTPγS binding could maximally inhibit adenylyl cyclase. We did not determine the Kᵣ values for the ligands in the adenylyl cyclase assay buffer so we could not construct an occupancy-response curve to determine whether spare receptors were present in the inhibition of adenylyl cyclase.

By the characterization of the binding affinity and efficacy at the δ opioid receptor of a wide range of opioids, the results of this study contribute to the assessment of opioid efficacy in stimulating G protein, a first step in the signal transduction cascade. An increased understanding of the mechanism of δ opioid receptor-mediated signal transduction at the cellular level will promote the use of δ opioid ligands as pharmaceutical tools and potential therapeutic agents.

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References


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