**Delta** Opioid Receptor Down-Regulation Is Independent of Functional G Protein yet Is Dependent on Agonist Efficacy

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**ABSTRACT**

Chronic treatment of C6 glioma cells stably expressing the rat delta opioid receptor (C6δ) with full agonists resulted in receptor down-regulation. Chronic (D-Ser²,L-Leu⁵)enkephalyl-Thr treatment caused a decrease in cell surface as well as a decrease in agonist-stimulated ([³⁵S]guanosine-5'-O-(3-thio)triphosphate binding. Treatment with full agonists for 12 hr resulted in a 90% decrease in receptor number that was paralleled by a decrease in the ability of agonist to stimulate ([³⁵S]guanosine-5'-O-(3-thio)triphosphate binding and inhibit forskolin-stimulated adenyl cyclase. Of the remaining receptors, a smaller fraction of receptors (41 ± 4 vs. 56 ± 4% in control) exhibited high affinity for agonist as compared to receptors in control membranes. Elimination of functional guanosine triphosphate binding protein (G protein) by Pertussis toxin pretreatment did not alter the ability of agonist to down-regulate receptor. We hypothesized that agonist affinity (not efficacy) would be a predictor of agonist's ability to down-regulate receptor. However, we found that only full agonists were able to down-regulate receptor number, G protein activation and adenyl cyclase inhibition. Chronic exposure to partial agonist 7-spiroindinoxymorphine, which has a very high affinity for the receptor, as well as morphine, did not cause receptor down-regulation. Taken together, these results suggest that full agonists alter receptor conformation such that the altered conformation is recognized by G protein as well as proteins involved in receptor down-regulation. In addition, down-regulation is independent of agonist-mediated G protein activation and subsequent downstream signaling.

Activation of the *delta* opioid receptor is suggested to play a role in multiple behavioral and physiological effects ranging from analgesia and mood-driven behavior 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. *Delta* opioids, acting at the *delta* opioid receptor, have been shown to mediate the inhibition of forskolin-stimulated adenyl cyclase (Evans et al., 1992), an increase in the production of inositol phosphates (Tsu et al., 1995), as well as modulation of ion channel opening (Tausiug et al., 1992).

The responsiveness of opioid receptors to opioids is altered by chronic exposure to agonist (for review, see Zadina et al., 1996). Agonist exposure leads to a loss of coupling to G proteins and subsequent effector protein interaction, as well as a decrease in the number of cell surface receptors. In addition, chronic agonist exposure elicits changes in post receptor, intracellular messenger pathways (for review, see Nestler, 1996). Many aspects of chronic agonist treatment have been characterized in NG108-15 cells which express the *delta* opioid receptor. More recently, *delta* opioid receptor down regulation has been characterized in human embryonic kidney (HEK293) cells, Chinese hamster ovary cells and Neuro2A cells stably expressing the mouse *delta* opioid receptor (Bot et al., 1997; Trapaidze et al., 1996; Chakrabarti et al., 1997). After the binding of agonist, a majority of receptors internalize within 30 min. The C terminal tail of the receptor (Thr³⁵³) is required for this internalization process (Trapaidze et al., 1996; Cvejic et al., 1996). Degradation of the receptor most likely accounts for the decrease in a majority of the cell surface receptors after long-term exposure to agonist. Thr³⁵³ may be phosphorylated or may be part of a recognition site for a cellular factor involved in the process of receptor down-regulation. After agonist exposure, the degree of phosphorylation of the human *mu* opioid receptor expressed in

**ABBREVIATIONS:** C6δ, C6 glioma cells stably expressing the rat *delta* opioid receptor; DSLET, [D-Ser²,L-Leu⁵]enkephalyl-Thr; SIOM, 7-spiroindinoxymorphine; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; SNC80, methyl ether of ((2S*,5R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-N,N-diethylbenzamide (BW373U86); G protein, GTP binding protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GTPγS, guanosine-5’-O-(3-thio)triphosphate; PTX, Pertussis toxin; A2 buffer, 128 mM NaCl, 2.4 mM KCl, 1.3 mM CaCl₂, 2.0 mM NaHCO₃, 3.0 mM MgSO₄, 10 mM NaHPO₄, 10 mM glucose, 8 mM theophylline, pH 7.4. PBS, phosphate-buffered saline.; HEPES, (N-[2-Hydroxyethyl]piperazine-N’-[2-ethane sulfonic acid])
Chinese hamster ovary cells was found to correlate with agonist efficacy (Yu et al., 1997).

We have stably expressed the rat mu and delta opioid receptor in C6 glioma cells and characterized the efficacy of several opioid ligands at the mu and delta opioid receptors (Emmerson et al., 1996; Clark et al., 1997). Furthermore, we found that the mu opioid receptor down-regulation in response to chronic agonist was dependent on agonist efficacy yet independent of the presence of a functional G protein. Pertussis toxin pretreatment did not prevent agonist stimulated-receptor internalization (Yabaluri and Medzihradsky, 1997). In addition, the efficacy of agonist to stimulate cAMP accumulation (Bot et al., 1997) was not necessary for agonist induced-receptor down-regulation. The ability of an agonist potency to produce the remaining response was identical suggesting a complex mechanism of receptor down-regulation. This is in contrast to the requirement for a functional G protein in agonist-mediated down regulation of the mu opioid receptor stably expressed in Neuro2A cells (Chakrabarti et al., 1997). In addition Chakrabarti et al. (1997) found that Pertussis toxin pretreatment did not eliminate delta opioid receptor-G protein coupling and therefore they were unable to evaluate down-regulation of the delta opioid receptor in the absence of functional G protein. Thus, the requirement for a functional G protein-coupled receptor in the process of receptor down-regulation is unclear.

In this study, we addressed both the functional state of the remaining receptors after chronic full and partial agonist treatment as well as the role of G protein in delta opioid receptor down-regulation. C6 glioma stably expressing the delta opioid receptor were used in this study. Although binding and either GTPγS binding (Breivogel, et al., 1997) or cAMP accumulation (Bot et al., 1997) have been evaluated after chronic agonist treatment, we evaluate coupling to both G protein and adenyly cyclase after agonist treatment. In addition, recent evidence from Chakrabarti et al. (1997) implicated the formation of a high affinity agonist/receptor/G protein complex as a necessary step in agonist-mediated receptor down regulation in Neuro2A cells stably expressing the delta opioid receptor. We wanted to evaluate the role of G protein in agonist-stimulated receptor down regulation in a different cell line.

We show that after chronic agonist treatment, agonist regulation of [35S]GTPγS binding and forskolin-stimulated cAMP accumulation was greatly attenuated; however, the agonist potency to produce the remaining response was identical to that in control cells. In addition, inhibitory G protein was not necessary for agonist induced-receptor down-regulation, indicating that agonist occupied receptor may be sufficient to trigger receptor internalization. The ability of an agonist to down-regulate receptor roughly correlated with its efficacy in the inhibition of adenyly cyclase and the stimulation of GTPγS binding.

**Materials and Methods**

**Materials.** [35S]GTPγS (1250 Ci/mmol) and [3H]naltrindole (33 Ci/mmol) were purchased from Du Pont (Boston, MA). The cAMP assay kit was purchased from Diagnostic Products (Los Angeles, CA). DPDPE was a generous gift from H. Mosberg (University of Michigan). Naltrindole, SNC80, DSLET and naltrexone were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI). Geneticin was purchased from Mediatech, Inc. (Herndon, VA). Pertussis toxin was purchased from List Biochemicals (Campbell, CA). Fetal bovine serum, Dulbecco's modified Eagles medium, Trizma and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell culture.** The rat delta opioid receptor was cloned by Meng et al. (1995) and was stably expressed in C6 glioma cells (clone C6613, Clark et al., 1997). Cells were grown to confluency under 5% CO2 in Dulbecco’s modified Eagles medium 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 day 3 and on the day before harvesting. Cells were harvested or passaged on day 5 or 6. Pertussis toxin treatment was carried out by addition of Pertussis toxin (100 ng/ml) at the time of media refreshment 24 hr before harvesting. The effect of DSLET exposure was evaluated by incubation of the cells in the presence of 1 μM DSLET for 0, 4, 12 or 24 hr. In cells treated with both Pertussis toxin and DSLET, the toxin was added 12 hr before agonist exposure.

**Membrane preparation.** Cells were washed four times with PBS (0.9% NaCl, 0.61 mM Na2HPO4, 0.38 mM KH2PO4, 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 EGTA, pH 7.4) at 37°C and pelleted by centrifugation at 200 × g for 3 min. The cells were resuspended in 20 ml (per 10 cm dish cells) buffer (128 mM NaCl, 2.4 mM KCl, 1.3 mM CaCl2, 2.0 mM NaHCO3, 30 mM MgSO4, 10 mM Na2HPO4, 10 mM glucose, pH 7.4) and incubated for 10 min at 37°C to remove any residual ligand. Cells were then pelleted and either 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) for the adenyly cyclase assay (see below) or else Dounce homogenized in volumes of hypotonic phosphate buffer (0.61 mM Na2HPO4, 0.38 mM KH2PO4, 0.2 mM MgSO4, pH 7.4). The resulting membranes were collected by centrifugation for 20 min at 20,000 × 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) using a bovine serum albumin standard. Samples were dissolved in 1 N NaOH for 30 min at room temperature before protein determination.

**Whole cell binding assay.** To evaluate the effect of agonist treatment on cell surface receptor number, 1 × 106 C6 cells were plated into 24-well dishes and grown for 3 days. Media was exchanged and cells were cultured in the presence of drug for 0, 4 and 12 hr. Media were removed and the cells were washed 5 × 1 min with room temperature DMEM. Five nM [3H]naltrindole were incubated with the cells in PBS containing 1% bovine serum albumin (0.3 ml total volume). Ten μM naltrindole were used to determine nonspecific binding. After a 30-min room temperature incubation, the cells were washed five times one minute with DMEM. One ml of trichloroacetic acid (5%) was added to each well and the plate was incubated for 10 min at 4°C. The contents of each well was washed five times with cold TCA before harvesting. The effect of DSLET exposure was evaluated by incubation of the cells in the presence of 1 μM DSLET for 0, 4, 12 or 24 hr. In cells treated with both Pertussis toxin and DSLET, the toxin was added 12 hr before agonist exposure.

**Receptor binding assay.** Ligand binding was carried out as described previously (Fischel and Medzihradsky, 1981). In brief, the assay medium for determination of [3H]naltrindole binding contained membrane protein (10–25 μg) diluted in either Tris-Mg buffer (50 mM Tris HCl, 5 mM MgCl2, pH 7.4) or Tris-Mg buffer with 100 mM NaCl, 100 μl water or unlabeled ligand (1 μM naltrindole final concentration for maximum specific displacement) and 100 μl [3H]naltrindole (0.01–3.5 nM) in a final volume of 2 ml. [3H]Naltrindole (0.04 nM) displacement by DSLET was measured in Tris-Mg buffer with 100 mM NaCl in the absence or presence of 10 μM GTPγS. 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.
concentrations of 50 mM Tris HCl, 100 mM NaCl, 5 mM MgCl2, ice-cold 50 mM Tris HCl buffer containing 5 mM MgCl2 and 100 mM NaCl and the reaction was terminated by diluting the sample with 2 ml of cold 8 mM theophylline. Inhibition of adenylyl cyclase activity was initiated by the addition of 50 mM Tris HCl, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (added fresh), 50 µM GDP and 50 pM [35S]GTPyS (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 & Schuell no. 32). The filters were then washed an additional three times with 2 ml of buffer. Filters were placed in vials and 400 µl ethanol and 4 ml Econo-Safe scintillation cocktail were added for liquid scintillation counting. Specific binding was defined by the difference between the [35S]GTPyS binding in the absence or presence of 50 µM unlabeled GTPyS. The experiment was performed three times in duplicate or triplicate.

Adenylyl cyclase assay. Cells were washed four times with PBS, collected and resuspended in A2 buffer (125 mM NaCl, 2.4 mM KCl, 1.3 mM CaCl2, 2.0 mM NaHCO3, 3.0 mM MgSO4, 10 mM Na2HPO4, 10 mM glucose, pH 7.4) as described for membrane preparation, followed by a 10-min of incubation at 37°C. The cells were pelleted and resuspended in A2 buffer containing 8 mM theophylline. 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 using a radioligand binding assay kit from Diagnostic Products (Los Angeles, CA). The experiment was performed three times in duplicate on three different batches of control and treated cells for the dose response curves and in two different batches of control and treated cells in triplicate for the evaluation of Pertussis toxin treatment on chronic agonist treatment.

Data analysis. [35S]GTPyS binding and adenylyl cyclase data from three experiments were combined and fit to a sigmoidal curve using GraphPad Prism (GraphPad, Inc., San Diego, CA). 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 Kd values (0.04 nM [3H]naltrindole) seven concentrations of competing ligand in duplicate were included in the binding assay. The individual experiments were normalized based on the amount of 0.04 nM [3H]naltrindole binding in control membranes and the data combined and analyzed using the one-site competition curve fit using Graph Pad Prism (GraphPad, Inc., San Diego, CA). Data obtained from three experiments in three different membrane preparations from control and DSLET-treated cells was combined and Kd values were calculated as IC50/Ka + [3H]H/L/Ka (Cheng and Prusoff, 1973) using 0.038 nM for the naltrindole Kd value.

[35S]GTPyS binding assay. Agonist stimulation of [35S]GTPyS binding was measured as described by Tian et al. (1994). Membranes (10–15 µg/tube) were mixed with ligand and assay buffer and preincubated for 10 min at 35°C. The experiment was initiated by the addition of [35S]GTPyS to yield a final volume of 100 µl with final concentrations of 50 mM Tris HCl, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (added fresh), 50 µM GDP and 50 pM [35S]GTPyS (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 & Schuell no. 32). The filters were then washed an additional three times with 2 ml of buffer. Filters were placed in vials and 400 µl ethanol and 4 ml Econo-Safe scintillation cocktail were added for liquid scintillation counting. Specific binding was defined by the difference between the [35S]GTPyS binding in the absence or presence of 50 µM unlabeled GTPyS. The experiment was performed three times in duplicate or triplicate.

Results

When C66 cells were exposed to either DSLET or DPDPE (1 µM each), there was a decrease in the number of cell surface receptors (fig. 1A). The half-time of receptor number decrease was approximately 3 hr. Although DSLET and DPDPE stimulated [35S]GTPyS binding to differing extents in these cells, where DSLET was more efficacious (data not shown), both were equally efficacious in the inhibition of forskolin-stimulated cAMP accumulation (Clark et al., 1997). We observed no difference in the receptor down-regulation caused by treatment with these two agonists. To evaluate receptor-G protein coupling, membranes were prepared from control cells and cells treated for either 0, 4 or 12 hr with DSLET. The stimulation of [35S]GTPyS binding by DSLET was evaluated and the maximal stimulation is shown in figure 1B. The maximal loss of agonist-stimulated [35S]GTPyS binding occurred within 4 hr.

What is the functional state of the remaining receptors after 12-hr exposure to agonist? To answer this question, we evaluated the stimulation of [35S]GTPyS binding and the inhibition of forskolin-stimulated cAMP accumulation by DSLET. Although greatly attenuated, the remaining receptors maintain a comparable ability to couple to effectors as as assessed by relative EC50 values in control and agonist-treated cells (fig. 2). The ability of DSLET to stimulate [35S]GTPyS binding in membranes from DSLET-treated cells was reduced 10-fold; however, the EC50 values were similar. In addition, Pertussis toxin pretreatment eliminated agonist-
stimulation (and EC50 values) for the membranes prepared from control concentration of DSLET was determined relative to control membranes. The action in control and DSLET-treated cells was 0.295

assay was performed in duplicate except that the forskolin-stimulation from three different cell treatments were averaged by normalizing the washed and membranes prepared as described in “Materials and Methods.” Data from three experiments were combined and the data fit to a one site binding hyperbola. Shown

Fig. 2. Reduced G protein coupling and adenylyl cyclase inhibition after chronic agonist treatment. A, C6 cells were incubated with (●) or without (●) 1 μM DSLET for 12 hr. In addition, some cells (open symbols) were also pretreated with 100 ng/ml Pertussis toxin. The cells were then washed and membranes prepared as described in “Materials and Methods.” Stimulation of 0.05 nM [35S]GTP-g binding by the indicated concentration of DSLET was determined relative to control membranes. The data were fit to a sigmoidal dose-response curve. The basal [35S]GTP-g binding in control, DSLET-treated and Pertussis toxin-treated cells was 6.7 ± 0.3, 5.7 ± 0.5 and 3.3 ± 0.1 pmol/mg, respectively. The maximum stimulation (and EC50 values) for the membranes prepared from control and agonist-treated cells are as follows: 438 ± 29% (79 nM) and 40 ± 5% (106 nM). Shown is the average results from two experiments performed on two different membrane preparations treated with Pertussis toxin and the average results from three experiments performed on three different membrane preparations for the control and DSLET-treated cells. B, after a 12-hr incubation with (●) or without (●) 1 μM DSLET, the cells were washed and the forskolin-stimulated cAMP accumulation was measured as described in “Materials and Methods.” Data from three experiments from three different cell treatments were averaged by normalizing the data relative to control membrane forskolin stimulation. Each individual assay was performed in duplicate except that the forskolin-stimulation was measured in triplicate. Forskolin-stimulated cAMP concentration in control and DSLET-treated cells was 0.285 ± 0.062 pmol/mg and 0.379 ± 0.049, respectively.

stimulated [35S]GTP-g binding. DSLET was maximally efficacious in the inhibition of forskolin-stimulated adenylyl cyclase in control membranes (fig. 2B) as well as SNC80, BW373U86 and DPDPE (Clark et al., 1997). All inhibited forskolin-stimulated adenylyl cyclase approximately 80%. Although smaller than the cAMP “overshoot” observed in C6 cells expressing the mu opioid receptor (Yabaluri and Medzhitovksky, 1997), an increase of approximately 30% was observed in agonist treated cells after forskolin stimulation. Forskolin-stimulated cAMP accumulation was inhibited by 72 ± 2 and

20 ± 4% in control and agonist-treated cells with EC50 values of 4 and 2 nM, respectively. The 95% confidence intervals for the EC50 values overlapped indicating that there is not a significant difference in DSLET potency to inhibit forskolin-stimulated adenylyl cyclase. Although the decreased cAMP accumulation in agonist-treated cells was only 28% of that of control cells, the inhibition was blocked by 10 μM NTI (n = 2, data not shown) indicating that the inhibition was mediated by the remaining delta opioid receptors. Thus, although the agonist-stimulated [35S]GTP-g binding was greatly attenuated and the agonist-mediated inhibition of forskolin-stimulated cAMP accumulation was decreased in tolerant cells, the potency of agonist in these cells was unchanged indicating that the remaining opioid receptors in chronic agonist-treated cells are functionally identical to receptors in control cells. This result was substantiated by the fact that the receptors remaining after chronic agonist treatment had identical affinity for antagonist [3H]naltrindole (table 1) and that only small changes in agonist binding were observed (table 2).

Proposed molecular mechanisms leading to receptor internalization and functional uncoupling from effecter proteins include 1) the formation of high affinity agonist/activated receptor/G protein complexes and/or 2) receptor conformational changes induced by agonists (Yu et al., 1997) including the dissociation of receptor dimers (Cvejic and Devi, 1997). Using Pertussis toxin-pretreated membranes, we were able to address the first hypothesis. To evaluate the role of G protein in the receptor down regulation process, cells were treated with Pertussis toxin (100 ng/ml) for 12 hr followed by incubation with agonist (1 μM DSLET, in the presence of Pertussis toxin) for an additional 12 hr. [3H]naltrindole binding revealed that Pertussis toxin treatment was unable to block receptor down regulation in agonist-treated cells. (fig. 3A). Identical results were observed in Neuro2a cells stably expressing the delta opioid receptor (Chakrabarti et al., 1997). To determine if inhibitory G proteins were necessary to observe the increased forskolin-stimulated cAMP accumulation in chronic agonist-treated cells, forskolin-stimulated cAMP accumulation was evaluated. As expected, inactivation of Gi/Go G proteins by Pertussis toxin pretreatment elimi-

### TABLE 1

[3H]Naltrindole equilibrium binding in membranes from control, agonist- and Pertussis toxin-treated C6 cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Kd (nM)</th>
<th>Bmax (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.037 ± 0.005</td>
<td>1746 ± 51</td>
</tr>
<tr>
<td>+ GTP-gS</td>
<td>0.039 ± 0.006</td>
<td>1855 ± 58</td>
</tr>
<tr>
<td>DSLET pretreatment</td>
<td>0.037 ± 0.01</td>
<td>257 ± 13</td>
</tr>
<tr>
<td>+ GTP-gS</td>
<td>0.046 ± 0.01</td>
<td>249 ± 12</td>
</tr>
<tr>
<td>Pertussis toxin pretreatment</td>
<td>0.034 ± 0.003</td>
<td>1962 ± 381</td>
</tr>
<tr>
<td>+ GTP-gS</td>
<td>0.035 ± 0.005</td>
<td>1827 ± 52</td>
</tr>
<tr>
<td>Pertussis toxin and DSLET pretreatment</td>
<td>0.042 ± 0.01</td>
<td>236 ± 21</td>
</tr>
</tbody>
</table>

C6 cells were incubated for 24 hr in the absence (control) or presence of Pertussis toxin (100 ng/ml). For the last 12 hr of the treatment, half of both control and Pertussis toxin-treated cells were incubated with 1 μM DSLET and membranes prepared as described in “Materials and Methods.” [3H]Naltrindole equilibrium binding was measured in the presence of 100 mM NaCl in the absence or presence of GTP-80 of 10 μM GTP-gS as described in “Materials and Methods.” Data from two experiments were combined and the data fit to a one site binding hyperbola. Shown is the mean and S.E.M. for the binding parameters. The average Kd from these measurements (0.035 nM) was used to calculate the Kd for DSLET shown in table 2.
nated the inhibitory effect of delta opioid agonist DSLET on cAMP accumulation (fig. 3B). Chronic DSLET elicited a forskolin-stimulated cAMP “overshoot” of approximately 30% as observed in figure 2B and a small, but antagonist reversible (data not shown) inhibition of cAMP accumulation. Pertussis toxin pretreatment eliminated the cAMP “overshoot” attributed to chronic agonist treatment (fig. 3B).

Previous results from Loh’s laboratory (Chakrabarti et al., 1997) found that Pertussis toxin-pretreatment in Neuro2A cells was unable to eliminate all Gi/Go G protein. To determine if all of the inhibitory G protein was eliminated by Pertussis toxin pretreatment, we evaluated DSLET affinity in membranes prepared from toxin-pretreated cells. Initially, tritiated naltrindole equilibrium binding was evaluated in membranes prepared from control, tolerant and Pertussis toxin-treated cells. Chronic DSLET treatment resulted in a 85% reduction in opioid binding sites as indicated by tritiated naltrindole equilibrium binding in membranes prepared from control and DSLET-treated cells. Affinity of antagonist for receptor was unchanged (table 1). These data indicate that the decrease in binding observed in figure 3A is a result of a decrease in the number of cell surface receptors and not due to a decreased ligand affinity. In addition, Pertussis toxin pretreatment did not significantly alter naltrindole binding parameters in either the absence or presence of GTPγS (table 1). The ability of DSLET to complete for [3H]naltrindole binding was assessed in these membrane preparations in the absence and presence of GTPγS to evaluate the degree of G protein coupling after chronic agonist treatment as well as after Pertussis toxin treatment (fig. 4; table 2). The data are presented on two graphs for clarity. DSLET competition curves reveal a high and low affinity binding site where the majority of the receptors (55%) binds DSLET with high affinity. The percent of high affinity binding sites decreases to 15% in the presence of GTPγS (fig. 4A). However, in membranes prepared from cells that had been pretreated with Pertussis toxin, GTPγS did not significantly alter DSLET binding affinity (fig. 4B; table 2). In contrast to the effect of Pertussis toxin in Neuro2A cells (Chakrabarti et al., 1997), we observe the expected functional elimination of inhibitory G protein after toxin treatment. Similar results were observed in cells pretreated with both Pertussis toxin and DSLET.

Interestingly, after chronic DSLET exposure, there was an apparent decrease from 56 to 41% of receptors that bind DSLET with high affinity (table 2). The fraction of high affinity binding sites was sensitive to guanine nucleotide indicating that substantial G protein coupling remains after chronic agonist treatment as would be hypothesized based on the observed remaining receptor-stimulated GTPγS binding and inhibition of adenyl cyclase.

Despite the inability to form high affinity agonist/receptor/G protein complexes in Pertussis toxin pretreated membranes, delta opioid receptors undergo down-regulation following chronic agonist exposure. These results were confirmed in an additional C6 glioma clone expressing approximately 0.5 pmol/mg delta opioid receptor. Pertussis toxin pretreatment was unable to block receptor down regulation caused by 12-hr DSLET pretreatment (data not shown).

We then hypothesized that the high affinity agonist binding complex was required for agonist-induced receptor down-regulation and evaluated the effect of chronic exposure to five agonists of differing efficacy to decrease receptor number, decrease agonist-stimulated GTPγS binding and to attenuate adenyl cyclase inhibition. DSLET and SNC80 are fully efficacious when evaluated by agonist-stimulated GTPγS binding and adenyl cyclase inhibition (fig. 2B) (Clark et al., 1997). DPDPDE is a full agonist when inhibition of adenyl cyclase is evaluated yet only ~60% as efficacious as DSLET in agonist-stimulated GTPγS binding (Clark et al., 1997). SIOM and morphine are partial agonists. SIOM has an efficacy of 70% for inhibition of adenyl cyclase with an EC50 of 10 nM (data not shown) and a much lower efficacy (18%) when agonist-stimulated GTPγS binding is evaluated (Clark et al., 1997). Morphine efficacy compared to DSLET for inhibition of adenyl cyclase (60%) was also higher than the 8% efficacy observed for stimulation of GTPγS binding (data not shown). The EC50 values for morphine-mediated adenyl cyclase inhibition and stimulation of GTPγS binding were 10 and 2 μM, respectively. Partial agonist activity was confirmed by inhibition of agonist-stimulated GTPγS binding by 100 μM naltrindole. Partial agonists SIOM (1 μM) and morphine (30 μM) were completely unable to down-regulate receptor and subsequent effector coupling (fig. 5). SIOM was maximally efficacious at 1 μM for inhibition of adenyl cyclase and stimulation of GTPγS binding. Chronic DSLET, SNC80 and DPDPDE pretreatment all maximally decreased receptor number (fig. 5A), decreased agonist-stimulated GTPγS binding (fig. 5B), as well as substantially attenuated.

### Table 2

<table>
<thead>
<tr>
<th>Conditions</th>
<th>DSLET Binding Affinities</th>
<th>Fraction of binding that is high affinity (± S.E.M.)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_1$ (nM)</td>
<td>$K_2$ (nM)</td>
</tr>
<tr>
<td>Control</td>
<td>0.9 (0.5–1.7)</td>
<td>114 (61–216)</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>1.9 (0.2–21)</td>
<td>294 (217–398)</td>
</tr>
<tr>
<td>DSLET pretreatment</td>
<td>0.9 (0.4–1.9)</td>
<td>379 (226–635)</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>1.2 (0.08–185)</td>
<td>490 (327–756)</td>
</tr>
<tr>
<td>Pertussis toxin pretreatment</td>
<td>0.9 (0.16–5.2)</td>
<td>240 (217–267)</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>0.16 (0.006–4)</td>
<td>264 (241–288)</td>
</tr>
<tr>
<td>Pertussis toxin and DSLET pretreatment</td>
<td>0.09 (0.006–1.1)</td>
<td>358 (311–411)</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>77 (37–163)</td>
<td>1261 (591–2688)</td>
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</tbody>
</table>

C6 cells were incubated for 24 hr in the absence (control) or presence of Pertussis toxin (100 ng/ml). For the last 12 hr of the treatment, half of both control and Pertussis toxin-treated cells were incubated with 1 μM DSLET and membranes prepared as described in "Materials and Methods." The ability of DSLET to compete for the binding of 0.04 nM [3H]naltrindole in these membranes was determined. The data from two (Pertussis toxin treatment) or three experiments were combined and $K_i$ values estimated as described in "Materials and Methods." The mean and 95% confidence intervals for the $K_i$ values are shown.
adenylyl cyclase inhibition (fig. 5C). Cross-tolerance was observed in that chronic DSLET treatment also attenuated SIOM receptor interaction and effector coupling. In contrast to the ability of DSLET, SNC80 and DPDPE to down-regulate receptor, after 12-hr exposure to SIOM or morphine, receptor binding and effector coupling were not significantly different from control.

Discussion

We clearly demonstrate two disparate findings: 1) that delta opioid receptor down-regulation does not require functional G protein/effector coupling however, 2) the ability of an agonist to down regulate the dOR correlates with agonist efficacy where partial agonists are ineffective. Thus we postulate that a similar conformation of the receptor is recognized by effector as well as proteins (possibly kinases) required for subsequent receptor down-regulation. The ability of partial and full agonists at the beta adrenergic receptor to stimulate adenylyl cyclase activity correlated with the agonist's ability to promote receptor phosphorylation by beta adrenergic receptor kinase (Benovic et al., 1988). Only ligand, receptor and kinase were reconstituted in these phosphorylation studies indicating the G protein is not required for receptor phosphorylation.

Previous results suggested that effector coupling is not required for dOR down-regulation. A mutant dOR, D95A dOR, which was unable to inhibit adenylyl cyclase, was able to partially down-regulate in response to chronic agonist although down-regulation was attenuated compared to wild-type receptor (Chakrabarti et al., 1997). The same study found that Pertussis toxin was unable to completely inactivate dOR-coupled G proteins in Neuro2A cells so they could
Down-Regulation Is Dependent on Agonist Efficacy

Fig. 5. [3H]Naltrexone binding and stimulation of [35S]GTPγS binding in membranes from agonist-treated cells, and inhibition of forskolin-stimulated adenyl cyclase in agonist-treated whole cells. A, C6 cells were treated for 12 hr with 1 μM DSLET, 1 μM SNC80, 5 μM DPDPE, 1 μM SIOM or 30 μM morphine. The cells were harvested, washed and crude membranes were prepared as described in “Materials and Methods.” Specific binding of 3 nM [3H]-NTI (saturating concentration) was measured at 25°C in 50 mM Tris, 5 mM MgCl2 and 100 mM NaCl as described in “Materials and Methods.” Shown is the mean ± S.E.M. of the combined data from three independent treatments, each assayed in duplicate. B, C6 cells were treated and membranes prepared as described in A. Membranes were preincubated for 10 min at 25°C in 50 mM Tris, 5 mM MgCl2, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 50 μM GDP in the absence (white bars) or presence (black bars) of 10 μM DSLET. [35S]GTPγS was added (0.05 nM) and binding was measured after 30 min at 25°C as described in “Materials and Methods.” Shown is the mean ± S.E.M. of the combined data from three independent treatments, each assayed in triplicate. C, C6 cells were treated as described in A. The cells were harvested, washed and resuspended in A2 buffer as described in “Materials and Methods.” Accumulation of cAMP in whole cells was measured (15 min at 37°C) in the presence of 10 μM forskolin (+FSK, white bars) or 10 μM forskolin plus 10 μM DSLET (+FSK/DSLET, black bars) or 10 μM SIOM (+FSK/SIOM, gray bars) as described in “Materials and Methods.” Shown is the mean ± S.E.M. of the combined data from three independent treatments, each assayed in duplicate. The data are expressed as the fraction of the average forskolin-stimulated cAMP accumulation in control cells, in the absence of agonist, for the individual treatment. The average forskolin-stimulated cAMP accumulation for the three treatments was 0.020 ± 0.003 pmol cAMP/min · μg protein.

not conclude that δOR down-regulation was independent of G protein coupling. In contrast, high affinity agonist binding was clearly eliminated after toxin treatment of the C6 cells (fig. 4). Possibly the δOR-G protein complex is more stable in Neuro2A cells and more resistant to Pertussis toxin than that in the C6 glioma cells. We have demonstrated by agonist binding (fig. 4) and loss of agonist-stimulated GTPγS binding (fig. 2A) that Pertussis toxin treatment eliminated functional G protein. Receptor down-regulation was apparently unchanged despite the lack of G, G, G protein. Pertussis toxin-sensitive G proteins have also been implicated in adenyl cyclase supersensitization after chronic agonist treatment and withdrawal (Avidor-Reiss et al., 1995). Because Pertussis toxin treatment decreased the ability of forskolin to stimulate adenyl cyclase in the C6 cells (fig. 3B), we could not ascertain the role of Go/Gi in adenyl cyclase supersensitization after removal of chronic agonist.

In contrast to mu opioid receptor expressed in Neuro2A cells, we have shown that the rat mu opioid receptor stably expressed in C6 glioma cells (C6μ cells) was able to undergo down-regulation in response to chronic treatment with a full agonist (Yabaluri and Medzihradsky, 1997). Interestingly, after Pertussis toxin pretreatment of C6μ cells, receptor down-regulation induced by partial agonists was attenuated (Yabaluri and Medzihradsky, 1997), yielding results not unlike those observed in this study for partial agonists morphine and SIOM without Pertussis toxin pretreatment. Possibly mu and delta ORs expressed in the C6 glioma cells exhibit differential coupling to G protein as observed with mu and delta ORs expressed in Neuro2A cells.

Identical rank order of efficacy for inhibition of adenyl cyclase was observed for ligands acting at the mouse delta opioid receptor expressed in human embryonic kidney 293 cells where DSLET = DPDPE > SIOM = morphine (Bot et al., 1997). However, in contrast to our observations after 12-hr agonist treatment, SIOM and morphine treatment for 3 hr produced a supersensitization to subsequent ligand exposure resulting in a leftward shift in the dose-response curve for adenyl cyclase inhibition (Bot et al., 1997). We found that after 12-hr SIOM or morphine pretreatment, the ability of DSLET and SIOM to inhibit adenyl cyclase was no different than the inhibition observed in control cells. However, our data confirm the lack of desensitization of the δOR by morphine and SIOM observed by Bot et al. (1997). Surprisingly, partial agonists in this system did not elicit a partial down-regulation as was observed for partial agonist salmeterol acting at the beta-2 adrenergic receptor (Kallal et al., 1998). Down-regulation of cell surface receptors occurred more slowly in the presence of the low intrinsic activity agonist.

Based on the similar affinity of agonist for receptor in control and chronic agonist-treated membranes (fig. 4; table 2), as well as the similar EC50 values for the stimulation of GTPγS binding and adenyl cyclase inhibition (fig. 2), it appears that the receptors remaining in the membrane have normal function. Thus the major effect of chronic agonist treatment is simply to decrease the number of receptors on the cell surface. However, we did not evaluate the levels of functional G protein after agonist treatment. Previously we had shown that pretreatment of SH-SY5Y cells with Tyr-d-Ala-Gly-(Me)Phe-Gly-ol (DAMGO) resulted in a 25% decrease in membrane G, G protein content (Carter and Medzi-
More recently, Breivogel et al. (1997) found thatafter short-term agonist treatment, the pool of G protein activated by δOR was rapidly reduced (with a half-time of 15 min) before a decrease in cell surface receptor number in NG108–15 cells treated with DSLET. The time course for loss of ligand binding and agonist-stimulated GTPyS binding in the C6δ cells (fig. 1) also supports the notion of a rapid loss of receptor-G protein coupling followed by a slower loss of receptor from the cell.

In summary, it appears that chronic treatment by a full agonist leads to a reduction in cell surface receptor number. G protein activation and subsequent downstream effector coupling are not required for the down-regulation. The decrease in cell surface receptors can account for the attenuated effector coupling observed in the tolerant cells. Additionally, partial agonists are ineffective at down-regulating the delta opioid receptor. Experiments are in progress to elucidate the biochemical mechanisms involved in the development of tolerance which we have characterized in the C6δ cells here and the C6μ cells previously (Yabaluri and Medzihradsky, 1997).

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References


Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


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