**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]enkephaly-Thr treatment caused a decrease in cell surface as well as a decrease in agonist-stimulated [³²P]guanosine-5'[^-O-(3-thio)-9'-O-(3-thio)]guanosine 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 [³²P]guanosine-5'[^-O-(3-thio)]guanosine triphosphate binding and inhibit forskolin-stimulated adenylyl 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 an 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 adenylyl 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 adenylyl cyclase (Evans et al., 1992), an increase in the production of inositol phosphates (Tan et al., 1995), as well as modulation of ion channel opening (Tausiag 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; Trapaizde 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 (Thr^{353}) is required for this internalization process (Trapaizde 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. Thr^{353} 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]enkephaly-Thr; SIOM, 7-spiroinidinoxymorphine; DPDPE, [d-Pen²,d-Pen⁵]enkephalin; SNC80, methyl ether of (+)-4-((α-L-Arg)⁹)-α-((2S*,5R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzy]-N,N-diethylbenzamide (BW373U86); G protein, GTP binding protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GTP-γS, 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 Na₂HPO₄, 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 ([35]S)GTPγS binding in membranes prepared from chronic agonist-treated cells was reduced yet the maximal inhibition of forskolin-stimulated adenylyl cyclase activity was unchanged 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 adenylyl 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 ([35]S)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 adenylyl cyclase and the stimulation of GTPγS binding.

Materials and Methods

Materials. [35]S(GTPγS (1250 Ci/mmole) and [3H]naltrindole (33 Ci/mmol) were purchased from Du Pont NEN (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). Genetecin 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 Genetecin (for subculture) or without Genetecin (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 lueling 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 of cells) 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, 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 adenyl cyclase assay (see below) or else Dounce homogenized in 10 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 with 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 × 105 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 overnight at 4°C. The contents of each well was mixed with 4 ml of scintillation cocktail and subject to scintillation counting.

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.
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 \([35S]GTP\gamma S\) binding by DSLET was evaluated and the maximal stimulation is shown in figure 1B. The maximal loss of agonist-stimulated \([35S]GTP\gamma S\) 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 \([35S]GTP\gamma S\) 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 assessed by relative EC_{50} values in control and agonist-treated cells (fig. 2). The ability of DSLET to stimulate \([35S]GTP\gamma S\) binding in membranes from DSLET-treated cells was reduced 10-fold; however, the EC_{50} values were similar. In addition, Pertussis toxin pretreatment eliminated agonist-
stimulation (and EC50 values) for the membranes prepared from control data were fit to a sigmoidal dose-response curve. The basal [35S]GTP concentration of DSLET was determined relative to control membranes. The 0.379 ± 0.049, respectively. Forskolin-stimulated cAMP concentration of DSLET was determined relative to control membrane forskolin stimulation. Each individual data from three different cell treatments were averaged by normalizing the values 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 quadruplicate. Forskolin-stimulated cAMP concentration in control and DSLET-treated cells was 0.285 ± 0.082 pmol/mg and 0.379 ± 0.049, respectively.

stimulated [35S]GTPγS 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 Medzhitov, 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]GTPγS 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 effector 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 elimina

![Fig. 2. Reduced G protein coupling and adenylyl cyclase inhibition after chronic agonist treatment. A, C66 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]GTPγS binding in control, DSLET-treated and Pertussis toxin-treated cells was 6.7 ± 0.5, 5.7 ± 0.5 and 3.3 ± 0.1 fmol/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 quadruplicate. Forskolin-stimulated cAMP concentration in control and DSLET-treated cells was 0.285 ± 0.082 pmol/mg and 0.379 ± 0.049, respectively.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[3H]Naltrindole Binding</th>
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<tbody>
<tr>
<td></td>
<td>Kd (nM)</td>
</tr>
<tr>
<td>Control</td>
<td>0.037 ± 0.005</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>0.039 ± 0.006</td>
</tr>
<tr>
<td>DSLET pretreatment</td>
<td>0.037 ± 0.01</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>0.046 ± 0.01</td>
</tr>
<tr>
<td>Pertussis toxin pretreatment</td>
<td>0.034 ± 0.003</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>0.034 ± 0.003</td>
</tr>
<tr>
<td>Pertussis toxin and DSLET pretreatment</td>
<td>0.035 ± 0.005</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>0.034 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.037 ± 0.01</td>
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</table>

C66 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 GTPγS at 37°C. 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 (0.038 nM) was used to calculate the Kd for DSLET shown in table 2.

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TABLE 1

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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 naltrexone 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 naltrexone 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 naltrexone binding parameters in either the absence or presence of GTPγS (table 1). The ability of DSLET to compete for [3H]naltrexone 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 adenylyl 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 adenylyl cyclase inhibition. DSLET and SNC80 are fully efficacious when evaluated by agonist-stimulated GTPγS binding and adenylyl cyclase inhibition (fig. 2B) (Clark et al., 1997). DPDPE is a full agonist when inhibition of adenylyl 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 adenylyl 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 adenylyl cyclase (60%) was also higher than the 8% efficacy observed for stimulation of GTPγS binding (data not shown). The EC50 values for morphine-mediated adenylyl 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 naltrexone. 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 adenylyl cyclase and stimulation of GTPγS binding. Chronic DSLET, SNC80 and DPDPE 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>$K_i1$ (nM)</th>
<th>$K_i2$ (nM)</th>
<th>Fraction of binding that is high affinity (± S.E.M.)</th>
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<tr>
<td>Control</td>
<td>0.9 (0.5–1.7)</td>
<td>114 (61–216)</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>+ GTPγS</td>
<td>1.9 (0.2–21)</td>
<td>294 (217–398)</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>DSLET pretreatment</td>
<td>0.9 (0.4–1.9)</td>
<td>379 (226–635)</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>Pertussis toxin pretreatment</td>
<td>1.2 (0.08–185)</td>
<td>490 (327–756)</td>
<td>0.17 ± 0.04</td>
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<td>Pertussis toxin pretreatment + GTPγS</td>
<td>0.9 (0.16–5.2)</td>
<td>240 (217–267)</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Pertussis toxin and DSLET pretreatment + GTPγS</td>
<td>0.16 (0.006–4)</td>
<td>264 (241–288)</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Pertussis toxin and DSLET pretreatment</td>
<td>0.09 (0.006–1.1)</td>
<td>358 (311–411)</td>
<td>0.09 ± 0.01</td>
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<tr>
<td>+ GTPγS</td>
<td>77 (37–163)</td>
<td>1261 (591–2688)</td>
<td>0.49 ± 0.1</td>
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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 δOR 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 δOR down-regulation. A mutant δOR, D95A δOR, 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 δOR-coupled G proteins in Neuro2A cells so they could

Fig. 3. Pertussis toxin pretreatment does not block delta opioid receptor down-regulation caused by chronic agonist treatment. C68 cells were incubated for 24 hr in the absence (control) or presence (+ PTX) 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 (+ DSLET) and (+ PTX/DSLET), respectively. A. [3H]naltrindole equilibrium binding was performed in Tris-Mg buffer on membranes prepared from these cells as described in “Materials and Methods.” The data were fit to a one-site binding hyperbola and the number of binding sites relative to control is shown. Shown is the mean ± S.D. from two experiments performed after two different cell treatments. The Bmax values in control membranes from these two experiments were 3.7 and 4.8 pmol/mg. B. The forskolin-stimulated cAMP accumulation (+ FSK) in these cells was determined in the absence (white bars) and presence (+ FSK + DSLET, black bars) of 10 μM DSLET as described in “Materials and Methods.” Data (performed in triplicate) from the same two cell treatments shown in A are expressed relative to control + FSK. The forskolin-stimulated cAMP accumulation (+ FSK) in these two experiments was 0.226 ± 0.004 pmol cAMP/μg membrane protein (n = 3) and 0.34 ± 0.02 pmol cAMP/μg membrane protein (n = 3).

Fig. 4. Agonist competition of [3H]naltrindole binding in membranes prepared from control, agonist- and Pertussis toxin-treated C68 cells. C68 cells were incubated for 24 hr in the absence (A) or presence (B) 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 (+ DSLET) and (+ PTX/DSLET), respectively. Membranes were prepared from the cells as described in “Materials and Methods.” Competition of 0.04 nM [3H]naltrindole binding by DSLET was measured in 50 mM Tris, 5 mM MgCl₂, and 100 mM NaCl in the absence (closed symbols) or presence (open symbols) of 10 μM GTPγS as described in “Materials and Methods.” The data are expressed as the percent of specific binding in the presence of [3H]naltrindole only. Shown is the mean ± S.E.M. of the combined data from three independent treatments, each assayed in duplicate.
Fig. 5. [3H]naltrindole binding and stimulation of [35S]GTPyS 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]GTPyS 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 washed, harvested 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/Gi 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 Gα G protein content (Carter and Medzi-
hradsky, 1993). More recently, Breivogel et al. (1997) found that after 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 GTPγS 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 δ 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 C6δ cells previously (Yabaluri and Medzihradsky, 1997).

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