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Regulation of Opioid Receptors in Rat Sensory Neurons in Culture

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SUMMARY

To determine whether opioid receptors in sensory neurons are regulated by chronic exposure to opioids, we assessed the binding of various opioid ligands to membranes derived from isolated rat dorsal root ganglia neurons grown in culture. Equilibrium binding of [^{3}H]diprenorphine onto membranes from cells grown for 13–15 days revealed a saturable binding site with a K_d value of 0.3 ± 0.2 nM and an approximate B_{max} value of 1300 ± 200 fmol/mg of protein. [^{3}H]Diprenorphine binding increased 3-fold from 1–15 days in culture. The μ receptors represent ~70 ± 11% of the [^{3}H]diprenorphine binding sites, as indicated by saturation binding of [^{3}H]DAMGO. The κ and δ receptors represent ~10 ± 3% and ~5 ± 2% of the [^{3}H]diprenorphine binding sites, respectively. Preexposure of neurons to 10 μM naloxone for 48 hr up-regulated the receptors by 40%, whereas incubation with 100 nM to 10 μM DAMGO for 48 hr resulted in a significant decrease in the B_{max} value of opioid receptors, with a maximum reduction of 70%. The identification of a high level of opioid receptors expressed in isolated sensory neurons and their modulation by opioids demonstrates that cultured sensory neurons are an excellent model with which to study opioid receptor regulation.

It is well established that intraspinal administration of opioids causes antinociception in both laboratory animals and humans (1, 2). The terminals of nociceptive sensory neurons that project to the superficial layers of the dorsal spinal cord represent one site that is proposed to mediate opioid analgesia at the level of the cord. Indeed, using various end points to measure the activity of sensory neurons, investigators have shown that opioids reduce the excitability of these cells. Opioids hyperpolarize sensory neurons (3, 4), reduce calcium entry (4–6), and inhibit the release of neurotransmitters from sensory nerve endings in the dorsal horn of the spinal cord (1, 7, 8).

Recent studies, however, suggest that the actions of opioids on sensory neurons are more complex and not limited to inhibitory effects (9, 10). Nanomolar concentrations of morphine can prolong the action potential duration of mouse sensory neuronal explants, whereas higher concentrations shorten the duration (9, 11). In an analogous manner, morphine can increase or decrease the evoked release of peptides from sensory neurons depending on the concentration of the opioid (12). The cellular mechanisms mediating the opposing actions of opioids on sensory neurons have not been elucidated. One possible explanation for the opposing actions of opioids is that different effects are mediated by different subtypes of receptors. Indeed, receptor-selective excitatory or inhibitory actions have been observed in opioid regulation of neurotransmitter release from rat trigeminal and spinal cord slices (7, 13). In rat DRG neurons, activation of μ- or κ- but not δ-opioid receptors reduces calcium currents (14, 15), whereas in mouse sensory neuronal cultures, low concentrations of κ agonists increase calcium conductance (11).

The purpose of the current study was to characterize opioid binding on isolated sensory neurons and to determine whether opioid receptors on these cells can be altered by exposure to agonists or antagonists. The use of primary cultures of sensory neurons as a model system has an advantage in that a relative homogeneous population of nondividing neuronal cells is used that are functionally important in mediating opioid-induced antinociception at the level of the spinal cord.

Although studies have been performed on the regulation of opioid receptors in proliferating cell lines (16) and in central nervous system tissue (17, 18), results are inconsistent between the various preparations. The up- and down-regulation of opioid receptors after chronic administration of opioids has been demonstrated in several cell lines, including NG108–15 cells (19), SK-N-SH cells (20), and SH-SY5Y cells (21). In various brain regions, the up-regulation of opioid receptors by antagonists has been observed (17, 18), whereas

ABBREVIATIONS: HBSS, Hanks' balanced salt solution; DRG, dorsal root ganglia; DAMGO, [D-Ala^2,N-MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; BSA, bovine serum albumin.

the effects of chronic exposure to agonist are equivocal. Down-regulation of opioid receptors in brain is observed only after exposure to nonselective agonists (22, 23), whereas selective opioid agonists produced either no change (24, 25) or an increase in the number of receptors (26). Given the different results in various preparations and the diverse actions of receptor-selective opioid agonists on sensory neurons, studies are needed to directly characterize opioid receptor binding and regulation on these neurons.

Our results demonstrate a high level (1000–2000 fmol/mg of protein) of opioid receptor binding in embryonic sensory neurons cultured for 2 weeks. Analysis of the relative abundance of opioid receptor subtypes in the cultures reveals a high content of μ receptors, an intermediate content of κ receptors, and a low content of δ receptors. Preexposure of the cultures to naloxone or DAMGO significantly modulated the level of opioid receptors in sensory neurons (this work has been presented in abstract form; Ref. 27).

Experimental Procedures

Materials. Timed pregnant Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). [³H]Diprenorphine, [³H]U69,593, and [³H]5-hydroxytryptamine were purchased from Amersham Corp. (Arlington Heights, IL). [³H]DAMGO and [³H]DP-DPE were from Chiron Mimotope Peptide Systems (San Diego, CA). Opioid peptides (DAMGO and DPDPE) were from Peninsula Laboratories (Belmont, CA). Routinely used chemicals were from Sigma Chemical (St. Louis, MO). Cell culture supplies were purchased from GIBCO-Bethesda Research Labs (Grand Island, NY), and nerve growth factor was from Harlan Bioproducts for Science (Indianapolis, IN). All procedures used in these studies were approved by the Animal Care and Use Committee at Indiana University School of Medicine (Indianapolis, IN).

Cell culture. DRG cultures were prepared as previously described (28). Briefly, ganglia were dissected from E15-E17 rat embryos and placed in sterile calcium-free, magnesium-free modified HBSS at 4°. After all of the ganglia were removed, they were incubated at 37° for 30 min in 3 ml of HBSS containing 250 mg/ml trypsin to dissociate the cells. After the incubation, 1 mg/ml DNase I was added to the solution, the ganglia were centrifuged at $200 \times g$ for 1 min, and the supernatant was aspirated. Ganglia were then resuspended in HBSS containing 2.5 mg/ml trypsin inhibitor. This solution was again centrifuged at $200 \times g$ for 1 min, and the supernatant was removed. The ganglia were washed once with fresh HBSS and then resuspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin, 50 μM 5-fluoro-2-deoxyuridine, 150 μM uridine, and 250 ng/ml nerve growth factor. The individual cells were dissociated by mechanical agitation through a fire-polished Pasteur pipette. A small amount of the cell suspension was removed and stained with trypan blue to determine cell viability (which routinely was ~90%). Viable cells were counted using a hemocytometer, and ~600,000 cells were placed into each 35-mm tissue culture dish, which had been precoated with 0.5 mg/ml rat tail collagen. The cells were maintained at 37° in a 5% CO_2/95% air atmosphere, and the medium was changed every 2 days. To determine whether opioid receptors in sensory neurons are modulated by chronic exposure to opioids, cells were pre-exposed to various opioids before binding studies. To account for potential variability between culture preparations, each set of experiments was replicated using cells from at least two different harvests. In addition, in each experiment, the control and treated cells are from the same harvest (i.e., one lot of cells obtained on the same day from embryonic rats dissected out of the same mother rat).

Preparation of sensory neuronal membranes. Sensory neuronal cultures were washed three times with phosphate-buffered saline composed of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.4. After washing, 1.5 ml of buffer was added to each well. The wells were scraped, and the cell suspension was removed. This solution was centrifuged at $2500 \times g$ for 10 min, the supernatant was aspirated, and the cells were resuspended in 1.5 ml of 5 mM Tris·HCl, pH 7.4, and incubated on ice for 10 min. The cells were then homogenized using a Dounce glass homogenizer (15 strokes). An equal volume of 0.64 M sucrose was added to the homogenate, and the solution was mixed and centrifuged at $28,000 \times g$ for 30 min. The supernatant was gently aspirated, and the membrane pellet was resuspended in 5 ml of 50 mM Tris·HCl, pH 7.4. To remove the residual opioids, the suspension was incubated at 37° for 15 min and then centrifuged at $28,000 \times g$ for 30 min. These procedures were repeated twice, and the pellet was stored at −80° until it was used in experiments. Routinely, these membrane fractions were stored for <2 weeks. On the day of binding experiments, the membrane pellet was resuspended in 50 mM Tris·HCl, pH 7.4. Protein concentrations were determined according to the method of Bradford (29), with BSA used as standard. The final concentration of membrane proteins in each reaction tube was 30–50 μg/ml.

Ligand binding studies. Membranes prepared as described above were incubated with [³H]diprenorphine (29 Ci/mmol), [³H]DAMGO (37.9 Ci/mmol), [³H]DPDPE (32.87 Ci/mmol), [³H]U69,593 (62.9 Ci/mmol), or [³H]5-hydroxytryptamine (96 Ci/mmol) for 2 hr at 4° in 50 mM Tris·HCl buffer containing 1% BSA, pH 7.4. The use of 1% BSA reduced the nonspecific binding of [³H]diprenorphine by ~50% compared with binding when membranes were incubated with 0.2% BSA in the buffer. The bound tritium-labeled ligand was separated from free ligand by rapid filtration through Whatman GF/B filters (Westbury, NY) presoaked with 1% polyethylenimine for 1 hr before filtration. Filters were washed three times with 3 ml of ice-cold 50 mM Tris·HCl buffer and dried overnight. Dried filters were placed in 10 ml of Ultima Gold scintillation cocktail (Packard Instrument, Meriden, CT), and radioactivity was determined by liquid scintillation counting. Tritium standard was included in every experiment so we could monitor the counting efficiency. In all experiments, nonspecific binding was determined by incubating membranes for 2 hr with the labeled ligand and 10 μM naloxone and was subtracted from total binding to obtain specific binding. In all instances, apparent K_d and B_{max} values were determined from Scatchard analysis of complete saturation binding data using the LIGAND computer program obtained from Dr. Peter J. Munson (National Institutes of Health, Bethesda, MD).

Statistics. A paired t test was used to compare the effects of 48-hr exposures to 10 μM naloxone or 10 μM DAMGO on the K_d and B_{max} values of opioid binding. Analysis of variance was used for the comparison of the effects of 100 nM to 10 μM DAMGO treatment for 5 or 48 hr. If a significant difference was obtained, data were further analyzed using Fisher's Protected LSD *post hoc* test.

Results

Characterization of [³H]diprenorphine binding. To initially determine whether opioid receptors are present on sensory neurons in culture, we examined the equilibrium binding of the nonselective opioid ligand, [³H]diprenorphine. Specific binding of [³H]diprenorphine to membranes prepared from these cultures increased over time of incubation, and the binding was maximal by 90 min (data not shown). Incubation of membranes from 13-day-old sensory neurons with various concentrations of [³H]diprenorphine results in a specific binding that saturates at 2 nM (Fig. 1). Scatchard analysis of diprenorphine binding for this representative experiment (Fig. 1, *inset*) indicates an apparent K_d value of 0.2 nM and a B_{max} value of 1000 fmol/mg of protein. Average
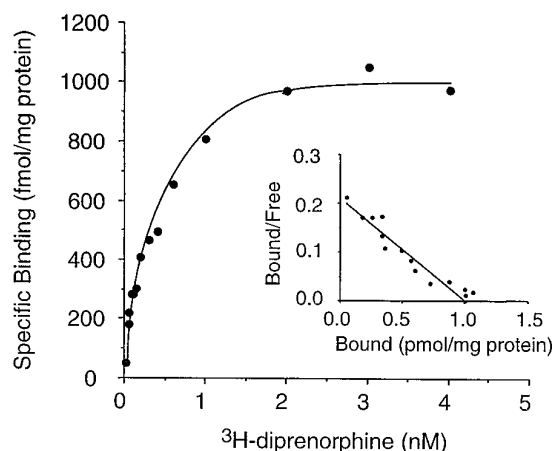
Fig. 1. Saturation binding of [³H]diprenorphine occurs in membranes from sensory neurons grown in culture for 13–15 days. Data are representative of five experiments. Specific binding was obtained by subtracting [³H]diprenorphine binding in the presence of 10 μM naloxone from binding in the absence of naloxone. *Inset*, Scatchard plot of the binding data from this experiment.

Fig. 2. Expression of opioid receptors in sensory neurons increases with the number of days in culture. Maximal specific binding (in fmol/mg of protein) is for three saturation binding experiments at each of the time points (*abscissa*) using 0.2–3 nM [³H]diprenorphine. Values are expressed as mean $\pm$ standard error.

values for K_d and B_{max} calculated from five independent experiments (separate cell preparations) were 0.3 $\pm$ 0.2 nM and 1300 $\pm$ 200 fmol/mg of protein, respectively. Analysis of the Scatchard plots with the use of LIGAND gave a best fit to a single line, suggesting that the binding in these membranes was predominantly to one site.

To confirm that binding of [³H]diprenorphine is on membranes derived from neurons, we plated 10,000 viable cells/well and grew them in the absence of nerve growth factor and mitotic inhibitors. In this manner, the non-neuronal cells would divide and grow to confluence, whereas the sensory neurons would not be likely to survive without nerve growth factor. Consequently, membranes from these cultures will be largely from non-neuronal cells. In the membrane preparation from these cultures, specific binding of [³H]diprenorphine was not detectable. This suggests that the opioid binding measured in our cultures is localized to neuronal membranes from cells grown in the presence of nerve growth factor.

The high affinity binding of [³H]diprenorphine to membranes of 13-day-old sensory neurons is displaced by naloxone in a concentration-dependent manner. The maximum displacement of 75 $\pm$ 2% of the total [³H]diprenorphine binding is observed when membranes are incubated with a naloxone concentration of $\geq$1 μM. This displacement of partial agonist binding by the antagonist strongly supports that diprenorphine binds to opioid receptors in the membranes prepared from sensory neurons.

To determine whether the level of opioid binding increases as a function of time in culture, we measured binding using [³H]diprenorphine at various concentrations (0.2–3 nM) on different days after harvesting of cells. As illustrated in Fig. 2, [³H]diprenorphine binding was detectable on the first day after plating (B_{max} = 380 fmol/mg of protein). At this time, the cells are round with few neuronal processes. The level of binding significantly increased ~3-fold from day 1 to day 15 in culture, reaching a B_{max} value of 1250 $\pm$ 140 fmol/mg of protein (three experiments). On day 15, the cells have formed a dense network of neurite processes. The [³H]diprenorphine binding on day 20 after plating was not significantly different
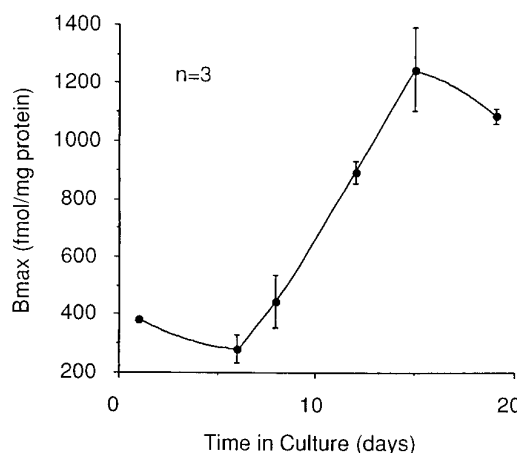
from that on day 15. Because the maximal number of binding sites were observed after ~2 weeks of growth in culture, all subsequent studies were performed on cells grown for 13–15 days.

Opioid receptor subtypes. To determine whether subtype-selective opioid ligands can displace [³H]diprenorphine binding, we performed competition binding studies using agonists selective for μ-, δ-, or κ-opioid receptors. DAMGO competes with 2 nM [³H]diprenorphine for membrane binding with a K_i value of 4.9 $\pm$ 2.8 nM, whereas DPDPE and U50488H exhibited K_i values of ~5 and ~18 μM, respectively. The displacement of [³H]diprenorphine binding by unlabeled DAMGO suggests that μ is the predominant type of opioid receptors present in cultured sensory neurons.

To quantitatively assess the abundance of various subtypes of opioid receptors in sensory neurons, saturation binding studies were performed using tritium-labeled selective opioid agonists. Scatchard analyses of a representative binding experiment of each opioid (under equilibrium conditions) are shown in Fig. 3. The μ-selective ligand [³H]DAMGO displayed high affinity binding with an apparent K_d value of 0.45 nM. The B_{max} value for [³H]DAMGO was 1500 fmol/mg of protein. The δ-selective ligand [³H]DPDPE bound with an apparent K_d value of 2.0 nM and a B_{max} value of 103 fmol/mg of protein, whereas [³H]U69,593, the κ-selective ligand, displayed a K_d value of 2.1 nM and a B_{max} value of 191 fmol/mg of protein. The experiment was performed in triplicate with similar results. Therefore, μ receptors represent ~70 $\pm$ 11% of the [³H]diprenorphine binding sites, whereas κ and δ receptors represent ~10 $\pm$ 3% and ~5 $\pm$ 2% of the [³H]diprenorphine binding sites, respectively.

Up-regulation of opioid receptors in sensory neurons. Because opioid receptors in brain are up-regulated by prolonged exposure to opioid antagonists (18, 30), we examined whether opioid receptors in isolated sensory neurons could also be up-regulated. Saturation binding studies with [³H]diprenorphine were performed in naloxone-treated cells and control cells obtained from the same harvest. As can be seen in Fig. 4A, 48 hr of exposure to 10 μM naloxone significantly increased [³H]diprenorphine binding over control levels. Similar experiments were repeated three times with cells
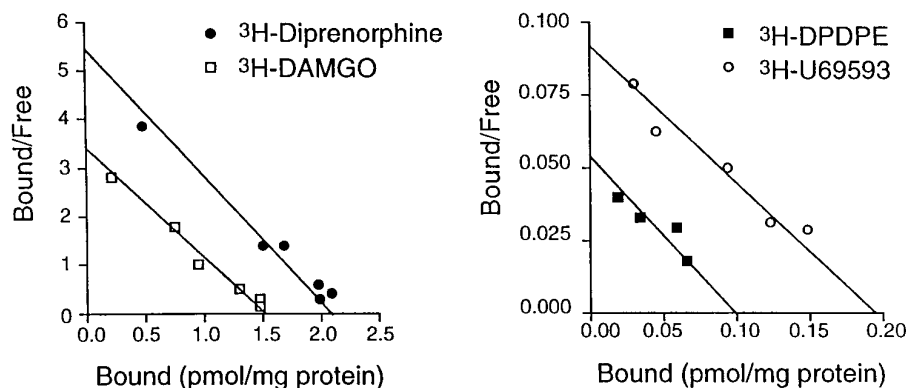
Fig. 3. Scatchard plots of the saturation binding of [³H]DAMGO, [³H]DPDPE, [³H]U69,593, and [³H]diprenorphine in membranes from sensory neurons grown in culture for 13–15 days. *Ordinate*, ratio of the bound to free ligand. *Abscissa*, amount of ligand bound (in pmol/mg of protein). Data are from a representative experiment performed in triplicate with similar results.
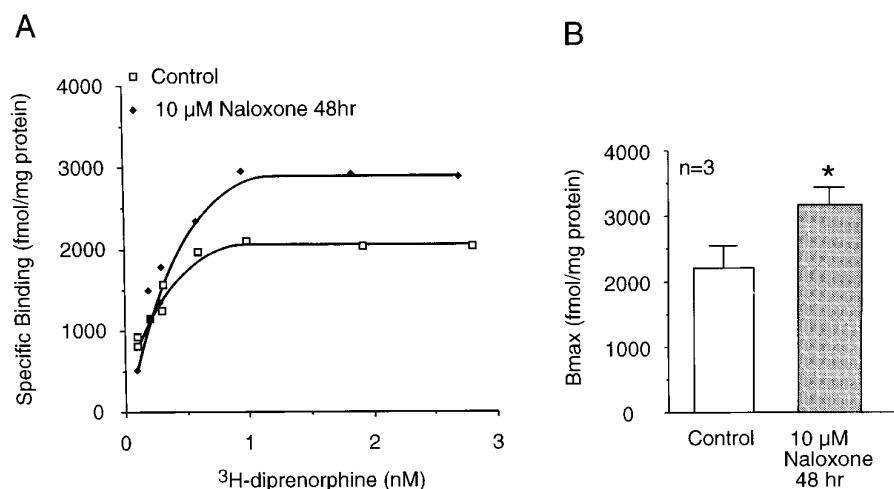
Fig. 4. Naloxone treatment results in up-regulation of opioid receptors in sensory neurons. Saturation binding studies of [³H]diprenorphine were performed using sensory neuron membranes prepared from cells that had been grown in culture for 13–15 days and exposed to 10 μM naloxone for 48 hr or from control cells that had been grown in the absence of naloxone. A, Binding data are representative of three experiments. *Ordinate*, specific binding (in fmol/mg of protein) obtained by subtracting [³H]diprenorphine binding in the presence of 10 μM naloxone from binding in the absence of naloxone. B, Mean ± standard error. B_{max} values were determined from three experiments using Scatchard analysis. □, Maximal binding in membranes from neurons not exposed to naloxone; ▨, maximal binding in membranes from cells exposed to 10 μM naloxone for 48 hr; ∗, statistically significant difference ($p < 0.05$) between control and opioid-treated neurons using paired t test.

derived from three different harvests. Pooling data from all three experiments generated B_{max} values for [³H]diprenorphine binding of 2300 ± 100 and 3200 ± 300 fmol/mg of protein for control and naloxone-treated cells, respectively (Fig. 4B). The naloxone-induced alteration in binding results in an increase in opioid receptor number without altering receptor affinity, because the K_d values for [³H]diprenorphine binding were not significantly different between control and naloxone-treated cells (0.11 ± 0.02 versus 0.21 ± 0.06 nM, respectively; $p = 0.189$). In contrast to [³H]diprenorphine binding, naloxone pretreatment did not significantly alter binding to the selective κ agonist [³H]U69,593. The B_{max} value for [³H]U69,593 binding in cells exposed to 10 μM naloxone for 48 hr was 188 ± 11 fmol/mg of protein, whereas binding in untreated cultures was 163 ± 13 fmol/mg of protein. Because μ and κ receptors represent the majority of the [³H]diprenorphine binding (see Fig. 3), the lack of an effect of naloxone treatment on κ binding suggests that the predominant up-regulation is occurring at μ receptors.

Down-regulation of opioid receptors in sensory neurons. To determine whether opioid receptors in sensory neurons are down-regulated by selective opioid agonists, we examined the effect on opioid binding of chronic exposure to DAMGO. We chose to study the μ agonist DAMGO because μ is the predominant type present in sensory neurons (see Fig. 3). As can be seen in Fig. 5A, the treatment of neuronal cultures with 10 μM DAMGO for 48 hr resulted in a significant reduction of [³H]diprenorphine binding (by 68%). B_{max}

values for [³H]diprenorphine binding from six studies using separate preparations of DRG cells were 1848 ± 300 and 656 ± 80 fmol/mg of protein for control and DAMGO-treated cells, respectively (Fig. 5B). The DAMGO-induced receptor down-regulation was due to change in opioid receptor number without any alteration in receptor affinity, because K_d values for opioid receptors from control and DAMGO-treated neurons were not significantly different (0.16 ± 0.04 nM for controls and 0.18 ± 0.03 nM for DAMGO-treated cells).

To demonstrate that the effect of DAMGO was selective for opioid receptors, experiments were performed to determine whether pretreatment with naloxone could attenuate DAMGO-induced down-regulation of binding. The treatment of neuronal cultures with 10 μM DAMGO and 10 μM naloxone for 48 hr completely attenuated the effect of DAMGO alone (Fig. 5B). The B_{max} value for [³H]diprenorphine binding from three studies using separate preparations of DRG cells was 2587 ± 229 fmol/mg of protein.

To confirm that the reduction of opioid receptors by prolonged exposure to DAMGO is not due to blocking of binding sites by the ligand itself, a portion of the cells were exposed to 10 μM DAMGO for 5 min before cell lysis and membrane preparation. The assumption is that any alteration of binding in these cells with 5-min drug treatment is due to the residual ligand in the membrane preparation. Cells exposed to DAMGO for 5 min displayed a similar level of [³H]diprenorphine binding compared with control cells (Fig. 6). Thus, the amount of residual DAMGO in the membrane preparation
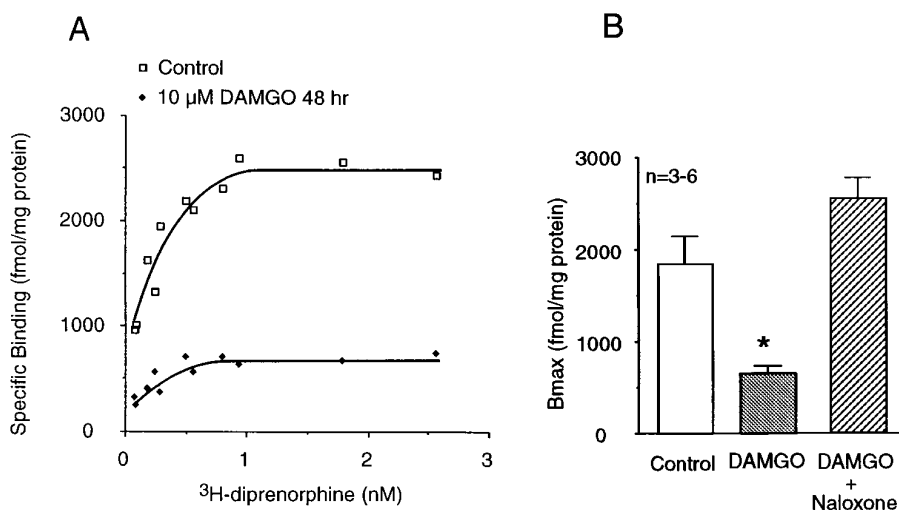
Fig. 5. Chronic exposure to DAMGO down-regulates opioid receptors in sensory neurons. Saturation binding studies of [³H]diprenorphine were performed using sensory neuron membrane prepared from cells that had been grown in culture for 13–15 days and exposed to 10 μM DAMGO for 48 hr and from control cells that had been grown in the absence of DAMGO. A, Binding data representative of three experiments. Ordinate, specific binding (in fmol/mg of protein) obtained by subtracting [³H]diprenorphine binding in the presence of 10 μM naloxone from binding in the absence of naloxone. B, Mean ± standard error. B_{max} values from six experiments (control and DAMGO) or three experiments (DAMGO plus naloxone) were determined using Scatchard analysis. □, Maximal binding in membranes from neurons not exposed to DAMGO; ▨, maximal binding in membranes from cells exposed to 10 μM DAMGO for 48 hr; ▨, maximal binding in membranes from cells exposed to both 10 μM DAMGO and 10 μM naloxone for 48 hr. ∗, Statistically significant difference ($p < 0.05$) between control and opioid-treated neurons using paired t test.

was not sufficient to interfere significantly with binding assays or to account for down-regulation after chronic exposure to the agonist.

To further substantiate that the effect of DAMGO was selective, [³H]5-hydroxytryptamine binding and binding to the κ-selective agonist [³H]U69,593 were measured in membranes from control cells and from cells exposed to 10 μM DAMGO for 48 hr. In contrast to [³H]diprenorphine binding, 48 hr of exposure to DAMGO did not significantly alter either [³H]5-hydroxytryptamine or [³H]U69,593 binding. B_{max} values for [³H]5-hydroxytryptamine binding from two studies using different sensory neuron preparations were 228 fmol/mg of protein for cultures not exposed to DAMGO and 206 fmol/mg of protein for cells exposed to the μ agonist. Maximal [³H]U69,593 binding was 163 fmol/mg of protein and 179 fmol/mg of protein for control and DAMGO-treated neurons, respectively.

Concentration-dependent effect of DAMGO on opioid binding. Although exposure of cells to 10 μM DAMGO for 48 hr decreases B_{max}, this concentration is several orders of magnitude greater than the affinity of this ligand for μ receptors (apparent $K_d = 0.45$ nM; Fig. 3). Consequently, we treated cells with lower concentrations of this opioid for 48 hr and assessed [³H]diprenorphine binding. As can be seen in Fig. 6A, DAMGO exposure resulted in a concentration-dependent down-regulation of [³H]diprenorphine binding. Treatment with 10 nM DAMGO for 48 hr did not reduce the B_{max} value compared with control, whereas 100 nM DAMGO reduced maximal binding by 25%. Higher concentrations of DAMGO further reduced the opioid binding, reaching a 70% decrease at 10 μM, which was the highest concentration tested. Exposure of cultures to 10 μM DAMGO for 5 min before membrane preparation did not alter binding compared with control cells, eliminating the possibility that the down-regulation was caused by masking of some of the opioid binding sites by residual DAMGO.

To determine whether receptor down-regulation could occur after shorter DAMGO exposure, cells were pretreated with various concentrations of this opioid for 5 hr before

opioid binding was measured. As in experiments with 48 hr of DAMGO exposure, 100 nM produced significant down-regulation of opioid receptors after a 5-hr exposure to DAMGO (Fig. 6B). The decrease in binding is also concentration dependent at 5 hr of exposure, when increasing concentrations of DAMGO were tested in three separate experiments. However, the magnitude of reduction with exposure to 1 or 10 μM of DAMGO for 5 hr was ~40% lower than that obtained with 48-hr exposure at the same concentration. When neurons were exposed to 1 μM DAMGO for 24 hr, the B_{max} value was reduced to a similar degree (~50%) as those with 48-hr treatment at the same concentration of DAMGO (data not shown).

Discussion

The results of this study demonstrate that abundant opioid binding sites exist on sensory neurons and that these receptors can be up- and down-regulated by chronic exposure to opioids. Indeed, the high abundance of opioid receptors in these neurons and their modulation by opioids establish sensory neuronal cultures as a model for the study of the regulation of opioid receptors and their potential role in opioid-induced antinociception.

We chose to examine binding using rat DRG neurons grown in culture because this provides a relatively homogeneous population of sensory neurons. Furthermore, opioid actions on sensory neurons may be important in mediation of opioid analgesia. Our cultures contain ~5% non-neuronal cells. Consequently, we examined binding on cells grown without nerve growth factor and mitotic inhibitors, thereby diminishing the number of sensory neurons. In this preparation, opioid binding measured by [³H]diprenorphine was not detectable, indicating that opioid receptors are not located on non-neuronal cells. The binding we measure in our neuronal cultures therefore is largely, if not exclusively, on sensory neurons.

Specific binding of opioid ligands to membranes prepared from sensory neurons in culture is saturable and of high

A

B
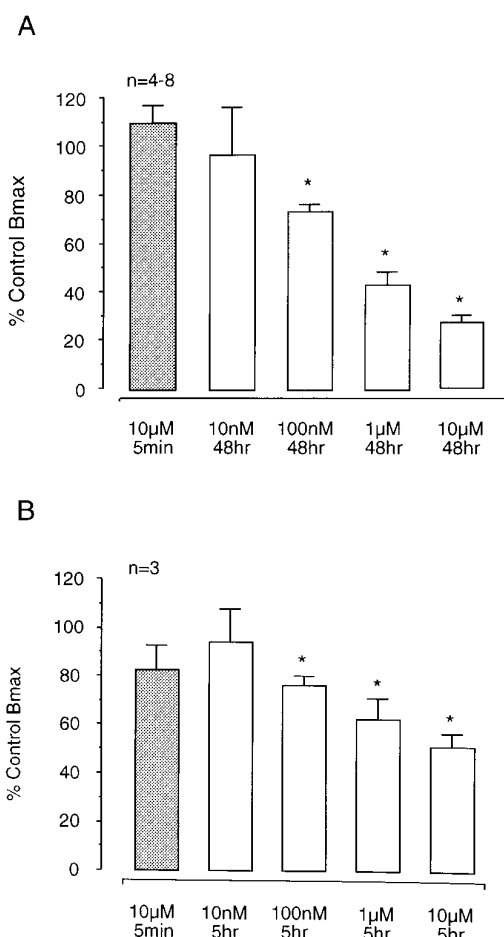
Fig. 6. Effect of various concentrations of DAMGO on opioid binding in sensory neurons. [^{3}H]Diprenorphine binding experiments were performed using sensory neuron membranes prepared from cells that had been grown in culture for 13–15 days and exposed to various concentrations of DAMGO for 5 min and (A) 48 hr or (B) 5 hr and from control cells that had been grown in the absence of DAMGO. B_{max} values are determined as a percentage of maximal binding in control cells using Scatchard analysis. In each experiment, control neurons and neurons treated with different concentrations of DAMGO were obtained from the same harvest. *Columns*, mean $\pm$ standard error for three to eight experiments. ▨, Binding after neurons were exposed to 10 μM DAMGO for 5 min; □, binding in membranes exposed to various concentrations of DAMGO for 5 or 48 hr; ∗, statistically significant difference ($p < 0.05$) between control and DAMGO-treated neurons using analysis of variance and Fisher's protected LSD.

affinity and can be displaced by naloxone. In addition, opioid receptors in isolated sensory neurons have similar affinities for selective ligands as those found in central nervous system. These observations confirm that the opioid receptors expressed by cultured sensory neurons have characteristics as opioid receptors *in vivo*. However, the binding of opioid receptors normalized to milligram of protein is higher in cultured rat sensory neurons than in spinal cord or primary cultures of embryonic brain from the same species (31, 32). This could be due to the presence of neuronal and non-neuronal cells in brain and spinal cord preparations that do not express opioid receptors. It is also possible that sensory neurons in culture contain higher levels of opioid receptors than do other types of neurons, although the calculated B_{max} values in our preparation could be an overestimation of spe-

cific binding secondary to the use of relatively high concentrations of naloxone to identify nonspecific binding.

Our data demonstrate that the μ receptor is the predominant subtype found in sensory neurons because DAMGO occupies 70% of [^{3}H]diprenorphine binding sites and this binding saturates at 2 nM. This value was obtained by estimating the B_{max} values from Scatchard analyses of three separate experiments. Furthermore, the apparent K_d value for DAMGO in these studies is ~0.5 nM, which corresponds to the affinity of this opioid for μ receptors (33). Consequently, the DAMGO binding measured is selective for μ receptors. There are significantly fewer δ and κ receptors (5% and 10%, respectively) observed in our sensory neuronal cultures. Indeed, the K_i values for displacement of [^{3}H]diprenorphine binding by δ and κ agonists were found to be in the micromolar range. Despite these high values, the K_d values for both δ- and κ-selective agonists were ~2 nM. The wide discrepancy between K_i and K_d values could result from the fact that most diprenorphine binding occurs at μ receptors, and the K_i values of δ and κ agonists represent, in part, nonspecific displacement at μ binding sites. In saturation binding studies, however, nanomolar concentrations of receptor-selective agonists are used and thus only high affinity binding is measured at the respective receptor subtypes.

The high μ binding in isolated sensory neurons is in agreement with immunohistochemical studies in the dorsal spinal cord and DRG (34). However, the low levels of δ receptor binding in our results are not consistent with the high level of δ receptor-like immunoreactivity detected by immunostaining experiments using dorsal spinal cord (35). This inconsistency might be accounted for by the fact that the ligand binding requires that the receptor form the appropriate tertiary structure in the membrane, whereas antibodies bind a specific amino acid sequence that may be independent of ligand binding. It is also possible that the neurons in culture have not fully expressed the δ receptors because of culture conditions or because they are derived from embryonic tissues. Further studies are needed to determined the reason for the difference in apparent expression of δ receptors in our model compared with the results of Arvidsson *et al.* (35).

In a similar manner, the low density of κ receptors in our cultures contrasts with the previous report by Attali and Vogel (32), which reported κ sites of 65–80% of total opioid binding sites in the rat spinal cord/DRG culture. There are a number of possible explanations for this discrepancy. First, cocultures of spinal cord slice and DRG contain various types of neurons, whereas our cultures contain only sensory neurons. Consequently, the κ receptor binding measured by Attali and Vogel could be localized on spinal cord neurons. It also is possible that the interaction between sensory neurons and their target neurons in spinal cord cocultures could enhance the expression of κ receptors. Finally, the number of κ receptors found in the cocultures could be overestimated, because κ binding was measured using a nonselective tritiated ligand (diprenorphine) in the presence of a high concentration of μ and δ ligands. [^{3}H]Diprenorphine could still bind to either μ or δ sites in this preparation, causing an overestimation of the number of κ sites.

Chronic treatment with opioid antagonists has been shown to increase the number of opioid receptors in brain, cell lines, and primary culture of mouse SC-DRG explants (18, 36, 37).

The increase in opioid binding after chronic administration of naltrexone or naloxone results from increases in the maximum binding capacity rather than from an increase in binding affinity. Our data show that the up-regulation of opioid receptors by the chronic use of naloxone is a direct effect on sensory neurons that results in a change in receptor number rather than altered affinity. Thus, our results in large part agree with the previous findings, despite the use of different experimental systems and procedures.

Our findings that exposure to opioid agonists results in a decrease in binding is the first evidence that opioid receptors in sensory neurons are down-regulated. This down-regulation is selective because chronic exposure to the μ-selective agonist DAMGO does not alter binding of either [³H]5-hydroxytryptamine or the κ-selective agonist [³H]U69,593. Furthermore, when sensory neurons are treated with both DAMGO and naloxone for 48 hr, the DAMGO-induced down-regulation is not observed. The observed decrease in the number of μ binding sites after DAMGO could reflect an alteration in the affinity state of the μ receptor from high to low. Indeed, Werling *et al.* (38) showed that chronic infusion of morphine into guinea pigs resulted in a decrease in the number of high affinity μ binding sites in cerebrocortical membranes. We did not examine whether there was an alteration in low affinity binding after chronic exposure to DAMGO. Our results clearly indicate a significant reduction in high affinity binding after DAMGO treatment and establish the use of isolated sensory neurons for further study of the mechanisms for opioid receptor down-regulation.

In previous studies, down-regulation of opioid receptors is produced only by high doses of nonselective and more potent agonists (etorphine, [D-Ala²,D-Leu⁵]enkephalin) that are associated with significant mortality (22, 23, 39, 40). Although studies have attempted to demonstrate the ability of morphine and several μ-selective agonists (e.g., fentanyl, PL017, sufentanyl, alfentanyl) to down-regulate opioid receptors (26, 41), the magnitude of reduction is either small or not observed. One possible explanation for the discrepancy between these studies and our results in isolated sensory neurons is that previous studies involve long-term *in vivo* administration of the lipophilic agonists before binding assays. Thus, it is not possible to eliminate the residual opioids in the membranes prepared from the brain, and this exogenous drug can interfere with opioid binding. For example, when [³H]etorphine was used to measure the residual amount after careful washing procedures, 34.6% of the ligand was still tightly bound to the membrane (22). Our experimental design circumvented this problem, as indicated by a parallel control in every experiment to affirm that residual DAMGO was eliminated and not interfering with our binding assay. We measure opioid bindings in neurons that were exposed to DAMGO for only 5 min before cell lysis and membrane preparation. If the washing procedures left behind a significant amount of DAMGO, the binding in these cells would be lower than that in control cells without DAMGO treatment. Because no difference was observed between neurons exposed to DAMGO for 5 min and control neurons, residual DAMGO could not affect our measurement of opioid bindings.

Another possible explanation for the difference between our results and those observed in brain tissue is that agonist-induced down-regulation could reflect neuronal development. This seems unlikely because all studies comparing binding in

cells pre-exposed to opioids had control cultures at the same stage in development. As such, the potential changes secondary to cell age are minimized.

The mechanism to account for opioid receptor regulation in sensory neurons remains unknown. Further studies are warranted to determine (a) the modulation of various subtypes of opioid receptors by selective ligands and (b) the effect of opioids on neurons with down-regulated opioid receptors. Because sensory neurons in culture express a large amount of opioid receptors and because these receptors are regulated by chronic exposure to opioids, these neuronal cultures represent an excellent model for studies of the mechanism of opioid receptor regulation.

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