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Antagonist-Induced Transient Down-regulation of δ-Opioid Receptors in NG108–15 Cells

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SUMMARY

According to current concepts, agonists can effect the down-regulation of cell surface receptors, whereas antagonists can cause their up-regulation. We have discovered that the opioid antagonists naltrexone, naloxone, and ICI174864 induce a transient down-regulation of δ-opioid receptors before up-regulation, in NG108–15 cells. The possibility of an apparent loss of sites due to blockade by residual antagonist was ruled out by several lines of evidence. The reduction in δ receptors was time, temperature, and antagonist concentration dependent. This down-regulation could not be induced by either the highly μ-selective opioid antagonist cyclic -D-Phe-Cys-Try-D-Trp-Arg-Thr-Pen-Thr-amide or the muscarinic antagonist atropine. In the same neurohybrid cells, the opioid agonist [D-Ala^2,D-Leu^5]enkephalin (0.1 μM, 60 min) effected a greater down-regulation of δ-opioid receptors. Similar qualitative changes in opioid binding of subcellular fractions were elicited with [D-Ala^2,D-Leu^5]enkephalin and naltrexone. However, the agonist was 2-fold more effective in reducing the heavy membrane population of receptors and 4-fold more potent in increasing the light membrane sites. Because heavy membranes are enriched in plasma membrane, whereas light membranes contain intracellular sites, these findings indicate that internalization occurs in both instances. Naltrexone and the δ-specific antagonists ICI174864 and naltrindole also diminished specific activities of two lysosomal enzymes, whereas opioid agonist-induced down-regulation was accompanied by an increase in their specific activities. Pretreatment of cell cultures with concanavalin A blocked both down-regulation and alterations in the lysosomal enzyme activities elicited by agonists and antagonists, suggesting that the latter is an opioid receptor-mediated process. The up-regulation of δ-opioid receptors by antagonists appears, then, to entail down-regulation that differs from that of agonists.

The phenomena of tolerance and physical dependence arising from chronic opioid exposure have not been defined (Ref. 1 and references cited therein). However, the development of neuroblastoma and neurohybrid cell cultures has provided a simplified model system for the study of opioid receptor adaptation (2–4). Upon long term agonist exposure to cells, at least two different time-dependent changes in δ receptors were reported to occur, i.e., desensitization (2, 3, 5), defined as a reduced ability of the agonist to inhibit adenylyl cyclase activity, and receptor down-regulation (4–6), designated as a reduction in the total number of cellular receptors.

Opioid receptor down-regulation occurs within 1 hr of medium supplementation of neurotumor cell cultures with enkephalins and persists for at least 48 hr (6). This reduction in receptor number is also elicited by opiate alkaloids that display an affinity for the receptor (5, 7). Nevertheless, enkephalin-induced down-regulation is partially blocked by a variety of nonselective opiate agonists and antagonists, by an unknown mechanism (4). For example, 50 μM morphine (which is not selective at this concentration) (8) was reported to reduce down-regulation induced by 0.1 μM DADLE by 73%.

In contrast, opioid receptor down-regulation has been difficult to document *in vivo*. However, antagonist-induced up-regulation has been well substantiated both *in vivo* (9, 10) and *in vitro* (Refs. 11 and 12 and references cited therein). In most cases, naltrexone or naloxone have been the antagonists of choice.

Here we report the discovery of a novel down-regulation of δ-opioid receptors induced by antagonists and we investigate its mechanism, comparing it with that of the agonist-mediated process. Upon short term treatment of NG108–15 cells with naltrexone, naloxone, and δ-selective opioid antagonists, decreased B_{max} values in homogenates and/or density gradient-purified preparations of cell surface receptors were observed. In contrast to the agonist-induced process, antagonist-induced

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ABBREVIATIONS: DADLE, [D-Ala^2,D-Leu^5]enkephalin; Con A, concanavalin A; CTAP, cyclic D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-amide; G protein, guanine nucleotide-binding regulatory protein; HM, heavy membrane; ICI174684, (allyl)$_2$Tyr-Aib-Aib-Phe-Leu-OH; LM, light membrane; QNB, quinuclidinyl benzilate; PBS, phosphate-buffered saline.

down-regulation was accompanied by a significant loss of lysosomal enzyme activity in NG108–15 cell homogenates.

Materials and Methods

Chemicals. Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO). ICI174864 was purchased from Cambridge Research Biochemicals (Wilmington, DE), whereas CTAP and [D-Ser,[2] L-Leu[5]]enkephalyl-Thr were obtained from Multiple Peptide Systems (San Diego, CA).

Cell cultures, treatment, and harvesting. NG108–15 cells were grown at 37° in a humidified CO_2 (10%) incubator in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 containing calf serum (10%). Cells were subcultured in 150-cm^2 flasks, and media were changed every 3 days. Cultures were treated with antagonists or agonists at varying concentrations and for the time periods indicated in the legends to the tables and figures. After a total of 5 days of growth, cells were harvested and washed twice with 35 ml of PBS (10 mM sodium phosphate, pH 7.75, 0.9% NaCl) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (buffer A). Additional washings (two or three times) were performed depending upon the type of Con A treatment (see below).

Con A pretreatment of cell cultures. The medium of 5-day-old confluent NG108–15 cells was changed to PBS, in the presence or absence of 0.25 mg/ml Con A (30 ml/flask), as described (13). Flasks were then maintained in the incubator at 37° for 20 min. Con A-treated cells were washed twice with PBS to remove unbound lectin, and then PBS with either naltrexone or DADLE was added. Incubation with DADLE took place at 37° for 60 min, whereas naltrexone treatment was for 5 min. Cells were harvested and washed twice with buffer A and twice with the same buffer without the divalent cations.

Subcellular fractionation. The fractionation protocol was adopted from the procedure of Sweat and Klee (14), with minor modifications. To facilitate subcellular fractionation, cells were washed twice with buffer A and were incubated at room temperature for 15 min with the same buffer containing 0.25 mg/ml Con A. This protocol differs from the Con A pretreatment described above in that it was performed at room temperature and after the cells had been treated with opioids and harvested. Cells were collected and rinsed twice with buffer A without Mg^{2+} and Ca^{2+}. Cells were then homogenized using a "cell cracker" (15). Briefly, cell suspensions were forced through a 36.83-μm precision bore opening in a stainless-steel block. In preliminary experiments, we demonstrated with light microscopy and enzyme marker studies that the aforementioned clearance affords homogenization of NG108–15 cell cultures that is optimal for the isolation of Golgi-enriched LMs and plasma membrane-enriched HMs. The fraction obtained from the cell cracker, referred to as cell homogenate, was either used directly (for binding or enzyme assays) or layered onto a sorbitol step gradient (20%, 32%, and 54%), which was centrifuged at 100,000 × g for 2 hr. The 20–32% sorbitol interface was recovered by centrifugation in 50 mM Tris·HCl, pH 7.4, at 150,000 × g, and this fraction was designated as LMs. The 32–54% sorbitol interface (HMs) was collected by similar centrifugation, after removal of lectin with 0.5 M α-methylmannoside.

Marker enzymes, i.e., ouabain-sensitive Na^+/K^+ ATPase and UDP-galactose transferase, were used to demonstrate that LMs were enriched in Golgi complexes, whereas HMs contained most of the plasma membranes (12, 16). β-Glucuronidase activity was measured with 4-methylumbelliferyl β-D-glucuronide as a substrate, whereas β-hexoseaminidase activity was estimated with 4-methylumbelliferyl N-acetyl β-D-glucosaminide (17).

Receptor binding assays. Homogenates or membrane fractions from NG108–15 cells were assayed for opioid binding activity as previously described (12). Preparations (200–600 μg of protein) were incubated with 1 nM [^{3}H]DADLE (30–46.9 Ci/mmol; NEN, DuPont, Boston, MA) at 25° for 60 min or with 1 nM [^{3}H]diprenorphine (47 Ci/mmol; Amersham, Arlington Heights, IL) at 37° for 20 min. Homologous competition binding assays were performed in the presence of 10–12 different concentrations of the corresponding unlabeled ligand. Muscarinic binding was measured with 0.5 nM [^{3}H]QNB (44.3 Ci/mmol; New England Nuclear, Boston, MA) at room temperature for 2 hr. Nonspecific binding was determined in the presence of 1 μM atropine and represented ≤20% of total binding. Incubations were terminated by addition of ice-cold 50 mM Tris·HCl, pH 7.4, samples were collected on Whatman GF/B filters, using a Brandel cell harvester (Gaithersberg, MD), and subjected to three 5-ml washes with buffer. In the [^{3}H]diprenorphine experiments, filters were presoaked in the same buffer containing 0.02% polyethylene glycol. Filters were then dried and counted in a Packard Tri-carb 1500 liquid scintillation analyzer.

Protein concentrations were determined by the method of Lowry *et al.* (18), with bovine serum albumin as a standard. Binding affinities were estimated by the LIGAND data analysis and curve-fitting program (19). Statistical analyses of the data were performed with the Student t test.

Results

Antagonist-induced down-regulation in NG108–15 cells. Short term treatment of NG108–15 cells with the opioid antagonist naltrexone (1 μM) resulted in down-regulation of δ-opioid receptor binding, as shown by homologous competition binding assays with [^{3}H]DADLE (Fig. 1). A time-course study with cell homogenates revealed that 2–5-min exposure to naltrexone gave optimum down-regulation (Fig. 2). Upon increasing of the interval of antagonist treatment to 30 min, B_{max} values were restored to control values, whereas up-regulation of δ-opioid binding appeared after at least 12 hr of exposure (Fig. 2). The down-regulation was reminiscent of that generated by agonist (0.1 μM DADLE, 1 hr) (Fig. 1). When the partial agonist [^{3}H]diprenorphine was used as radioligand, two high affinity binding sites were detected in homogenates from control and naltrexone-treated NG108–15 cells (Table 1). This is

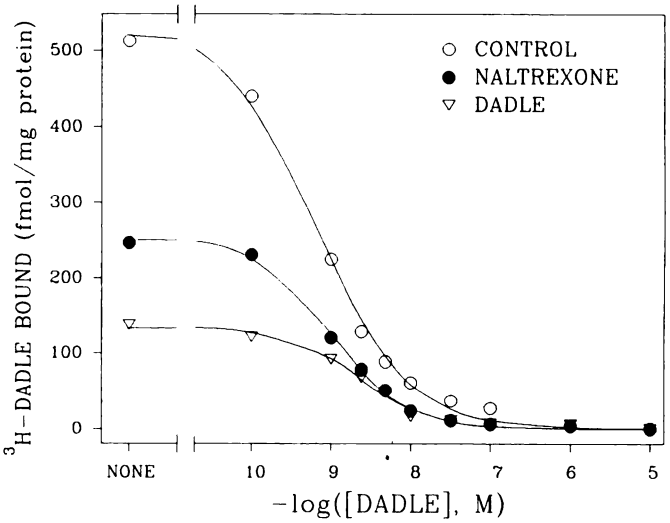

Fig. 1. [^{3}H]DADLE homologous competition binding curves obtained from NG108–15 cell homogenates. Cultures were treated with naltrexone (1 μM, 5 min) or DADLE (0.1 μM, 60 min), as described in Materials and Methods. The concentrations of unlabeled DADLE required to reduce [^{3}H]DADLE binding by 50% in the assay were as follows: control, 0.9 nM; naltrexone-treated, 1.0 nM; DADLE-treated, 1.7 nM. These values were determined graphically and correspond to K_d values, which were estimated using the LIGAND computer program (see the legends to Figs. 2 and 3). *Curves* are representative of three curves each for control and treated cell cultures.

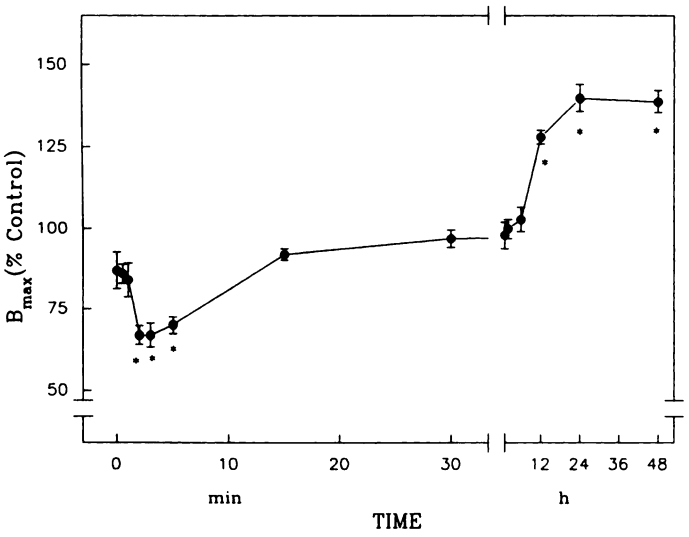

Fig. 2. Changes in [^{3}H]DADLE binding to cell homogenates from NG108–15 cells treated with 1 μM naltrexone for various periods of time. Before homogenization, cultures were washed five times with buffer A, as described in Materials and Methods. Each *point* is the mean of three determinations of B_{max} values (expressed as percentage of control, which ranged from 591 ± 45 to 731 ± 31 fmol/mg of protein) from homologous competition curves. Binding parameters were estimated using the LIGAND program. K_d values ranged from 2.6 ± 0.4 to 3.9 ± 0.3 nM. *, Different from control ($p < 0.05$).

TABLE 1

Agonist and antagonist binding parameters for membrane preparations after *in vitro* and *in vivo* naltrexone treatment

For *in vitro* experiments, 20,000 × *g* membrane pellets from NG108-15 cell-free homogenates were treated with 1 μM naltrexone for 5 min and then washed five times with 50 mM Tris·HCl, pH 7.4. Binding assays were carried out as described in Materials and Methods (three experiments). For *in vivo* experiments, homogenates were prepared from NG108-15 cells that had been treated with 1 μM naltrexone for 5 min, as described in Materials and Methods. [^{3}H]diprenorphine binding data fit a two-site model better than a single-site model (three experiments).

Treatment	[^{3}H]DADLE		[^{3}H]Diprenorphine	
	K_d	B_{max}	K_d	B_{max}
	nM	fmol/mg of protein	nM	fmol/mg of protein
In vitro				
Control	1.8 ± 0.03	475 ± 41	1.1 ± 0.2	565 ± 51
Naltrexone	1.9 ± 0.06	586 ± 68	1.2 ± 0.09	704 ± 29
In vivo				
Control	2.4 ± 0.06	784 ± 23	0.01 ± 0	32 ± 3.2
			12 ± 1.2	752 ± 57
Naltrexone	3.9 ± 0.3	593 ± 35[a]	0.4 ± 0.2	42 ± 3.6
			23 ± 6.7	586 ± 20[a]

[a] Treated versus controls, $p < 0.05$.

most likely due to the fact that we were measuring binding in total homogenates, which contain not only plasma membrane receptors but also intracellular sites. In another study, we found that some of the intracellular sites have a high affinity component that we are able to resolve. The statistically insignificant 22% increase in [^{3}H]diprenorphine binding of the *in vitro* experiment parallels the increase seen for [^{3}H]DADLE binding under the same conditions and serves to support our argument that naltrexone blockade of sites is not occurring. If it were, we would have seen a decrease in binding.

Antagonist-induced down-regulation of δ-opioid receptors was dose dependent. However, treatment of NG108–15 cells for 5 min with different concentrations of naltrexone, naloxone, diprenorphine, or the muscarinic antagonist atropine gave variable results, depending upon the drugs tested (Fig. 3). IC_{50} values (concentrations at which the ligands induce half of the maximal receptor down-regulation) for naltrexone and naloxone, as determined by [^{3}H]DADLE binding to cell homogenates (B_{max} values), were similar (1–2 nM) (Fig. 3). The values are 1 order of magnitude less than the K_d values for naltrexone and naloxone for these receptors (19.2 ± 7.7 and 13.4 ± 5.2 nM, respectively), measured in the same preparations. This is consistent with the notion that receptor occupation by antagonist is driving this down-regulation. Interestingly, naltrexone and naloxone did not induce more than 40–50% reduction in receptor density at any concentration studied, whereas 0.1 μM DADLE was reported to cause >80% down-regulation in mem-

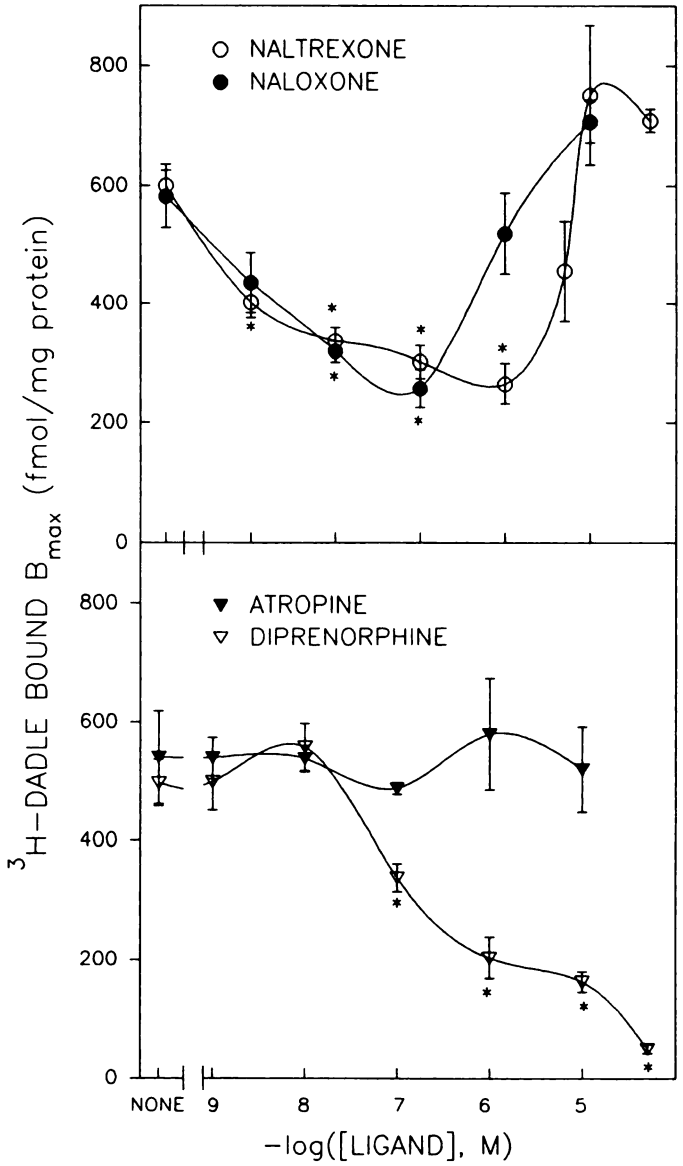

Fig. 3. [^{3}H]DADLE binding to homogenates from NG108–15 cells treated with different concentrations of antagonists. Cell cultures were supplemented with various concentrations of naltrexone, naloxone, diprenorphine, or atropine. After 5 min, cells were harvested and homogenates were prepared as described in Materials and Methods. K_d values ranged from 2.2 ± 0.1 to 6.3 ± 0.4 nM. IC_{50} values were determined graphically. *, Treated cells were significantly different from controls ($p < 0.05$). Data are the mean ± standard error of three experiments.

brane fractions from N4TG1 cells (4) and 58–65% down-regulation in these studies (Fig. 1 and see below). Moreover, at concentrations of >1 μM, naltrexone and naloxone returned [^{3}H]DADLE binding to control densities, presumably due to an acceleration of up-regulation. Diprenorphine, which is a partial agonist, exhibited an IC_{50} value of 200 nM for down-regulation, whereas the K_d for its lower affinity site was 17-fold lower (Table 1). In contrast to naltrexone and naloxone, diprenorphine caused down-regulation of δ-opioid binding at all concentrations studied, but to a lesser extent and less potently than did the typical agonists etorphine and DADLE (4, 5). Atropine treatment did not affect [^{3}H]DADLE binding.

Because 1 μM naltrexone binds to both δ- and μ-opioid receptors, specificity requirements for down-regulation were considered. If NG108–15 cells were treated with the δ-selective opioid peptide antagonist ICI174864 (5 min), a concentration-dependent decrease of [^{3}H]DADLE binding (B_{max} values) to homogenates was observed (Fig. 4). In contrast, if cell cultures were pretreated with similar concentrations of this antagonist for 48 hr, an up-regulation of δ-opioid binding was detected in homogenates, as shown in Fig. 4. Again, exposure to the highly selective δ-opioid antagonist ICI174864 had no effect on the affinity of [^{3}H]DADLE binding.

In contrast to naltrexone and naloxone, neither short nor long term treatment with the highly μ-selective (8) antagonist CTAP (1 μM) caused down- or up-regulation of δ-opioid binding in NG108–15 cell homogenates. Corresponding B_{max} values for [^{3}H]DADLE were 739 ± 31, 724 ± 39, and 733 ± 32 for control, 5-min-treated, and 48-hr-treated cells, respectively. At a 1 μM concentration, CTAP has insignificant affinity for δ sites, which is not the case for naltrexone or naloxone. Both naltrexone and naloxone display nanomolar affinity for δ sites.

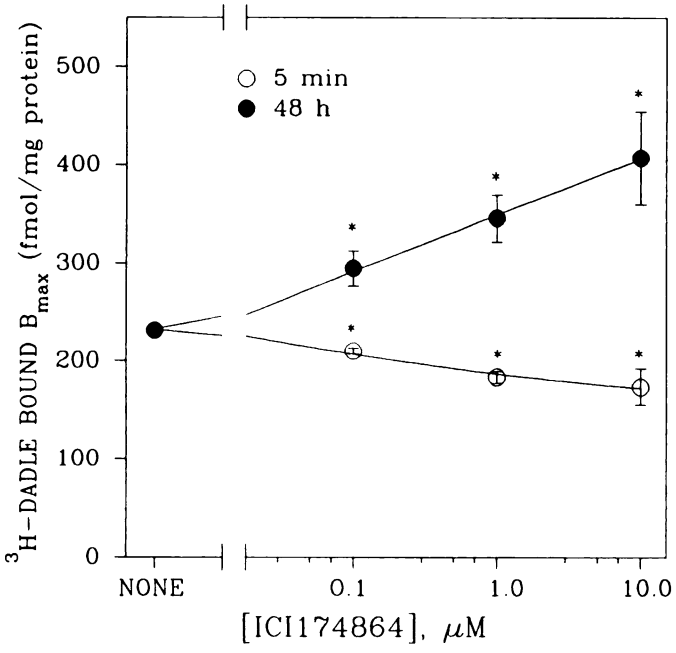

Fig. 4. [^{3}H]DADLE binding parameters of homogenates from NG108–15 cells treated with ICI174864. Cell cultures were supplemented with various concentrations of ICI174864 for 5-min or 48-hr periods. Homogenates were prepared as described in Materials and Methods. K_d values ranged from 1.3 ± 0.09 to 2.1 ± 0.03 nM. *, Treated cells were significantly different from controls ($p < 0.05$). Data are the mean ± standard error of three experiments.

Because NG108–15 cells also express muscarinic receptors (20), it was of interest to test the selectivity of the naltrexone effect on cholinergic binding. However, muscarinic binding measured with [^{3}H]QNB was not affected by either short term (93 ± 5.1% of control) or long term (91 ± 2.9% of control) exposure of cell cultures to naltrexone.

Evidence to exclude the possibility of antagonist masking of opioid binding sites. Data in Figs. 1 and 2 show that down-regulation was induced by an opioid antagonist and an agonist without significant changes in affinity. The fact that K_d values for down-regulated opioid receptors did not undergo a change regardless of whether naltrexone or DADLE was the inducer, in both these experiments (Figs. 1 and 2) and those reported below, indicates the induction of an authentic down-regulation of δ-opioid receptors. In contrast, "apparent" receptor down-regulation due to the presence of residual agonists has been detected in studies with opioid (21) and other systems and is accompanied by a significant decrease in receptor affinity, with little or no change in density. We observed 4–10-fold increases in K_d values when we failed to wash our membranes adequately after treatment of cells with opioids.

In addition to the constancy of K_d values for opioid binding in down-regulated cells, several other lines of evidence rule out the possibility that antagonists were simply masking sites, instead of inducing down-regulation. When naloxone and naltrexone concentrations were elevated to >1 μM, down-regulation was abolished (Fig. 3). Moreover, receptor down-regulation is a process that requires intact cells. Therefore, opioid antagonists would not be expected to cause the same effect in a cell-free preparation. Accordingly, naltrexone did not diminish binding to 20,000 × g membrane preparations *in vitro*. Upon incubation of membranes for 5 min with 1 μM naltrexone, followed by five washes with buffer, K_d and B_{max} values for both [^{3}H]DADLE and [^{3}H]diprenorphine were unchanged (Table 1). When NG108–15 cells were pretreated for 5 min with 1 μM naltrexone at 4°, a temperature at which receptor-mediated endocytosis is arrested, [^{3}H]DADLE B_{max} values for treated cell homogenates (701 ± 50 fmol/mg of protein) were not statistically different from controls (548 ± 37 fmol/mg of protein).

Con A blockade of both agonist- and antagonist-induced down-regulation in NG108–15 cells. The findings described above raised the question of whether antagonists elicit down-regulation by a process of endocytosis similar to that for agonists. Because it has been demonstrated that pretreatment of cells with Con A blocks agonist-induced sequestration of β-adrenergic receptors (Ref. 13 and references cited therein), this procedure was used with NG108–15 cells before agonist- and antagonist-induced down-regulation. As shown in Fig. 5, if cells were pretreated with Con A the inhibition of δ-opioid binding by naltrexone or DADLE was abolished and B_{max} values were similar to those in control cells. K_d and B_{max} values were determined for the agonist [^{3}H]DADLE, and comparable results were obtained with the partial agonist [^{3}H]diprenorphine in these experiments (Fig. 5).

Subcellular distribution of δ-opioid receptors after down-regulation. It has been established that long term incubation of cells with agonists alters the sedimentation behavior of β-adrenergic receptor-containing membranes (Ref. 13 and references cited therein). This phenomenon could be due to a redistribution of receptor sites within the cell. Therefore, we examined the effects of agonist (DADLE) or antagonist

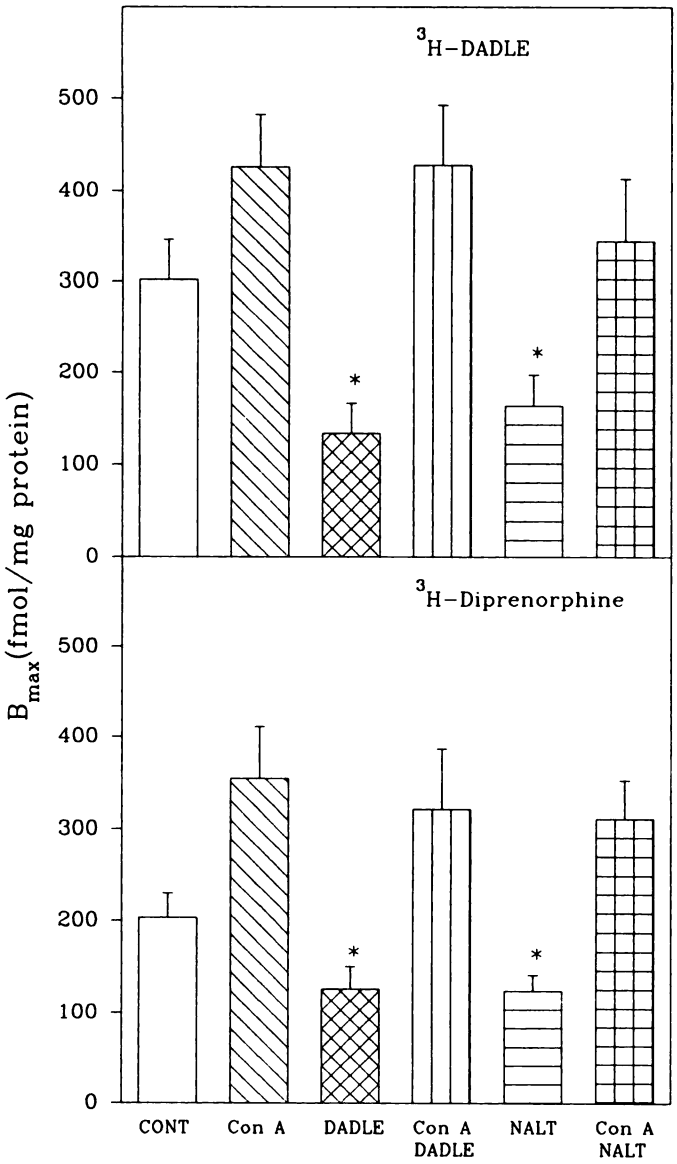

Fig. 5. Blockade by Con A of both agonist- and antagonist-induced down-regulation in NG108–15 cells. Controls (*CONT*) were run in the absence of opioids and Con A pretreatment. Cell cultures were pretreated with Con A (0.25 mg/ml) in PBS, as described in Materials and Methods. DADLE (0.1 μM, 60 min) or naltrexone (*NALT*) (1 μM, 5 min) was added either with or without Con A pretreatment of the cultures. [^{3}H]DADLE (*top*) and [^{3}H]diprenorphine (*bottom*) B_{max} values were measured in homogenates, prepared with the cell cracker, from control and treated cells. K_d values ranged from 0.5 ± 0.09 to 2.1 ± 0.3 nM. *, Values for DADLE and naltrexone treatment in the absence of Con A were significantly different from all others ($p < 0.05$). Data represent the mean ± standard error from three experiments.

(naltrexone) treatment on δ-opioid binding distribution in subcellular fractions from NG108–15 cells. In our previous studies, we prepared cell homogenates using a Polytron, followed by sorbitol gradient centrifugation (12, 16). Here cells were disrupted by a cell cracker (15). The purity of the subcellular fractions was assessed with marker enzymes. The data revealed that the cell cracker afforded a better resolution of LMs and HMs than previously achieved (16). Activity of the Golgi marker UDP-galactosyltransferase was not detectable in the HM preparation, whereas the specific activity of the plasma membrane marker ouabain-sensitive Na^+/K^+-ATPase was 3.9 times higher for HMs (74 ± 3.8 nmol/mg/min) than for LMs (19 ± 2.7 nmol/mg/min).

Membrane preparations of LMs and HMs were examined for δ-opioid binding, with [^{3}H]DADLE, before and after the cells were treated with 1 μM naltrexone for 5 min. No changes were observed in K_d values of control and treated cells. B_{max} values for LM binding in naltrexone-treated cells were elevated by 51%, whereas a 29% decrease in the density of δ-opioid binding sites was estimated for HMs (Fig. 6). Upon pretreatment of NG108–15 cells with DADLE (0.1 μM, 60 min), the distribution of δ-opioid binding in the subcellular fractions displayed a similar pattern, but one that differed quantitatively, in comparison with the effects produced by naltrexone. The agonist

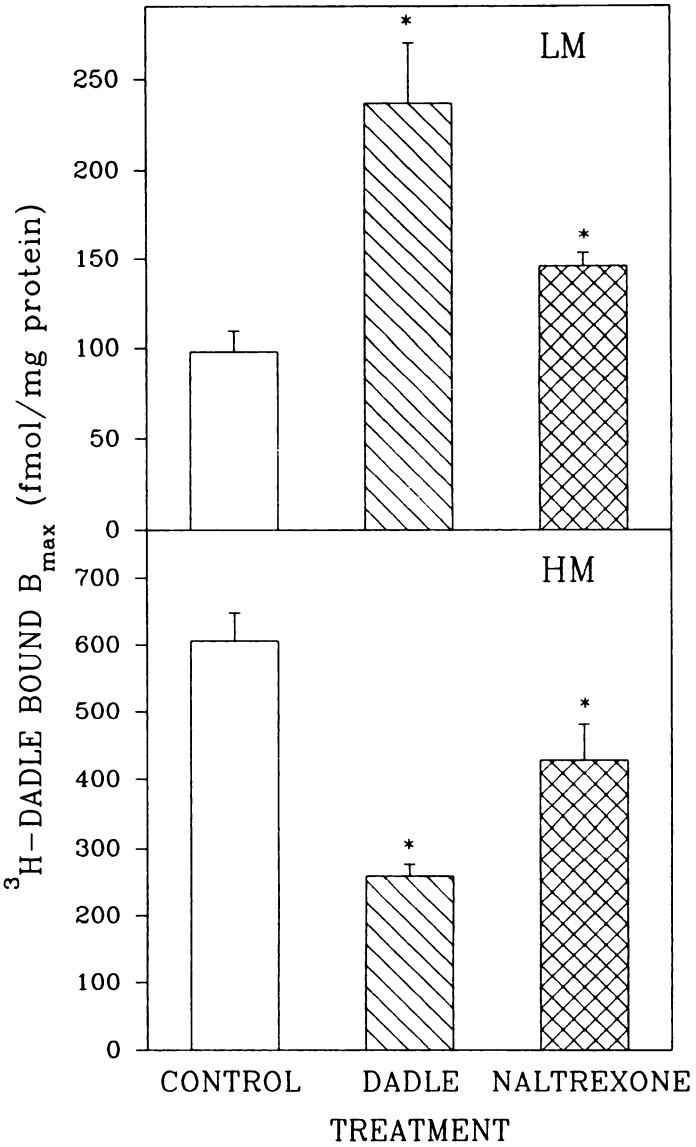

Fig. 6. [^{3}H]DADLE binding parameters of subcellular fractions from naltrexone- or DADLE-treated NG108–15 cell cultures. Cells treated with opioids, as described in Materials and Methods, were homogenized with the cell cracker. Membrane fractions were resolved by sorbitol density gradient centrifugation. K_d values ranged from 2.4 ± 0.3 to 2.9 ± 0.2 nM for DADLE pretreatment and from 1.2 ± 0.2 to 2.0 ± 0.2 nM for naltrexone-exposed cells. Protein levels in subcellular fractions did not change upon opioid treatment. *, Significant difference for DADLE- or naltrexone-treated versus control ($p < 0.05$). Data represent the mean ± standard error from three to six experiments.

increased the density of [^{3}H]DADLE binding sites in LMs by >2-fold and decreased HM B_{max} values by 58%. K_d values were not affected.

Lysosomal enzyme activity changes after opioid agonist and antagonist treatment of NG108–15 cell cultures. Antagonist-induced up-regulation of opioid receptors is not blocked by the protein synthesis inhibitor cycloheximide in NG108–15 cells (12). This mechanism of up-regulation may entail termination of receptor degradation, a process that may involve lysosomal proteases. Therefore, we tested lysosomal enzyme activities in homogenates from control and opioid antagonist-treated NG108–15 cells. Previous time and concentration dependency studies with naltrexone indicated that 5-min exposure and 1 μM concentrations of several δ antagonists evoked optimal inhibition of β-glucuronidase and β-hexoseaminidase activities (12). Upon 5-min treatment with naltrexone or the δ-selective opiate alkaloid antagonist naltrindole (0.1 nM), activities of both β-glucuronidase (46% and 40%, respectively) and β-hexoseaminidase (42% and 31%, respectively) were reduced (Table 2). The δ-selective peptide antagonist ICI174864 elicited a concentration-dependent inhibition of both lysosomal enzymes after a short term treatment of the cell cultures (Fig. 7). The antagonists elicited significant attenuation of enzyme activities at the same concentrations (0.1–10 μM) at which down-regulation was observed (Fig. 4). This loss in enzyme activity persists after up-regulation (12).

These findings raised the question of whether supplementation of NG108–15 cell culture medium with an opioid agonist would produce comparable effects on lysosomal enzymes. Specific activities of β-glucuronidase and β-hexoseaminidase in homogenates from cell cultures treated with the opioid agonist DADLE (0.1 μM, 60 min) are shown in Table 2. In contrast to the antagonist-induced decrease, there was a significant increase, of 21–23%, in the activities of both enzymes in homogenates from DADLE-treated cells. These findings suggest that agonists and antagonists are acting via different mechanisms, with respect to the modulation of lysosomal enzymes.

Noteworthy was the observation that 1 μM naltrexone influenced neither β-glucuronidase nor β-hexoseaminidase activities *in vitro*. Upon incubation of a 20,000 × *g* membrane preparation from NG108–15 cells with antagonist and lysosomal substrates, enzyme activities did not differ from controls (β-glucuronidase, 4.7 ± 0.3 versus 4.8 ± 0.2 nmol/min/mg; β-hexoseaminidase, 49 ± 6.5 versus 50 ± 5.9 nmol/min/mg).

If opioid-induced changes in lysosomal enzyme activities occur independently of opioid receptor down-regulation, then alterations in enzyme activity may persist when this down-regulation is inhibited by Con A. However, Con A pretreatment also blocked both agonist- and antagonist-induced changes in lysosomal enzyme activities (Table 2). Interestingly, it also elevated lysosomal enzyme activity 1.8-fold. This change in enzyme activity was not observed when Con A was added after the cells had been harvested (β-glucuronidase, 4.0 ± 0.06 versus 4.3 ± 0.09 nmol/min/mg; β-hexoseaminidase, 46 ± 0.7 versus 46 ± 2.8 nmol/min/mg of protein).

TABLE 2

Lysosomal enzyme specific activities in NG108-15 cell homogenates

In experiments 1–4, NG108-15 cells were treated with naltrexone (1 μM, 5 min), the specific δ-opioid antagonist naltrindole (0.1 nM, 5 min), or DADLE (0.1 μM, 60 min). Cultures were washed with PBS, and cell-free homogenates were prepared as described in Materials and Methods. In experiments 5–7, the cells were pretreated with Con A (0.25 mg/ml, 20 min) and washed several times, and then naltrexone (1 μM, 5 min) or DADLE (0.1 μM, 60 min) was added. Cells were then washed and total homogenates were prepared as described above. Three to nine experiments were performed.

Experiment	Treatment	β-Glucuronidase	β-Hexoseaminidase
		nmol/min/mg	*nmol/min/mg*
1	Control	4.8 ± 0.3	48 ± 3.4
2	Naltrexone	2.6 ± 0.09*	28 ± 2.1*
3	Naltrindole	2.9 ± 0.2*	33 ± 2.6*
4	DADLE	5.9 ± 0.12*	58 ± 4.8*
5	Con A pre-treatment	8.4 ± 0.2	78 ± 2.4
6	Con A + naltrexone	8.5 ± 0.07	81 ± 4.0
7	Con A + DADLE	7.9 ± 0.1	78 ± 3.6

* Different from control, $p < 0.05$.

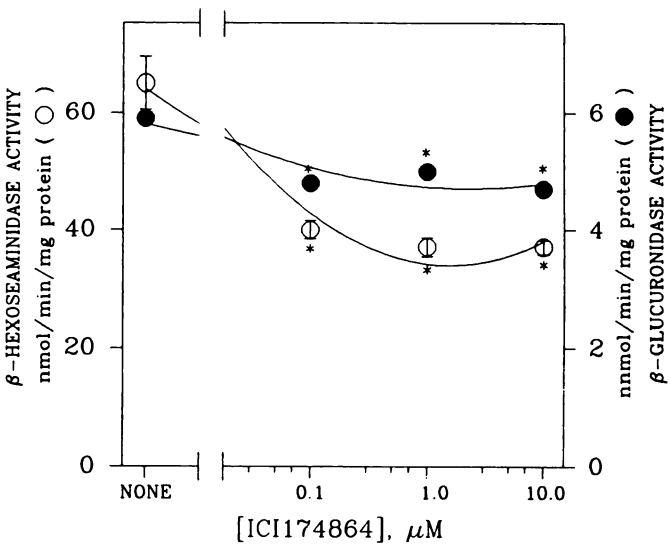

Fig. 7. ICI174864 concentration dependence of lysosomal enzyme specific activities in NG108–15 cell homogenates. Cell cultures were treated with various concentrations of ICI174864 for 5 min, and then β-glucuronidase or β-hexoseaminidase activities were measured as described in Materials and Methods. *, Treated cells were significantly different from controls ($p < 0.05$). Data are the mean ± standard error of three experiments.

Discussion

In this study, a novel opioid antagonist-induced down-regulation of δ-opioid binding in NG108–15 cells was detected and compared with that elicited by an agonist. As shown in Figs. 2, 3, and 4, antagonist-induced down-regulation is a time-, temperature-, and concentration-dependent process. Inasmuch as the highly μ-selective (8) antagonist CTAP was not effective, most likely only δ-opioid receptors are involved here and inducing antagonists must display δ affinity. This is consistent with the correlation between IC_{50} values for naltrexone- and naloxone-induced down-regulation and K_d values observed for these antagonists in binding assays and further supports the requirement for receptor occupation in this process (Fig. 3).

Several lines of evidence indicate that the observed decrease in the density of δ-opioid receptors after short term treatment with antagonists reflects antagonist-induced down-regulation of the receptors, rather than the presence of residual drug during binding assays. 1) Authentic down-regulation of δ-opioid binding was corroborated by the constancy of the K_d values of [^{3}H]DADLE and [^{3}H]diprenorphine binding in NG108–15 cell

homogenates (Figs. 1 and 3; Table 1). 2) Down-regulation of receptors occurs only in intact cells. This effect was not reproduced after addition of 1 μM naltrexone to cell-free preparations. 3) It is obvious from Fig. 3 that naltrexone elicits its maximal effects at a 1 μM concentration. At higher concentrations the transient down-regulation effect was not detected after 5 min. If the receptor loss was attributed to the presence of residual ligand not removed in the five washings, then at higher drug concentrations receptor losses should have increased. Instead, binding activity reverted to control values at concentrations of ≥1 μM for naltrexone and 1–10 μM for naloxone. These findings exclude the explanation that [^{3}H]DADLE or [^{3}H]diprenorphine binding is prevented by residual antagonist. 4) The partial agonist diprenorphine down-regulates the receptor to a greater extent than do the typical antagonists naltrexone, naloxone, and ICI174864, and this drug does not appear to reverse binding to control values or to cause up-regulation at concentrations used in these studies. 5) Treatment of NG108–15 cells with 1 μM naltrexone at 4° (a temperature at which receptor internalization is impeded) does not lead to down-regulation of δ opioid binding. 6) Con A pretreatment of cells blocks agonist- and antagonist-induced opioid receptor down-regulation, consistent with its effect on β-adrenergic receptors exposed to agonists (13). 7) Comparable changes in the subcellular distribution of opioid receptors upon naltrexone or DADLE addition to cell cultures also support this notion. Both opioids caused a loss in the HM population of receptors and an increase in LM sites. Because HMs are enriched in plasma membrane receptors, whereas LMs contain the bulk of the intracellular sites, it is reasonable to assume that internalization occurs in both instances, consistent with findings for opioids (22) and other receptor systems.

Although agonist- and antagonist-induced opioid receptor down-regulation processes possess some common features, there appear to be differences. Firstly, there are quantitative variations in opioid receptor density reduction. Secondly, the opposite effects seen on lysosomal enzyme activities (Table 2; Fig. 7) for agonists and antagonists may also have mechanistic implications. A possible involvement of lysosomes in the down-regulation of δ-opioid receptors has been investigated in NG108–15 cells (22) and other neuroblastoma cell lines (23). Evidence has been gained to suggest involvement of lysosomes in the degradation of the internalized opioid peptide-receptor complex. Because low levels of the opiate alkaloid analog naltrindole and the opioid peptide ICI174864 (1 μM) elicited a similar loss of lysosomal activity, a mechanism entailing lysosomotropic effects is unlikely. The elevation of the intracellular pH seen with acidophilic weak bases, such as 50 μM chloroquine, could not be achieved by low levels (0.1 nM) of naltrindole used in these experiments. Moreover, it is unlikely that a peptide such as ICI174864 would be taken up by lysosomes by passive diffusion. In contrast to antagonist action, DADLE treatment of NG108–15 cells caused an increase in the specific activity of the two lysosomal enzymes studied. Moreover, Con A prevents lysosomal enzyme changes. In summary, these data reveal the existence of a putative mechanism of modulation of lysosomal enzyme activity by opioid agonists and antagonists involved in δ receptor down- and up-regulation. Differential effects of DADLE and naltrexone on lysosomal enzyme activities are consistent with their ultimate physiological actions. Because agonist-induced down-regulation may lead to degradation of peptides and/or receptors by lysosomal enzymes, it is reasonable that agonists would enhance hydrolase activity. In contrast, antagonists may elicit their up-regulation by blocking receptor degradation via a mechanism involving the suppression of lysosomal enzyme activity. This is supported by the persistence of antagonist-induced loss of lysosomal enzyme activity, in NG108–15 cell homogenates, accompanying up-regulation after 48 hr (12).

Inhibition of opioid agonist binding by guanine nucleotides is a well documented phenomenon, which is known to reflect G protein coupling to the opioid receptor and, therefore, is a criterion of receptor functionality. Binding sensitivity to GTP analogs has been correlated with opioid inhibition of adenylyl cyclase and opioid stimulation of GTPase activity in NG108–15 HMs (12, 14, 16). In control LMs, the absence of opioid effects on adenylyl cyclase and GTPase activities is paralleled by opioid binding insensitivity to guanine nucleotides. Upon short term exposure of NG108–15 cells to naltrexone, enhanced sensitivity of LM agonist binding to a GTP analog ensues (12, 16). This enhancement may be interpreted as being due to the presence in LMs of greater amounts of newly synthesized and/or recycled intracellular opioid receptors *en route* to the cell surface along with G proteins (Refs. 12 and 16 and references cited therein). Once these newly synthesized and/or recycled receptors reach the cell surface, up-regulation is achieved.

Recently, it has been demonstrated that β-adrenergic receptors can be down-regulated by 1-hr antagonist treatment in human leukocytes and murine lymphoma cells *in vitro* (24). Moreover, the clinical drugs pindolol and celiprolol down-regulated the β-adrenergic receptor by a mechanism different from the one required by typical agonists (25, 26). Although Molinoff and co-workers referred to these compounds as "atypical" agonists, their inability to affect adenylyl cyclase could be interpreted to indicate that pindolol and celiprolol are antagonists. It is possible that opioid antagonists also down-regulate their receptors by a mechanism different from that of agonists. Observed quantitative differences in the receptor density reduction, as well as differential effects on lysosomal enzyme activities, support this notion.

Although cultured cells are a good model to study the processes involved in receptor adaptation, it is important to establish that antagonist-induced down-regulation can also occur *in vivo*. In preliminary studies, administration of naltrexone (10 mg/kg, intraperitoneally) to rats resulted in 57% lower [^{3}H][D-Ser^2,L-Leu^5]enkephalyl-Thr B_{max} values in hindbrain, but not in striatum, hippocampus, or cortex after 60 min. Longer naltrexone exposure (6–48 hr) caused up-regulation of δ-opioid binding in all four regions.

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