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Opiate Receptor Down-Regulation and Desensitization in Neuroblastoma × Glioma NG108-15 Hybrid Cells Are Two Separate Cellular Adaptation Processes

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SUMMARY

Chronic treatment of neuroblastoma × glioma NG108-15 hybrid cells with the opiate agonist etorphine resulted in a decrease in both opiate receptor density (receptor down-regulation) and opiate ability to inhibit prostaglandin E_1 (PGE_1)-stimulated increases in cyclic AMP levels (receptor desensitization). Opiate receptor down-regulation and desensitization were homologous as indicated by the lack of apparent change in muscarinic, α_2-adrenergic, and PGE_1 receptor binding and also retention, albeit modulation, of the ability of carbachol and norepinephrine to inhibit PGE_1-stimulated increases in cyclic AMP levels after 24 hr of etorphine treatment. PGE_1-stimulated increases in cyclic AMP levels remained identical in etorphine-treated and control cells. Several lines of evidence indicate that receptor desensitization and receptor down-regulation in NG108-15 cells are two separate cellular adaptation processes. (a) With an agonist which appears to be efficiently coupled, i.e., an agonist whose apparent K_d value is much larger than its apparent IC_{50} value for regulation of cyclic AMP levels (K_i), the concentration of ligand required to produce half-maximal down-regulation is analogous to its K_i value, whereas the concentration of ligand required to produce half-maximal desensitization is related to its K_d value; (b) receptor desensitization precedes receptor down-regulation; (c) only opiate agonists could produce receptor down-regulation, whereas both opiate agonists and partial agonists could desensitize post-receptor occupancy events. Still further evidence for dissociability of these processes was obtained by incubating NG108-15 cells with etorphine at 30° for 2 hr. Under these conditions, there was a decrease in etorphine's ability to regulate adenylate cyclase while [^{3}H]diprenorphine binding remained unaltered. IC_{50} values of D-Ala^2-D-Leu^5-enkephalin's competition for [^{3}H]diprenorphine binding to intact cells increased 19.6-fold after etorphine treatment for 90 min, while naloxone IC_{50} values remained unaltered. This apparent increase in IC_{50} values was much lower, about 2-fold, when receptor binding was carried out in membranes isolated from cells treated with etorphine chronically. Furthermore, analysis of [^{3}H]etorphine binding to such membranes in the presence of 10 mM Mg^{2+} indicated a loss of receptor binding sites with no change in apparent affinity, whereas [^{3}H]diprenorphine binding revealed no significant alteration in either B_{max} or K_d values. Therefore, during opiate receptor desensitization, a reduction of agonist high-affinity site occurs with no apparent alteration in total receptor number.

INTRODUCTION

In neuroblastoma × glioma NG108-15 hybrid cells, opiate agonists decrease intracellular adenosine cyclic AMP levels, at least in part, by inhibiting adenylate cyclase activity (1–3). Analogous to other receptor-adenylate cyclase interactions, opiate inhibition of adenylate cyclase activity required the presence of GTP, suggesting involvement of the *N*-component (G/F subunit) of adenylate cyclase and the monovalent cation Na^+ (4). The ability of opiate agonists to stimulate GTPase activity (5) further indicates a similarity in the mechanism of action between a stimulatory receptor, such as the *beta*-adrenergic receptor and the inhibitory opiate receptor.

When hybrid cells were chronically exposed to mor-

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phine, a loss of opiate regulation of adenylate cyclase was observed (6, 7). On the basis of the ability of the opiate antagonists, naloxone, to increase cyclic AMP levels above control values in such preparations, Sharma *et al.* (7) have suggested that this loss of opiate activity was due to a concomitant increase in adenylate cyclase activity which could be modified by unsaturated fatty acids in the growth medium (8). Hence, the opiate receptor appeared to remain coupled to adenylate cyclase after chronic treatment. This does not conform to observations in which chronic exposure to agonist produced a decrease in cellular response (receptor desensitization), and a decrease in cell surface receptor density (receptor down-regulation) in a number of hormone and neurotransmitter systems (9–15). Hence, the reported failure of opiates to desensitize their receptor system in NG108-15 cells implies a marked difference from other receptor systems.

Clearly, chronic treatment with opiates produces desensitization to the drugs in animal and human studies. Tolerance to opiate agonists is a frequent side effect from repeated administration of the drugs. Although tolerance to opiate agonists was observed routinely, a receptor density decrease was reported not to be the mechanism for cellular adaptation. In fact, morphine pellet implantation produced an increase in opiate receptor number in synaptic membrane preparations (16, 17). Parallel increases in opiate receptor binding were observed after naloxone pellet implantation. Apparently, the opiate receptor in rodents does not down-regulate during chronic administration of morphine.

Recently, however, we have been able to demonstrate opiate receptor desensitization and down-regulation in NG108-15 cells after chronic etorphine treatment (18). The increase in cyclic AMP levels and adenylate cyclase activity during chronic opiate treatment could not account for the loss of opiate receptor activity after continuous treatment with agonist. Similar to *beta*-adrenergic receptor desensitization and down-regulation, the loss of opiate activity in the hybrid cells is a multiple-step process. Receptor desensitization occurs prior to down-regulation of opiate receptors. In agreement with our findings, Chang *et al.* (19) also reported down-regulation of opiate receptors in neuroblastoma N4TG1 cells. Hence, the opiate receptor in neuroblastoma clonal cell lines undergoes cellular regulation in a manner analogous to that reported with other receptors. Since the NG108-15 and N4TG1 cell lines contain a homogeneous population of opiate receptors, the *delta* subtype (20, 21), it is advantageous to study the cellular adaptation processes involved in chronic opiate treatment with neuroblastoma cells. Therefore, in the present communication, evidence for the existence of opiate receptor desensitization and down-regulation is presented, and these processes are further characterized. Our results indicate that they are distinct cellular processes in response to chronic opiate treatment.

MATERIALS AND METHODS

Cell culturing. The initial stock cultures of neuroblastoma × glioma NG108-15 cells were generous gifts of Dr. B. Hamprecht (Physiologischemishes Institut des Universitat, Wurzburgh, Federal Republic of Germany). The hybrid cells were cultured in Dulbecco's modified Eagle's medium containing HAT (0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine) plus 10% fetal calf serum in a humidified atmosphere of 10% CO_2 and 90% air. The cells were detached from the growing surface by the addition of Saline D (NaCl, 137 mM: KCl, 5 mM: Na_2PO_4-$7H_2O$, 0.17 mM; KH_2PO_4, 0.22 mM; glucose, 6 mM; sucrose, 59 mM; pH 7.0) for propagation or experimentation. Cells in confluency with passage number between 20 and 30 were used in all experimentation.

Chronic treatment with opiates. NG108-15 cells were treated with various concentrations of sterile opiate ligands for 24 hr in growth medium containing 10% fetal calf serum. For opioid peptides which are metabolically labile in serum, cells were initially cultured in regular growth medium. On the day of treatment, growth media with serum were removed and cells were washed three times with 10 ml of a chemically defined medium as outlined by Bottenstein and Sato (22) supplemented with HAT. We have determined that NG108-15 cells remained viable for up to 2 weeks in this defined medium.[2] Opioid peptides then were added chronically to cells in the Sato medium. Prior to harvesting cells, identical concentrations of opioid drugs were added to nontreated control cells. Incubations then were carried out at 37°C for 5 min, since opiate receptor binding reached steady-state within 5 min (23). Bound opiate ligands were removed by washing the pelleted cells twice with 10 ml of Saline D at 37° and once with 10 ml of Krebs-Ringer-Hepes[3] buffer (NaCl, 110 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1.8 mM; glucose, 25 mM; sucrose, 55 mM; and Hepes, 10 mM at pH 7.4) at 37.

Opiate receptor binding. Opiate receptor binding to intact cells in suspension was carried out as described (23). Briefly stated, incubations were carried out at 37° for 20 min with 10^6 cells in 1.0 ml of Krebs-Ringer-Hepes buffer (KRHB) at pH 7.4 in the presence of 4–5 nM [^{3}H] diprenorphine. Nonspecific binding was determined by the addition of 5 μM nonradioactive diprenorphine or etorphine. Incubations were terminated by collecting the cells on Whatman GF/B filters, and excess radioactivity was removed by washing the filters three times with 5 ml of KRHB at 0°. After incubating at 20° overnight in 5 ml of Liquiscint (National Diagnostics, Somerville, N. J.), radioactivity on the filters was determined by liquid scintillation counting.

Opiate receptor binding to hybrid cell membranes was carried out with the 22,000 × *g* × 20 min pellets of cellular homogenates. Preparation of the membrane pellets was as described previously (23). Opiate ligands bound to chronically treated cells were removed by the following procedure. Membrane pellets from four culture flasks T-150 cm^2 were resuspended in 40 ml of 25 mM Hepes buffer at pH 7.7 containing 100 mM NaCl and 10 μM phenylmethylsulfonyl fluoride at 37°. The membrane suspension was then incubated at 37° for 10 min. Dissociation studies have indicated that, under these conditions, 70% of the bound etorphine would be dissociated. Membranes were pelleted by centrifugation at 22,000 × *g* for 20 min. This procedure was repeated again. The final membrane pellets were resuspended in 20 ml of 25 mM Hepes (pH 7.7 at 4°) to remove residual NaCl. Membranes were repelleted by centrifugation at 22,000 × *g* for 20 min. The final pellets were suspended in 25 mM Hepes (pH 7.7) for receptor binding assays. Membranes equivalent to 250–350 μg of protein were used in each assay. Opiate receptor binding assays with membrane preparations were carried out in a manner similar to the procedures with intact cells except that incubations were carried out at 24° for 90 min to attain equilibrium.

Assays of other receptors. *Alpha*$_2$-adrenergic, muscarinic, and PGE_1 receptor binding assays were carried out with the membrane pellets prepared as described in the previous section. Incubations were carried out at 24° for 90 min using [^{3}H]rauwolscine (*alpha*$_2$-adrenergic), QNB (muscarinic), and [^{3}H]PGE_1 to label the binding sites. Nonspecific

[2] M. T. Griffin, P. Y. Law, and H. H. Loh, manuscript in preparation.

[3] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGE_1, prostaglandin E_1; ZK62711, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone; QNB, quinuclidinyl benzilate; DAME, D-Ala^2, Met^5-enkephalinamide; DADL, D-Ala^2, D-Leu^5-enkephalin, GPA1657, 2-methyl,5-phenyl-9-methyl-2′-hydroxy-6,7-benzomorphan; GPA2163, 2-propynyl,5-phenyl-9-methyl-2′-hydroxy-6,7-benzomorphan.

binding of [^{3}H]rauwolscine, [^{3}H]QNB, and [^{3}H]PGE$_1$ were determined with 5 μM phentolamine, QNB, and PGE$_1$, respectively.

Measurement of cyclic AMP levels. The effect of opiate ligands on cyclic AMP levels was determined by measuring the conversion of the [^{3}H]adenine-labeled ATP pools to cyclic AMP, as described previously (23, 24). The measurements were made either with NG108-15 cells in suspension, 0.5 × 10^6 cells/0.5 ml of KRHB at pH 7.4, or with hybrid cells seeded into 17-mm wells and cultured for 4 days. Incubations with cell suspensions were carried out at 37° for 20 min in KRBH; incubations with cells cultured in 17-mm plates were for 10 min at 37° in incubation medium (24). After termination of incubations with 0.27 N perchloric acid and after addition of [^{32}P]cyclic AMP as an internal standard, radioactive cyclic AMP was separated from other ^{3}H-labeled nucleotides by the double-column chromatographic methods outlined by White and Karr (25). When the hybrid cells were treated with opiates chronically, a concentration of opiate identical with that used in chronic treatment was added to cells during harvesting in order to minimize the increase in cyclic AMP production due to the absence of opiate agonist or partial agonist subsequent to the treatment phase.

Adenylate cyclase measurement. Adenylate cyclase activity in membrane preparations was determined by measuring the production of cyclic AMP from [α-^{32}P]ATP as described previously (24). Radioactive cyclic AMP was separated from other nucleotides by the double-column techniques described by White and Karr (25).

Protein determinations. Concentrations of protein in membrane preparations were measured by the method of Lowry *et al.* (26). The hybrid cells' membranes and the intact cells were initially dissolved with a solution of 1% sodium dodecyl sulfate/1 N NaOH/H_2O (1:1:1) heated at 60° for 20 min. This enabled the complete solubilization of all proteins in NG108-15 hybrid cells. Bovine serum albumin was used as the protein standard and was treated with the identical concentrations of sodium dodecyl sulfate and NaOH.

Materials. [^{3}H]Adenine (16 Ci/mmole), [^{3}H]QNB (33 Ci/mmole), and [^{3}H]rauwolscine (83 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Etorphine (46 Ci/mmole), [^{3}H]diprenorphine (9 Ci/mmole), [^{3}H]PGE$_1$ (46 Ci/mmole), and [α-^{32}P]ATP (17 Ci/mmole) were purchased from Amersham (Arlington Heights, Ill.). [^{32}P]Cyclic AMP was prepared by enzymatic conversion of [α-^{32}P]ATP using NG108-15 membranes. All reagents used in the present studies were purchased from Sigma Chemical Company (St. Louis, Mo.). Nonradioactive QNB and ZK62711 were generous gifts from Dr. A Blume (Roche Institute, Nutley, N. J.) and Schering (Berling, Federal Republic of Germany), respectively. Etorphine and diprenorphine were obtained from the National Institute of Drug Abuse (Bethesda, Md.). Other opiate alkaloids were a generous gift from Dr. T. M. Cho (University of California, San Francisco). Opioid peptides were purchased from Peninsula Laboratories (Belmont, Calif.). The D-Ala^2 analogues and the *N*-CH$_3$-D-Ala^2 analogues of enkephalins were generous gifts from Wellcome Research Laboratories (Research Triangle Park, N. C.).

RESULTS

Time- and concentration-dependent decrease in opiate receptor activity. In our previous report using [^{3}H]diprenorphine as the opiate ligand, a time-dependent decrease in opiate receptor binding in NG108-15 cells was observed after chronic treatment with 10 nM etorphine (18). Scatchard analysis (27) of [^{3}H]diprenorphine binding to cells treated with etorphine for 24 hr showed a decrease in the maximal number of binding sites (B_{max}) with no apparent decrease in receptor affinity (K_d) (18). We have now observed this effect at 3 hr, but not at 1 or 2 hr, after addition of etorphine (Table 1). Therefore, [^{3}H]diprenorphine binding can be used to monitor opiate receptor down-regulation following chronic treatment with various drugs.

The degree of opiate receptor down-regulation is dependent on the concentration of ligand used during the incubation period. When NG108-15 cells were treated with various concentrations of etorphine for 24 hr, different amounts of [^{3}H]diprenorphine binding to intact cells were observed (Fig. 1a). The decrease in [^{3}H]diprenorphine binding could not entirely be due to residual etorphine because the current washing procedure removed >70° of etorphine associated with cells after 24 hr of opiate treatment (Table 2). From the data presented in Fig. 1a, the concentration of etorphine required to produce 50% of maximal down-regulation of opiate receptors in NG108-15 cells was 1.35 ± 0.07 nM (n = 3). As noted in a previous report (18), only 60% of total diprenorphine specific binding could be reduced maximally by chronic etorphine treatment, even after prolonging incubations. The amounts of [^{3}H]diprenorphine bound to cells treated with 10 nM etorphine for 24, 48, and 72 hr were 41.0 ± 3.1%, 42.6 ± 6.8%, and 35.1% ± 2.7% of the control cells.

TABLE 1

[^{3}H]Diprenorphine binding to neuroblastoma × glioma NG108-15 hybrid cells after chronic etorphine treatment

[^{3}H]Diprenorphine binding to intact hybrid cells was carried out as described under Materials and Methods. For binding incubations subsequent to chronic exposure to etorphine for a short duration (1–3 hr), 10 nM etorphine was added to control cells prior to harvesting and to etorphine-treated cells in order to prevent reversion of receptor from the chronic state back to the control state. K_d values were obtained from the equation $K_{d_{app}} = K_d (1 + [E_0]/K_d)$, where $K_{d_{app}}$ is the dissociation constant of [^{3}H]diprenorphine in the presence of 10 nM etorphine [E_0], which has the K_d[1] value of 22.2 nM (23). The K_d and B_{max} values were obtained by regression analysis of Scathchard plots of binding data. Concentrations of 0.1 nM–10 nM [^{3}H]diprenorphine were used in the binding assays. The values in parentheses represent the 95% confidence limits of the analysis.

	K_d	B_{max}
	nM	*fmoles/mg protein*
Control (n = 4)	2.4 ± 0.2	549 ± 46
Etorphine, 10 nM		
1 hr	2.3 (2.0–2.6)	550 (480–620)
2 hr	2.4 (1.2–3.6)	495 (250–740)
3 hr	3.3 (2.4–4.2)	400 (290–510)
24 hr	2.8 (1.1–4.5)	220 (86–350)
72 hr	2.9 (1.7–4.1)	190 (110–270)

The apparent disappearance rate constant of the receptor was determined under conditions where maximal down-regulation was attained. As shown in Fig. 1b, the rate of down-regulation is dependent on the concentration of agonist used. The disappearance rate of [^{3}H]diprenorphine binding follows first-order kinetics, and hence rate constants could be obtained from the slope of the lines in Fig. 1b. The disappearance rate constants were determined to be −0.227 ± 0.016 hr^{-1} and −0.431 ± 0.033 hr^{-1} for 10 nM and 100 nM etorphine, respectively. The $t½$ for loss of the opiate receptor in the presence of 100 nM etorphine is 1.6 ± 0.1 hr, which is significantly different from $t½$ for loss of the receptor in the presence of 10 nM etorphine, which is 3.1 ± 0.2 hr ($p < 0.01$).

Opiate receptor desensitization is likewise dependent on the time and concentration of opiate used during the "chronic" incubation. When NG108-15 hybrid cells were incubated with various concentrations of etorphine for

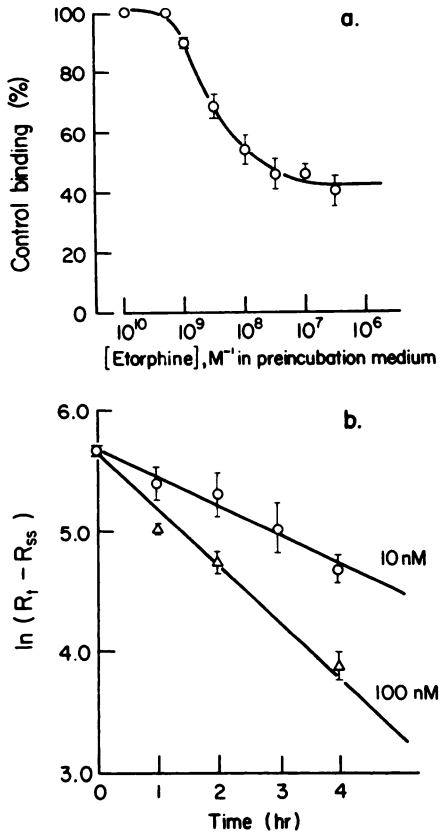

FIG. 1. *Concentration- and time-dependent decreases in [^{3}H]diprenorphine binding*

NG108-15 hybrid cells were treated (a) with various concentrations of etorphine for 24 hr or (b) with 10 nM (○) or 100 nM (△) etorphine for various time periods. [^{3}H]Diprenorphine binding to intact cells after removal of excess etorphine was determined as described under Materials and Methods. The amount of [^{3}H]diprenorphine specifically bound to control cells in a was 449 ± 22 fmolees/mg of protein (n = 13). In b, R_t denotes receptor concentration at time t, and R_{ss} denotes receptor concentration at the steady-state level, which was 195.0 ± 18 fmolees/mg of protein (n = 8).

24 hr and the ability of 1 μM etorphine to regulate PGE_1-stimulated increases in cyclic AMP levels was measured, a concentration-dependent decrease in etorphine activity was observed (Fig. 2, Curve *II*). The amount of etorphine required to produce 50% of maximal desensitization was 14.0 ± 5 nM (n = 3). This concentration of etorphine is significantly higher than that required to down-regulate half-maximally (Fig. 1a; Table 3), and it is higher than that required to produce maximal increases in cyclic AMP production in the presence of naloxone after chonic opiate treatment (Fig. 2, Curve *I*). Complete receptor desensitization by etorphine required full occupancy of the opiate receptor population. When hybrid cells were incubated with 1 nM etorphine for 24 hr, partial desensitization was observed (Fig. 2). The same degree of desensitization was observed even after 72 hr of 1 nM etorphine incubation.

There appears to be a relationship between the coupling efficiency (K_d/K_i) of opiate ligands and their potencies to desensitize and to down-regulate opiate receptors in NG108-15 hybrid cells. As summarized in Table 3,

TABLE 2

Amount of etorphine remaining bound to NG108-15 hybrid cells after 24 hr of chronic exposurre

NG108-15 cells (3.5 × 10^6) were treated with 10 nM [^{3}H]etorphine (specific activity 10 Ci/mmole) for 24 hr. Afterward, cells were harvested and etorphine was removed by the washing procedure described in the text. After each wash, aliquots of cells were collected on Whatman GF/B filters for determination of bound radioactivity. The authenticity of radioactivity was determined by extracting etorphine from hybrid cells and separating the extract on Silica Gel G in solvent mixture of 1-butanol/acetic acid/water (6 = 1.5:2:5) as described (28). The values represent averages of duplicate determinations.

Treatment	Etorphine bound	
	Amount	% Remaining
	fmoles/mg protein	
Before wash	224	100
1st wash (20 ml Saline D, 37°)	144	64.0
2nd wash (20 ml Saline D, 37°)	125	55.9
3rd wash (20 ml KRHB, 37°)	60.5	26.9

both etorphine and Sandoz FK33824, two of the opiate agonists which appear to be "efficiently coupled" (23), i.e., $K_d/K_i \gg 1$, required much higher concentrations of ligand to desensitize them to down-regulate the opiate receptor. In contrast, the amount of etorphine required to desensitize levorphanol activity half-maximally was determined to be 1.2 ± 0.19 nM (n = 3). Levorphanol is a partial agonist in inhibiting PGE_1-stimulated increases in cyclic AMP levels in NG108-15 cells, and it has a K_d/K_i ratio of 1.6 ± 0.19 (23). Agonists which have a coupling efficiency close to unity have similar EC_{50} values for

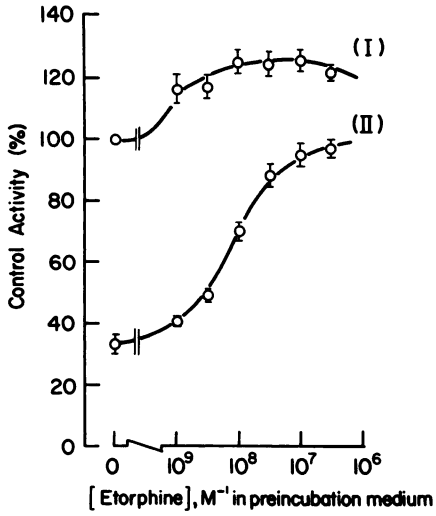

FIG. 2. *Etorphine concentration-dependent desensitization of the opiate receptor in NG108-15 cells*

Hybrid cells, cultured on 17-mm plates, were treated with various concentrations of etorphine for 24 hr. PGE_1 (5 μM)-stimulated increases in [^{3}H]cyclic AMP levels were determined in the presence of 100 μM naloxone (Curve *I*) or 1 μM etorphine (Curve *II*) as described under Materials and Methods. The values represent the average ± standard error of the mean from three separate passages of cells. Quadruplicate 17-mm plates were used in each treatment with individual etorphine concentrations. The control value for PGE_1-stimulated [^{3}H]cyclic AMP production was 1.52 ± 0.06 × 10^5 cpm/mg of protein per 10 min.

TABLE 3

Agonist concentration-dependent desensitization and down-regulation of opiate receptors in NG108-15 cells

Neuroblastoma × glioma NG108-15 hybrid cells were cultured on either 100-mm or 17-mm plates and were treated with various concentrations of these three opiate agonists for 24 hr. Afterward, cells on 100-mm plates were harvested, and [^{3}H]diprenorphine binding was determined as described under Materials and Methods. The ability of 1 μM etorphine, 1 μM GPA1657, or 10 μM Sandoz FK33824 to regulate cyclic AMP levels in cells cultured on 17-mm plates after corresponding opiate agonist treatment was also determined. The ED_{50} values for desensitization or for down-regulation were then obtained by regression analysis of log-logit plots. The values presented are determinations from three separate passages of cells. Intrinsic activity, K_d, and K_i values are from ref. 23.

Opiate agonist	Intrinsic activity	K_D	K_i	EC_{50} for	
				Desensitization	Down-regulation
		nM	nM	nM	nM
Etorphine	1.0	22.2 ± 2.7	2.2 ± 0.7	14.3 ± 5.0	1.35 ± 0.07[a]
GPA1657	0.96	158 ± 16	109 ± 11	105 ± 18	142 ± 38
Sandoz FK33824	1.01	2460 ± 164	154 ± 28	474 ± 45	173 ± 33[a]

[a] EC_{50} values for desensitization and for down-regulation were statistically significantly different ($p \leq 0.025$).

desensitization and down-regulation, as exemplified by the values of GPA1657 (Table 3).

Reversibility of opiate receptor down-regulation and desensitization. After neuroblastoma × glioma NG108-15 hybrid cells were treated with 10 nM etorphine for 24 hr, the chronic effect could be reversed by removing the ligand. In order to ensure complete removal of receptor-bound etorphine, hybrid cells were first incubated with 10 μM naloxone for 5 min at 37°; naloxone was subsequently removed by washing the cells with Dulbecco's modified Eagle's medium. Opiate activity was measured at 1, 2, 4, 8, and 24 hr subsequently. As shown in Fig. 3, both [^{3}H]diprenorphine binding sites and etorphine-mediated inhibition of PGE_1-stimulated cyclic AMP production reappeared in a time-dependent manner. There was a 1-hr lag in the increase to a detectable level of [^{3}H]diprenorphine binding, whereas etorphine inhibition of PGE_1-stimulated cyclic AMP production recovered immediately (Fig. 3). The ability of etorphine to regulate cyclic AMP levels was fully restored after 8 hr, but [^{3}H]diprenorphine binding did not reach its maximal level until 24 hr after etorphine removal. Since the doubling time of NG108-cells under the present growth conditions is approximately 24 hr, the reappearance of etorphine activity and diprenorphine binding presumably represents opiate receptor recovery in etorphine-treated cells and not in newly divided cells. The amount of binding increase after etorphine removal was the same when the cells were cultured in either medium containing 10% fetal calf serum or medium without serum for 6 hr. There was no apparent difference in the rates of appearance of etorphine activity in cells treated with 10 nM etorphine for 3 hr (when there is 50% down-regulation of opiate receptor) or in cells treated with etorphine for 24 hr. Therefore, the recovery of etorphine activity was independent of the concentration of receptors in neuroblastoma × glioma NG108-15 hybrid cells.

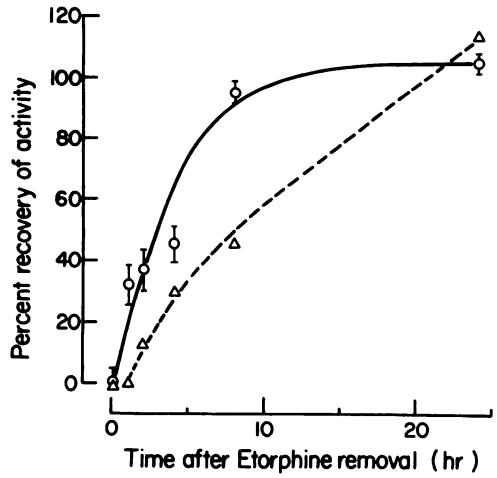

FIG. 3. *Time-dependent recovery of opiate receptor activity after receptor down-regulation and desensitization*

NG108-15 cells cultured on 100-mm plates for binding studies or on 17-mm plates for cyclic AMP measurements were treated with 10 nM etorphine for 24 hr. Recovery from the chronic opiate effect was initiated by removing etorphine bound to cells as described in the text. The ability of 10 nM etorphine to inhibit the PGE_1 (5 μM)-stimulated [^{3}H]cyclic AMP increase (○) or [^{3}H]diprenorphine (5 nM) binding (△) was measured at various times. At 0 time, the amount of [^{3}H]diprenorphine bound was 210 fmoles/mg of protein. Ten nanomolar etorphine did not inhibit PGE_1-stimulated cyclic AMP level increases. At full recovery, [^{3}H]diprenorphine binding was 590 fmoles/mg of protein, and 10 nM etorphine inhibited cyclic AMP production by 44%. In control cells with the same passage numbers, [^{3}H]diprenorphine binding was determined to be 525 fmoles/mg of protein, and 10 nM etorphine inhibited cyclic AMP levels by 42%. The values for [^{3}H]diprenorphine binding were averages from two separate incubations with etorphine; cyclic AMP measurements were averages from four separate incubations with etorphine prior to recovery.

Relationship between intrinsic activity and ability of various ligands to attenuate opiate receptor activity. Based on the concentration-dependent studies (Fig. 1 and 2), opiate receptor desensitization and down-regulation appear to involve the coupling state of the receptor. In the case of *beta*-adrenergic receptors, it has been suggested that the intrinsic activity of the ligand is dependent on its ability to induce the receptor into the coupling state (29). If opiate receptors act in a similar manner, then one would predict that the ability of various opiates to induce chronic effects should correspond to their intrinsic activities. Table 4 summarizes the results of the experiments to test this. When NG108-15 cells were incubated with various ligands for 24 hr, only agonists, i.e., ligands with intrinsic activities >0.9 compared with that of etorphine, were able to down-regulate the opiate receptor. It is of interest to note that not only

TABLE 4

Chronic treatment of NG108-15 hybrid cells with opiate alkaloids and opiate peptides: relationship between intrinsic activity and ability to down-regulate the opiate receptor

Neuroblastoma × glioma NG108-15 hybrid cells cultured on 100-mm plates were incubated with various opiate alkaloids or opioid peptides for 24 hr at 37° under growth conditions. The concentrations of opiate ligands used are given after each ligand and were approximately 5 times the K_d values for each ligand. Before termination of incubations, identical concentrations of opiate ligands were added to control plates, and the cells were incubated at 37° with ligands for 5 min. Afterward, excess opiate ligands were washed away, and [^{3}H]diprenorphine binding was carried out as described under Materials and Methods. Control binding was 497.0 ± 2.4 fmoles/mg of protein (n = 39). The values represent the average ± standard error of the mean from three separate passages of cells. Intrinsic activities of opiate ligands were from ref. 23.

Treatment	Intrinsic activity		% Control binding
Opiate alkaloids			
Etorphine, 10 nM	1.00		41.0 ± 7.7
Ethylketocyclazocine, 500 nM	0.96		41.3 ± 1.3
GPA1657, 500 nM	0.96		64.9 ± 7.9
Cyclazocine, 50 nM	0.62		91.8 ± 2.9
Diprenorphine, 20 nM	0.70	95.7 ± 13.7	108 ± 8.0[a]
Levorphanol, 300 nM	0.65		96.8 ± 6.7
Metazocine, 600 nM	0.69		105 ± 3.4
Morphine, 100 μM	0.69	98.2 ± 6.3	92.6 ± 4.7[a]
N-Allylnormetazocine, 200 nM	0.40	100 ± 10	93.3 ± 4.8
Nalorphine, 300 nM	0.18		103 ± 2.9[a]
Oxymorphone, 400 nM	0.68		98.1 ± 2.9
GPA2163, 500 nM	0		98.7 ± 7.7
Naloxone, 20 μM	0	100 ± 5.7	95.8 ± 4.1[a]
Opioid peptides			
Leu^5-enkephalin, 20 nM	0.94		50.6 ± 2.4[a]
Met^5-enkephalin, 20 nM	0.95		47.6 ± 5.8[a]
D-Ala^2,Met^5-enkephalin, 10 nM	0.98	78.8 ± 3.5	50.6 ± 2.6[a]
DAME, 15 nM	0.98		53.3 ± 8.9
DADL, 5 nM	0.92		51.5 ± 8.9
Sandoz FK33824	1.01		41.4 ± 3.7
N-CH$_3$-D-Ala^2,D-Met^5-enkephalin, 500 nM	0.94		50.4 ± 4.9
N-CH$_3$-D-Ala^2,D-Leu^5-enkephalin, 500 nM	0.94		52.7 ± 4.7
Dynorphin 1–13, 300 nM	0.96	83.9 ± 1.9	65.1 ± 2.7[a]
α-Neoendorphin, 100 nM	0.97		27.9 ± 2.6[a]
B_h-endorphin, 100 nM	0.99		65.2 ± 3.2[a]

[a] Incubations were carried out in Sato medium (22).

could opiate peptides induce down-regulation, as suggested by Chang *et al.* (19) in their studies with N4TG1 cells, but the ability of opiate ligands to induce down-regulation is related to their intrinsic activities. Agonists induce down-regulation of the opiate receptor in NG108-15 hybrid cells, and partial agonists and antagonists do not (Table 4). Increases in either incubation time or the concentration of ligand did not produce further down-regulation. Because incubations were carried out in growth medium which contains 10% fetal calf serum, some of the metabolically labile opioid peptides did not produce down-regulation under these conditions (Table 4). However, when hybrid cells were washed free of fetal calf serum and incubations were carried out in a chemically defined medium as outlined by Bottenstein and Sato (22), down-regulation of opiate receptors was observed with the metabolically labile peptides. In such a medium, Leu^5- and Met^5-enkephaline could produce down-regulation to the same extent as the metabolically stable analogue, DADL. Incubations in serum-free medium with partial agonists or antagonists did not alter their absence of effect (Table 4).

The ability of opiate agonists to induce down-regulation can be blocked by the presence of a partial agonist or antagonist in the incubation medium. As shown in Table 5, the addition of naloxone or levorphanol decreased the magnitude of etorphine down-regulation of the opiate receptor, although chronic incubation with naloxone alone did not produce any decrease or increase in [^{3}H]diprenorphine binding to intact cells. Similar results were observed with levorphanol and were concentration-dependent (Table 5).

The ability of various opiate ligands to induce desensitization is also related to their intrinsic activity. When NG108-15 hybrid cells were treated for 24 hr with 5 nM DAME, the most potent *delta* agonist in this system, the ability of DAME to produce cyclic AMP levels was attenuated (Fig. 4a); the K_i of DAME increased from 0.51 nM in control cells to >1 μM in chronically treated DAME cells. Concurrently, the ability of a partial agonist such as levorphanol to regulate cyclic AMP levels was completely abolished. The ability of an agonist to desensitize opiate receptors in hybrid cells completely was not

TABLE 5

Effect of partial agonists and antagonists on etorphine-dependent down-regulation of the opiate receptor

Neuroblastoma × glioma NG108-15 hybrid cells were treated with opiate agonists, partial agonists, and antagonists for 24 hr under growth conditions. Five minutes prior to harvesting, opiate ligand concentrations in all petri dishes were equalized. Excess ligands were removed by washing the cells repeatedly as described under Materials and Methods. The values represented the average ± standard error of the mean of cells from three separate passage numbers. The values for etoorphine-treated cells are from 10 separate incubations.

Treatment	Specific binding	% Control
	fmoles/mg protein	
Control	530 ± 9.5	100
+ Naloxone, 20 μM	539 ± 6.9	102 ± 1.3
+ Levorphanol		
200 nM	516 ± 25	97.3 ± 4.7
2000 nM	464 ± 29	87.6 ± 5.5
+ Etorphine, 10 nM	214 ± 9.5	40.4 ± 1.8
+ Naloxone, 20 μM	480 ± 24[a]	90.6 ± 4.5
+ Levorphanol		
200 nM	223 ± 32	42.1 ± 6.1
2000 nM	385 ± 21[a]	72.6 ± 3.9

[a] $p < 0.005$ compared with etorphine-treated cells.

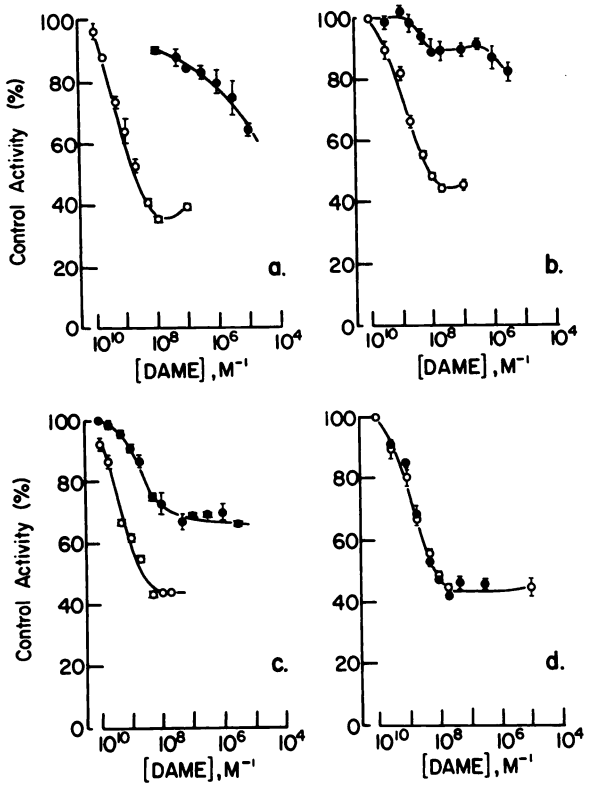

FIG. 4. *Relationship between the intrinsic activity of opiate ligands and opiate receptor desensitization in NG108-15 cells*

PGE_1 (5 μM)-stimulated increases in cyclic AMP levels in suspensions of hybrid cells were inhibited by various concentrations of DAME in control cells (○) or in cells treated chronically with opiates (●). Hybrid cells were treated for 24 hr with (a) 5 nM DAME, (b) 1.0 μM Sandoz FK33824, (c) 100 nM cyclazocine, or (d) 1 μM naloxone. Control activity in the absence of added opiates was determined to be $2.3 \pm 0.09 \times 10^5$ cpm/mg of protein/20 min ($n = 9$).

dependent on the ligand's affinity for receptor. As shown in Fig. 4b, Sandoz FK33824, an active opioid peptide with the lowest affinity for opiate receptors and lowest potency in NG108-15 cells (23), also produced desensitization. When the hybrid cells were desensitizied with a partial agonist, such as cyclazocine (Fig. 4c), the ability of DAME to regulate cyclic AMP levels was partially retained and cyclazocine's activity was completely abolished. The IC_{50} value of DAME in cyclazocine-treated cells was determined to be 1.43 nM, and maximal inhibition level of DAME was reduced to 39% from a control value of 52%.

The inability of cyclazocine to desensitize opiate agonist activity completely in NG108-15 hybrid cells was related to its intrinsic activity rather than the presence of multiple receptors in hybrid cells. This was illustrated by partial desensitization of opiate effect with ketaocyclazocine, a pure agonist in this system. When hybrid cells were treated with 30 nM ketocyclazocine for 24 hr, there was a 4-fold decrease in ketocylazocine's potency and a 36% decrease in maximal inhibition level (Table 6). In this desensitized state, levorphanol's ability to inhibit cyclic AMP production was abolished; however, the magnitude of decrease in effects of Leu^5-enkephalin and Sandoz 33824 was the same as that of ketocyclazocine (Table 6).

When hybrid cells were treated with naloxone, an

TABLE 6

Opiate inhibition of cyclic AMP production in NG108-15 cells after chronic ketocyclazocine (KC) treatment

Neuroblastoma × glioma NG108-15 hybrid cells were treated with 30 nM ketocyclazocine or saline for 24 hr. The ability of various opiate agonists and partial agonists to inhibit PGE_1-stimulated increase in AMP levels was determined in cell suspensions as described under Materials and Methods. The amount of [^{3}H]cyclic AMP produced in the absence of any added opiate was 2.60×10^5 cpm/10^6 cells. IC_{50} values were obtained from regression analysis of inhibition curves determined with 12 different concentrations of opiate. The values in parentheses represent the fold increase in IC_{50} values of each agonist chronic ketocyclazocine treatment.

Opiates	IC_{50}		Maximal inhibition	
	Control	+ Chronic KC	Control	+ Chronic KC
	nM	nM	%	%
Ketocyclazocine	53	200 (3.8)	59.6 ± 0.7	38.1
Levorphanol	295	—	31.0 ± 0.8	5.8 ± 2.6
Leu^5-enkephalin	3.4	14.1 (4.1)	60.4 ± 0.1	44.8 ± 0.6
Sandoz FK33824	210	670 (3.2)	61.1 ± 0.8	43.3 ± 1.7

opiate antagonist, there was no measurable alteration in opiate effect in NG108-15 cells (Fig. 4d). After complete removal of naloxone, the ability of DAME to inhibit cyclic AMP production in control and naloxone-treated cells was identical. Moreover, the ability of naloxone to antagonize etorphine inhibition in control and chronic naloxone-treated cells remained the same. The EC_{50} values for naloxone for the reversal of 0.1 μM etorphine inhibition of PGE_1-stimulated increases cyclic in AMP levels in control and naloxone-treated cells were 3.4 μM and 3.8 μM, respectively.

Homologous desensitization of the opiate receptor in NG108-15 hybrid cells. Su *et al.* (30) have defined homologous desensitization as that desensitization which is characterized by a specific loss of response to that agonist used during chronic incubation without alteration in the response to other stimuli. In contrast, when cells treated with one ligand become desensitized to other stimuli, heterologous desensitization has occurred. The data summarized in Table 7 indicate that in neuroblastoma × glioma NG108-15 hybrid cells, chronic opiate treatment produced homologous desensitization. After chronic treatment with 10 nM etorphine, although there was 66.3% decrease in [^{3}H]diprenorphine binding, there was no measurable decrease in [^{3}H]QNB (muscaranic receptor), [^{3}H]rauwolscine (*alpha*$_2$-adrenergic receptor), or [^{3}H]PGE_1 binding to the membrane preparations.

When the ability of muscarinic or *alpha*$_2$-adrenergic agonists to inhibit cyclic AMP production was determined in etorphine-treated cells and compared with that in control cells, some alteration in muscarinic and *alpha*$_2$-adrenergic receptor activity was observed. As summarized in Table 7, after chronic incubation of NG108-15 cells with 10 nM etorphine for 24 hr, there was no alteration in the IC_{50} values of norepinephrine to inhibit PGE_1-stimulated increases in cyclic AMP levels. However, there was a significant decrease in the maximal inhibition level (Table 7). A decrease in the maximal inhibition level was even more pronounced in carbachol inhibition of cyclic AMP production, about 27%; further-

TABLE 7

Effect of chronic etorphine treatment on other receptors' activities in neuroblastoma × glioma NG108-15 hybrid cells

Neuroblastoma × glioma NG108-15 hybrid cells were treated with 10 nM etorphine for 24 hr. Receptor binding was carried out with membrane preparations of the hybrid cells as described under Materials and Methods. In measuring carbachol or norepinephrine inhibition of PGE_1-stimulated increases in cyclic AMP levels and in measuring PGE_1-stimulated increases in cyclic AMP levels in chronically treated cells, 10 nM etorphine was present in the incubation mixture in order to prevent the spontaneous increases in cyclic AMP levels due to the absence of opiate agonist. The values presented are the average standard error of the mean of determinations from three separate passages of cells. The numbers in parentheses are the % changes in receptor binding after chronic etorphine treatment.

Receptor	Specific binding		
	Control	Chronic etorphine	
	fmoles/mg protein		
Opiate, [^{3}H]diprenorphine, 5.9 nM	596 ± 18.7	200 ± 12	(−66.3 ± 2.9)
Muscarinic, [^{3}H]QNB, 3.6 nM	38.6 ± 1.3	34.5 ± 1.2	(−10.2 ± 4.6)
Alpha$_2$-adrenergic, [^{3}H]rauwolscine, 1.5 nM	59.9 ± 6.5	62.6 ± 4.6	(+7.9 ± 16.7)
Prostaglandin, [^{3}H]PGE_2, 1.2 nM	7.3 ± 0.2	6.8 ± 0.4	(−6.5 ± 8.0)
Receptor	**Regulation of cyclic AMP level**		
	Control	Chronic etorphine	
Alpha$_2$-adrenergic, norepinephrine			
IC_{50} μM	2.08 ± 0.36	1.92 ± 0.50	
n_H	1.50 ± 0.17	1.40 ± 0.16	
Maximal inhibition, %	50.0 ± 1.9	41.0 ± 2.3[a]	
Muscarinic, carbachol			
IC_{50}, μM	0.99 ± 0.31	2.12 ± 0.04[a]	
n_H	1.55 ± 0.16	0.90 ± 0.10[a]	
Maximal inhibition, %	49.3 ± 2.3	35.9 ± 2.3[a]	
Prostaglandin, PGE_1			
EC_{50}, μM	0.103 ± 0.018	0.108 ± 0.022	
n_H	1.15 ± 0.23	1.16 ± 0.16	
Maximal activity, cpm/mg protein/20 min	2.96 ± 0.04 × 10^5	2.85 ± 0.02 × 10^5	

[a] $p \leq 0.05$ compared with controls.

more, the potency of carbachol was also decreased after chronic opiate treatment, with a 2-fold increase in the IC_{50} value and a decrease in the Hill coefficient. In the same cells, PGE_1 receptor activity was not altered (Table 7). These significant decreases in carbachol- and norepinephrine-modulated activities were observed only after 24 hr of etorphine incubation, a time at which down-regulation of the receptor was maximally observed. When NG108-15 cells were treated with 10 nM etorphine for 3 hr, a time at which the opiate receptor effects on cyclase were desensitized but the receptor loss was not yet complete (18), muscarinic agonist- and *alpha*-adrenergic agonist-mediated inhibition of PGE_1-stimulated increases in cyclic AMP levels remained unchanged as compared with controls.

Decreased opiate receptor affinity for agonists during receptor desensitization. As summarized in Table 1 and in previous studies (18), there is a time-dependent decrease in [^{3}H]diprenorphine binding during chronic etorphine treatment. Decreases in [^{3}H]etorphine binding were observed prior to the decrease in [^{3}H]diprenorphine binding (data not shown). The decrease in etorphine binding could be due to a decrease in receptor affinity for agonist. In other receptor systems, such as the *beta*-adrenergic receptor, receptor affinity for agonist has been demonstrated to decrease during the initial phase of receptor desensitization (10, 30). Apparently, opiate receptor desensitization follows a mechanism similar to that described for the *beta*-adrenergic receptor.

When hybrid cells were treated with 10 nM etorphine for 90 min, there was a 15% decrease in [^{3}H]diprenorphine binding and the ability of 1 μM etorphine to inhibit PGE_1-stimulated increases in cyclic AMP was attenuated by 30% (18). When the ability of DADL to compete for [^{3}H]diprenorphine binding to intact cells was determined, a decrease in DADL affinity after chronic etorphine treatment was observed (Fig. 5). In these binding experients, 10 nM etorphine was present in all reaction mixtures to prevent reversion of the opiate receptor from the chronically treated state to its original state. That this rever-

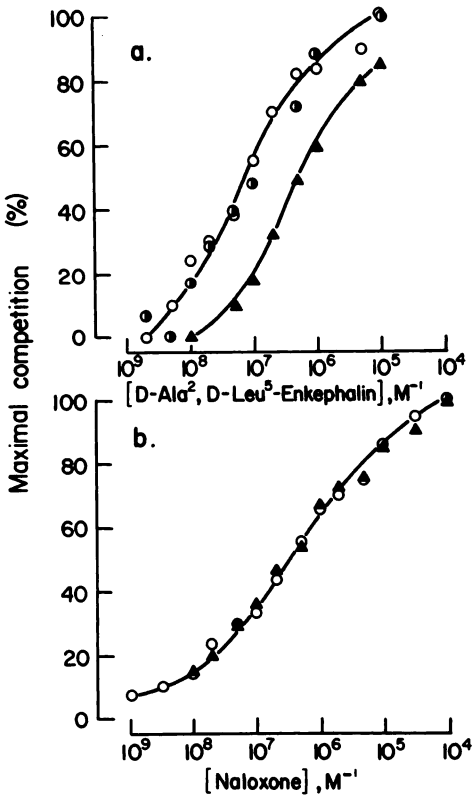

FIG. 5. *Competition for [^{3}H]diprenorphine binding to intact NG108-15 cells with various concentrations of (a) DADL or (b) naloxone*

Hybrid cells were treated with 10 nM etorphine for 90 min. The ability of DADL or naloxone to compete for 5 nM [^{3}H]diprenorphine was determined in control cells (○), in 10 nM etorphine-treated cells (▲) or in cells treated with etorphine and then washed free of opiate (◑). Values represented are averages from two separate experiments.

sion occurs was clearly demonstrated by the observation that DADL affinity in etorphine-treated but washed cells was analogous to that in control cells (Fig. 5). The incubation time for the binding assay was reduced from 20 min to 10 min at 37° in order to eliminate any acute etorphine effect on receptor affinity. From the data represented in Fig. 5a, the apparent dissociation constant for DADL in control cells and in the presence of etorphine was determined to be 14.0 ± 2.1 nM (n = 5). After 90 min of etorphine incubation, the apparent dissociation constant of DADL was calculated to be 273 ± 47 nM (n = 3), an approximately 20-fold decrease in receptor affinity for this agonist. The ability of the antagonist naloxone to compete for [^{3}H]diprenorphine was unchanged under these conditions (K_d = 52 ± 9.2 nM) (Fig. 5b).

When opiate receptor binding was carried out with the 22,000 × g × 20 min membrane pellet prepared from cells treated with 10 nM etorphine for 180 min, an apparent decrease in etorphine affinity was observed. The apparent dissociation constant of etorphine in control versus etorphine-treated membranes was 0.94 ± 0.25 nM versus 2.0 ± 0.32 nM, respectively (n = 3; not statistically significant). This increase in the apparent K_d for etorphine was considerably smaller than the measured difference when experiments were carried out with whole cells (Fig. 5).

Apparent decrease in opiate number during receptor desensitization. Thus far, our studies have suggested that receptor down-regulation and receptor desensitization might be two different cellular processes. However, during incubation at 37°, it is difficult to separate these processes completely. In current studies, we observed that, by lowering the incubation temperature to 30°, the opiate receptor down-regulation and receptor desensitization could be resolved.

As summarized in Table 8, exposure of NG108-15 cells to 100 nM etorpnine for 2 hr at 30° abolished the ability of Leu^5-enkephalin to inhibit adenylate cyclase activity. In membranes prepared from control cells, 50 nM enkephalin inhibited PGE_1-stimulated adenylate cyclase by 20 ± 4.5%. Although this magnitude of enkephalin inhibition in thrice-washed membranes was lower than that obtained with nonwashed membranes, this was not caused by the absence of GTP or Na^+, two of the components required for opiate receptor regulation of adenylate cyclase activity (4). Nevertheless, under identical assay conditions, membranes prepared from cells treated with etorphine did not exhibit any enkephalin-mediated inhibition of adenylate cyclase activity (Table 8). The PGE_1-stimulated adenylate cyclase activities in etorphine-treated membranes were identical in the presence of naloxone, naloxone ± enkephalin, or no addition of opiates (Table 8). The specific activities of adenylate cyclase in control and etorphine-treated cells in the presence of all agents tested, with the exception of enkephalin, were identical. Hence, by repeatedly washing the membrane to remove excess bound etorphine during a 2-hr treatment, the reported increase in adenylate cyclase activity after chronic opiate treatment was eliminated (7).

TABLE 8

Effect of etorphine treatment at 30° on adenylate cyclase activity in neuroblastoma × glioma NG108-15 hybrid cells

Neuroblastoma × glioma NG108-15 hybrid cells were treated with saline or 100 nM etorphine for 2 hr at 30°. After etorphine incubations, hybrid cells were harvested and membranes were prepared as described under Materials and Methods. Adenylate cyclase activity in the presence of various agents was determined as outlined under Materials and Methods. The values presented are the averages from four different preparations of membrane from four different passages of cells thus treated.

	Adenylate cyclase activity	
	Control	+Etorphine
	pmoles/mg protein/min	
Basal	2.05 ± 0.14	1.93 ± 0.15
+PGE_1, 10 μM	17.6 ± 0.76	18 ± 0.81
+Leu^5-enkephalin, 50 nM	14.6 ± 0.8	18.5 ± 0.2
+Leu^5-enkephalin, 50 nM +Naloxone, 10 μM	18.4 ± 0.88	18.4 ± 0.86
+GppNHp, 10 μM	6.0 ± 0.32	6.1 ± 0.41

From our hybrid cell studies with whole cells or membranes treated with 10 nM etorphine for 90–180 min at 37° (Fig. 5), there was a minimal decrease in number of binding sites, and a reduction of agonist binding affinity was observed. Hence, one would predict that membranes prepared from cells treated with 100 nM etorphine for 2 hr at 30° would produce a reduction in agonist affinity. Instead, an unexpected decrease in etorphine binding sites was observed. Analogous to intact cell binding data, membranes isolated from cells treated with 100 nM etorphine for 2 hr at 37° demonstrated a 43% decrease in [^{3}H]diprenorphine binding and a 73% decrease in [^{3}H]etorphine binding (Table 9). When the same analysis was made of membranes from cells treated with 100 nM etorphine for 2 hr at 30°, no reduction in [^{3}H]diprenorphine binding was observed, although [^{3}H]etorphine binding was reduced by 44% (Table 9). Since this reduction of etorphine binding could be due to a reduction of either receptor affinity or density, receptor saturation studies were carried out with 15 concentrations of [^{3}H]etorphine, 0.1 nM–10 nM. These saturation studies revealed a reduction in the amount of [^{3}H]etorphine specifically bound at every concentration of ligand used. Scatchard analysis of [^{3}H]etorphine binding, carried out in the presence of Mg^{2+}, indicated a linear plot, with a decrease in B_{max} but not in apparent K_d values for [^{3}H]etorphine. In contrast, [^{3}H]diprenorphine binding remained unchanged in the same membrane preparations. Therefore, there was a reduction of [^{3}H]etorphine high affinity binding sites during receptor desensitization.

DISCUSSION

The current findings support our previous conclusions that opiate receptors of the *delta* subtype in neuroblastoma × glioma NG108-15 hybrid cells undergo desensitization and down-regulation. Desensitization is characterized by the loss of opiate agonist or partial agonist ability to regulate cyclic AMP levels. Down-regulation is characterized by a decrease in the number of binding

TABLE 9

Temperature-dependent opiate receptor down-regulation in NG108-15 cells

Neuroblastoma × glioma NG108-15 hybrid cells were treated with 100 nM etorphine for 2 hr at either 37° or 30°. Membranes were prepared and washed free of etorphine as described under Materials and Methods. [^{3}H]Diprenorphine (5 nM) and [^{3}H]etorphine (1.5 nM) binding experiments were then carried out. The values presented are the average ± standard error of the mean. The values in parentheses are the numbers of different passages of cells used. The apparent dissociation constants, K_d, and the maximal binding sites, B_{max}, were obtained from Scatchard analysis of binding data. All binding assays were carried out in the presence of 10 mM $MgCl_2$.

Preincubation	37°		30°	
	Control	+Etorphine	Control	+Etorphine
[^{3}H]Diprenorphine binding				
fmoles/mg of protein	594 ± 26 (n = 3)	336 ± 11[a]	517 ± 42 (n = 4)	471 ± 50
% decrease		43.4 ± 4.3		8.9 ± 9.7
K_d, nM	—	—	1.95 (n = 2)	2.13
B_{max}, fmoles/mg of protein		—	564	545
[^{3}H]Etorphine binding				
fmoles/mg of protein	227 ± 4.5 (n = 3)	32.8 ± 19.8[a]	223 ± 40 (n = 5)	117 ± 15[b]
% decrease		73.4 ± 7.9		44.3 ± 6.0
K_d, nM	—	—	1.63 ± 0.11 (n = 6)	1.89 ± 0.18 (n = 4)
B_{max}, fmoles/mg of protein			374 ± 19	164 ± 13[a]

[a] $p \leq 0.005$ compared with control values.
[b] $p \leq 0.05$ compared with control values.

sites with no apparent alteration in the dissociation constant.

In measuring the decrease in opiate receptor binding capacity during chronic opiate treatment, artifactual results could be obtained if the opiate ligand used to treat hybrid cells was not removed completely. Were this the case, a lower amount of [^{3}H]diprenorphine would be bound to treated cells than to control cells. The possibility of retained ligand is unlikely because (a) an identical amount of opiate ligand was added to control cells to occupy the same fraction of binding sites occupied in chronically treated cells 5 min prior to harvesting; (b) there was a time-dependent decrease in [^{3}H]diprenorphine binding when cells were treated with the same concentration of drug (Fig. 1); (c) the ED_{50} value for down-regulation corresponds to agonist K_i values and not K_d values (Table 3); (d) not all opiate ligands used produced decreases in [^{3}H]diprenorphine binding (Table 4); and (e) the presence of exogenous ligand would decrease the affinity of [^{3}H]diprenorphine binding but not the number of binding sites, as indicated in Table 1 and in a previous report (18), since opiate ligand is interacting with a single binding site in NG108-15 cells (23). Even if none of the bound etorphine were removed, based on the K_d of etorphine of 22 nM in intact cells (23), it was calculated that 10 nM etorphine would occupy only 30% of the total receptor population. As indicated in Table 2, >70% of [^{3}H]etorphine initially bound was washed away from chronically treated cells. Only 60 fmoles/mg-protein of etorphine remained. If one assumed that all of the remaining etorphine was irreversibly bound to high-affinity sites, a maximal 10% decrease in opiate binding sites should occur. Experimental data indicated that ≥60% of binding sites was abolished by 10 nM etorphine treatment. Together with all other observations, these data clearly suggest that the reduction in [^{3}H]diprenorphine binding could not be due to residually bound etorphine, but must be due to the opiate receptor being down-regulated.

Apparently, the ability of the opiate ligand to promote down-regulation is directly related to its ability to modify adenylate cyclase activity. In Table 4, the relationship between intrinsic activity of ligand and its ability to down-regulate the receptor is clearly established. Only agonists can down-regulate the opiate receptor, and the amount of ligand required to down-regulate the opiate receptor half-maximally is related to its K_i value in altering adenylate cyclase activity (Fig. 1; Table 3). Possibly, receptor down-regulation is dependent on the coupling between adenylate cyclase and opiate receptor. However, coupling between adenylate cyclase and receptor is not the only criterion in receptor down-regulation, because partial agonists can inhibit adenylate cyclase but cannot down-regulate the opiate receptor (Table 4). Since there is a difference in the ability of partial agonists and agonists to promote receptor down-regulation, receptor down-regulation must lie beyond the receptor coupling state. Su *et al.* (30) have postulated that, in *beta*-adrenergic receptor down-regulation, there exists a further activated receptor species, *HR**, whose existence is a prerequisite for receptor down-regulation. This model is consistent with our findings and implies that down-regulation must be a property of the agonist-receptor complex rather than related to the percentage of receptor in the coupling state.

On the basis of our current study and previously reported studies from other laboratories (18), we conclude that receptor desensitization and receptor down-regulation are two different cellular adaptation processes. The rate of appearance of desensitization is faster than that of down-regulation; receptor desensitization and receptor down-regulation require different concentrations of agonist, and receptor desensitization to opiates is not a consequence of a decrease in number of binding sites. Additionally, it is possible to separate receptor desensitization and receptor down-regulation (Tables 8 and 9). By lowering the incubation temperature from 37° to 30°, we observed receptor desensitization but not receptor

down-regulation. The ability of partial agonists to desensitize opiate receptor in NG108-15 cells but not to down-regulate them (Table 4; Fig. 5) offers still further evidence for two separate cellular adaptation processes.

Apparently, upon exposure to opiate agonists, there are at least three cellular processes occurring to counteract the chronic presence of opiates. Receptor desensitization followed by receptor down-regulation are two which have been studied and discussed thus far. The third cellular process is the increase in adenylate cyclase activity during chronic opiate exposure, as concluded from our concentration-dependent studies. The concentration of etorphine required to increase adenylate cyclase activity maximally was much lower than that needed to produce maximal receptor desensitization (Fig. 2). The EC_{50} value of GPA1657 to produce a half-maximal increase in cyclic AMP production after 24 hr of incubation was 28 nM, which was lower than the amount of GPA1657 needed to produce down-regulation or desensitization (Table 3). Furthermore, in neuroblastoma N18TG2 cells, one of the parent cell lines of NG108-15, an increase in adenylate cyclase activity was not observed after chronic etorphine treatment (24), whereas receptor desensitization and receptor down-regulation were observed with N18TG2 cells. Thus, these data suggest that the increase in adenylate cyclase activity is a third independent cellular adaptation process.

Opiate receptor desensitization and down-regulation in NG108-15 cells are homologous. Chronic treatment of hybrid cells produced decreases in opiate receptor binding, but not in muscarinic, *alpha*$_2$-adrenergic, or prostaglandin receptor binding (Table 7). Although some decrease in muscarinic *alpha*$_2$-adrenergic receptor inhibition of cyclic AMP production was observed after 24 hr of etorphine treatment, none was induced with 3 hr of etorphine treatment. Furthermore, as with other receptor systems for which homologous desensitization occurs, cellular cyclic AMP has little or no effect on the desensitization process (10). By treating hybrid cells chronically with dibutyryl cyclic AMP or with an adenylate cyclase stimulator such as cholera toxin or forskolin, opiate receptor activity or chronic opiate action remained identical with that of nontreated cells.[2] Therefore, even with a slight decrease in muscarinic and *alpha*$_2$-adrenergic receptors' activities after prolonged etorphine treatment, insensitivity toward cyclic AMP and 3 hr of etorphine treatment indicate that opiate receptor down-regulation and desensitization are homologous.

Opiate receptor desensitization, like down-regulation, was related to the intrinsic activities of the ligands (Fig 4; Table 6). One could argue that these observations imply the presence of multiple opiate receptors in NG108-15 hybrid cells, particularly since tolerance studies provided some of the early evidence in support of multiple opiate receptors in mammalian tissues (31, 32). However, the ability of ketocyclazocine to desensitize partially the activity of all agonists tested to the same extent does not support this conclusion (Table 6). Our previous study of pA_2 values of naloxone and agonist activity in the presence of a partial agonist also indicate a homogeneous population of opiate receptors in the NG108-15 system.

After incubation with 100 nM etorphine for 2 hr at 30°, there was a 44% decrease in [^{3}H]etorphine binding in etorphine-treated membranes. Scatchard analysis revealed a decrease in the number of etorphine sites (Table 9), although [^{3}H]diprenorphine binding was unaffected. This apparent disappearance of etorphine binding sites could not be due to the presence of unremoved etorphine, for then a parallel decrease in [^{3}H]diprenorphine binding would have been observed. It is also unlikely that [^{3}H]etorphine and [^{3}H]diprenorphine are labeling different binding sites, for data obtained thus far indicate that this clonal cell line contains a homogeneous population of opiate receptors, the *delta* subtype (20, 21, 23). Saturation binding with [^{3}H]dihydromorphine, [^{3}H]DAME, and [^{3}H]diprenorphine yielded the same number of sites in hybrid cells. Moreover, etorphine and diprenorphine are structurally similar, and binding sites indicated that these two ligands are interacting with the same site. Thus, the decrease in etorphine binding sites after exposure to opiate at 30° could not be due to the disappearance of opiate receptor per se. However, because desensitization is thought to be due to the progressive inability of an agonist to induce the "high-affinity state" of the receptor, presumably related to its coupling to adenylate cyclase, one might anticipate a decrease in the high-affinity states after chronic agonist treatment, with an over-all reduction of agonist potency. This was confirmed by a 19-fold decrease in receptor affinity for DADL in intact desensitized cells (Fig. 5). This reduction of receptor affinity might have required the presence of all coupling factors, such as GTP and Na^+, since minimal reduction in receptor affinity for agonist was observed with membranes prepared from desensitized cells when saturation and competition binding studies were performed in the presence of Mg^{2+} only. Since there was no reduction in [^{3}H]diprenorphine binding sites, the reduction of [^{3}H]etorphine binding sites that was observed in the presence of Mg^{2+} suggested that a greater proportion of opiate receptors was in a low-affinity state which could not be trapped by using the radioligand binding methods employed. Perhaps the use of higher concentrations of [^{3}H]etorphine or equilibrium dialysis techniques would permit detection of this low-affinity state.

The apparent decrease in opiate receptor binding sites could account for the differences in rates of opiate receptor down-regulation in N4TG1 cells reported by Chang *et al.* (19) and our findings in NG108-15 cells. Chang *et al.* (19) reported a complete down-regulation of [^{125}I]DADL binding within 1 hr of treatment with 100 nM DADL. With 100 nM etorphine treatment, down-regulation of opiate receptors in NG108-15 cells did not reach maximal levels until 8 hr after initiation of treatment. Although this discrepancy could be due to the use of different clonal cell lines or different agonists, it seems to us that the differences reflect the use of radioactive agonist as the ligand for opiate receptor labeling by Chang *et al.* (19). Since desensitization can occur rapidly (18), an initial reduction in opiate receptor binding observed using agonists as radioactive ligands could reflect this process rather than down-regulation.

In summary, we have demonstrated that chronic opiate treatment of neuroblastoma × glioma NG108-15 cells produces homologous receptor desensitization and receptor down-regulation, and that these are two different

cellular adaptation processes. Furthermore, there is at least one more cellular adaptation process in response to chronic opiate treatment, i.e., the concomitant increase in adenylate cyclase activity first reported by Sharma *et al.* (7). The exact mechanisms for receptor desensitization and down-regulation remains to be elucidated, but it is apparent that formation and disposal of the receptor-ligand complex is the key event in cellular adaptation.

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