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N_i-Coupled Receptors in Cultured Neural Hybrid Cells: Cell Specificity for Dibutyryl Cyclic AMP-Induced Down-Regulation But Not Morphological Differentiation

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SUMMARY

Opiate, muscarinic, and α_2-adrenergic receptors and the N_i-coupled response of adenylate cyclase (AC) inhibition were examined in neuroblastoma × glioma NG108-15 (108 CC15) and neuroblastoma × Chinese hamster brain NCB-20 clonal hybrid cells, induced to differentiate with 1.0 mM dibutyryl cAMP (dBcAMP). Scatchard analysis of binding of the opiate agonist ^{3}H-(D-Ala^2,D-Leu^5)enkephalin (DADLE) and the antagonist [^{3}H] diprenorphine to dBcAMP-treated NCB-20 cell membranes indicated an 80% reduction in opiate receptor density relative to untreated cells (B_{max} = 47 ± 11 fmol/mg of protein versus 220 ± 48 fmol/mg of protein), with no change in ligand affinities. Binding of the muscarinic cholinergic antagonist [^{3}H]quinuclidinyl benzilate and the α_2-adrenergic agonist [^{3}H]-*p*-aminoclonidine to dBcAMP-treated NCB-20 membranes was also reduced by 50% and 28%, respectively. In contrast, treatment of NG108-15 cells with dBcAMP did not down-regulate opiate, muscarinic, or α_2-adrenergic receptor sites. Opiate and α_2-adrenergic receptor sites were not down-regulated in the N18TG2 neuroblastoma clone, the common parent of both the hybrid cells, and the apparent source of these receptors. The C6BU-1 parent of the NG108-15 hybrid showed poor specific binding of all ligands examined. dBcAMP was very potent in inducing opiate receptor site down-regulation of NCB-20 cells, with an ED_{50} after 4 days treatment of 8 μM. The time course of loss of [^{3}H]DADLE and [^{3}H]quinuclidinyl benzilate specific binding was similar, and maximum down-regulation was achieved after 2 days. In contrast, neither higher concentrations of dBcAMP (5.0 mM) nor longer treatment times (7 days) resulted in down-regulation of receptor sites on NG108-15 cells. Coupling of opiate receptors to AC was also selectively altered in differentiated NCB-20 cells. Prostaglandin E_1-stimulated AC was maximally inhibited by 1 μM DADLE in membranes from undifferentiated cells to different degrees (30% in NCB-20 and 54% in NG108-15). dBcAMP treatment had no effect on opiate inhibition of AC in NG108-15 cells but reduced by 50% the maximum opiate inhibition of AC in NCB-20 cells. These data indicate that the signal for receptor down-regulation which was triggered by dBcAMP in the NCB-20 cell comes uniquely from the Chinese hamster brain cell NCB-20 parent. The differences between NCB-20 and NG108-15 cells in the regulation of N_i-coupled receptors provides an example of dBcAMP-induced heterologous down-regulation with unique cell specificity, which is unrelated to the morphological differentiation process triggered by dBcAMP, which is common to both cells.

The mouse neuroblastoma hybrid cells, NG108-15 and NCB-20, derived from the fusion of the mouse neuroblastoma cell, N18TG2, to rat glioma (C6BU-1) and to fetal Chinese hamster brain cells, respectively, have been extensively employed to investigate cellular mechanisms of neurotransmitter receptor regulation underlying tolerance to and dependence on opiate and cholinergic agents. Specific membrane receptors are expressed on these cells which may be either linked in an inhibitory manner (N_i) to AC (opiate, muscarinic, cholinergic, α_2-adrenergic) or positively coupled (N_s) (PGE_1, adenosine) to this effector system.

Both cell lines possess many properties of mature neurons which can be enhanced by inducing differentiation with various agents that increase intracellular cAMP levels (1). Morphologically, differentiated cells produce neurite-like processes with increased somal and nuclear size and a highly differentiated ultrastructure (2, 3). Like mature neurons, neuroblastoma hybrids contain enzymes involved in the synthesis and degradation of neurotransmitters and exhibit electrically excitable membranes following depolarizing stimuli (4–9).

The induction of differentiation elicits many changes in the normal, neuronal function of these hybrid cells. For instance, endogenous opioid peptide content, neurotransmitter synthesis and release, synapse formation, and action potentials generated by hormonal and electrical stimulation are all increased in

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ABBREVIATIONS: N_i, inhibitory guanine nucleotide-binding-regulatory protein; AC, adenylate cyclase; [^{3}H]DADLE, ^{3}H (D-Ala^2, D-Leu^5)enkephalin; dBcAMP, N^6, 2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; [^{3}H]DPN, [^{3}H]diprenorphine; [^{3}H]PAC, [^{3}H]*p*-aminoclonidine; PGE_1, prostaglandin E_1; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate; [^{3}H]RAUW, [^{3}H]rauwolscine; N_s, stimulatory guanine nucleotide-binding-regulatory protein.

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differentiated NG108-15 cells (1, 10–14). Although many studies dealing with the effects of differentiation on morphology, neurotransmitters, and ion channel function have been conducted using NG108-15 cells, there have been few reports on receptor-effector coupling, or the pharmacological properties of receptors, following cell differentiation. Opiate, muscarinic cholinergic, and α_2-adrenergic receptor agonists decrease basal and PGE_1-stimulated intracellular cAMP levels, and inhibit AC activity in murine neuroblastoma hybrid cells (15–23). Chronic exposure of cells to opiates, norepinephrine, or acetylcholine analogues results in a return of AC activity, and removal of agonist or antagonist challenge increases activity to above-normal levels (19, 20, 24–27). This regulation of AC by opiates has been proposed as a model for narcotic tolerance and dependence (28) and may be associated with opiate receptor desensitization.

Desensitization or tachyphylaxis is a cellular adaptive process in which the cells' responsiveness to a hormone or transmitter is lost or attenuated subsequent to agonist treatment. This phenomenon is shared by many receptor-coupled systems (23, 29–33). Homologous or agonist-specific desensitization is characterized by a diminished response only to a receptor-specific hormone (33–35) and has been observed in frog erythrocytes, mammalian lung, and cultured cell lines (33–38). Heterologous desensitization, in contrast, is characterized by an attenuated responsiveness to other receptor agonists in addition to the desensitizing hormone, and occasionally to activators of AC such as guanine nucleotides and fluoride (33, 39, 40). The molecular events responsible for agonist-induced desensitization have been the subject of much investigation (33, 41). In homologous desensitization or down-regulation, a portion of the receptors appears to be sequestered out of the plasma membrane and recovered in an intracellular compartment, an event coincident with an uncoupling of the receptor-effector system (33, 42–44). Heterologous desensitization, in contrast, is primarily associated with functional alterations of the receptor-effector system (36, 45) and appears not to involve receptor sequestration (33, 37, 41). In some β-adrenergic receptor systems, both forms of desensitization appear to be correlated with phosphorylation of the β-adrenergic receptor (46, 47). Previously, NG108-15 cells have been shown to undergo a rapid and specific desensitization of muscarinic cholinergic receptor-mediated inhibition of AC (30). Chronic opiate treatment of NG108-15 cells also results in down-regulation of opiate receptors (28).

In this study, regulation of three N_i-coupled receptor systems was examined in NG108-15 and NCB-20 hybrid cells following differentiation by dBcAMP. Down-regulation of opiate, muscarinic cholinergic, and α_2-adrenergic receptor sites (B_{max}) was observed in differentiated NCB-20 cells, but not in differentiated NG108-15 cells. AC activities determined in parallel also revealed an uncoupling of opiate receptors on differentiated NCB-20 cells, but not on differentiated NG108-15 cells. The results reported here thus provide a cell-specific example of heterologous receptor down-regulation. Since both hybrids share one common parental cell, which seems to be the source of the N_i-coupled receptors, these neuronal hybrid cultures provide a model system in which genetic aspects of receptor regulation may be studied. A preliminary communication of these findings has been published (48).

Materials and Methods

Cell growth and differentiation. NG108-15 and NCB-20 neuroblastoma cell hybrids, obtained at passage 14 from Dr. M. Rasenick (University of Illinois), were grown as previously described (15). Briefly, cells were cultured in Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), glutamine (1 mM), hypoxanthine (0.1 mM)-aminopterin (1 μM)-thymidine (20 μM), and penicillin-streptomycin-neomycin, in a humidified atmosphere of 10% CO_2 in air at 37°. The N18TG2 and the C6BU-1 cells (obtained from Drs. M. Nirenberg, National Institutes of Health, Bethesda, MD, and K. Braas, Johns Hopkins University, Baltimore, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with serum and glutamine, but in the absence of hypoxanthine-aminopterin-thymidine. Cells were subcultured in 150-cm^2 flasks (Corning) every 5 days and the medium was changed every 2–3 days to maintain stock cultures. For experiments, cells were seeded in 100 × 20 mm Petri dishes (Corning) at an initial cell density of 5×10^5 cells/culture dish. Cells between passage 17 and 30 were routinely used.

Cell differentiation was induced by the addition of dBcAMP to a 1-day-old culture seeded at an initial cell density of 5×10^5 cells/dish. Unless otherwise indicated, a concentration of 1.0 mM dBcAMP was used and treatment was continued for 4 days. Since dBcAMP inhibits cell growth by about 50% (see Fig. 2), the number of culture dishes routinely treated with dBcAMP was twice the number employed for untreated controls.

Preparation of membranes. Membranes were prepared as previously described (31). Briefly, 5-day-old confluent, untreated, or dBcAMP-treated cultures were washed once in D_1-saline (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2PO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose), harvested in the same buffer by gentle agitation, and prepared for use either for receptor binding or for AC assays. For binding assays, freshly harvested cells were resuspended in 50 mM Tris-HCl buffer (pH 7.4 at 0°), homogenized using a Brinkman Polytron PT 10/35 instrument (setting 5.5, 15 sec), and centrifuged three times (10 min at 46,000 × g) with intermediate resuspension in the same buffer.

For AC assays (49), untreated or dBcAMP-treated monolayers were washed twice by centrifugation (5 min, 1,500 × g) with ice-cold D_1-saline to remove residual dBcAMP. Cell pellets were resuspended in buffer containing 5.0 mM Tris-HCl (pH 7.4), 0.32 M sucrose, and 1.0 mM $MgCl_2$, homogenized with a Teflon glass homogenizer (15 strokes, 2,000 rpm), and centrifuged at 600 × g to remove nuclei and unbroken cells. The supernatant was centrifuged for 10 min at 40,000 × g to obtain a particulate membrane fraction. Cell membranes were adjusted to a protein concentration of approximately 3 mg/ml and stored frozen in aliquots in liquid nitrogen for up to 6 months. Immediately before use, membranes were thawed and resuspended in 50 mM Tris-HCl buffer for measurement of AC activity. Protein was determined by the Bio-Rad method using bovine serum albumin as standard (50). Cell titers were routinely determined using a hemocytometer.

Receptor binding assays. Binding of the opiate ligands, [^{3}H]DPN and [^{3}H]DADLE, to freshly prepared undifferentiated and differentiated cell membrane fractions was measured in 50 mM Tris-HCl buffer (pH 7.4) as described previously with some modification (31). Nonspecific binding, defined as binding which occurred in the presence of 10 μM morphine sulfate, accounted for 5–10% of the total binding observed. All incubations were carried out at 23° for 90 min except where indicated.

Binding of the muscarinic cholinergic antagonist, [^{3}H]QNB, to undifferentiated and differentiated cell membranes was determined by a modification of a procedure described previously for brain tissue (51). Incubations of [^{3}H]QNB with cell membranes were carried out in 50 mM Tris-HCl, pH 7.4, for 90 min at 37° except where indicated. Nonspecific binding using 1.0 μM atropine sulfate represented 20% of the total ligand bound.

Binding of the α-agonist, [^{3}H]PAC, was determined in 50 mM Tris-HCl, 1.0 mM $MgCl_2$, pH 8.4, at 24° for 40 min as previously described (23). Nonspecific binding was defined by parallel incubations carried

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out in the presence of 100 μM norepinephrine, and accounted for 25% of the total ligand bound. Incubations of cell membranes with the α-antagonist, [^{3}H]RAUW, was performed at 24° for 40 min (23). Nonspecific binding was defined by parallel incubation with 100 μM norepinephrine and represented 10–30% of total ligand bound.

In saturation experiments, the range of ligand concentrations used was as follows: [^{3}H]DPN (30 pM–5 nM), [^{3}H]DADLE (0.1–5.0 nM), [^{3}H]QNB (2.0–60.0 pM), [^{3}H]PAC (0.2–20 nM), [^{3}H]RAUW (0.2–20 nM). For all ligands, assay volume was adjusted to 0.5 ml, except for [^{3}H]QNB, where the incubation volume was increased to 2.5 ml. The protein concentration of untreated or dBcAMP-treated membranes was ~0.2 mg/tube. After incubation, the assay tube contents were filtered under vacuum over Whatman GF/B filters, using a Brandel modified cell harvester, and rinsed three times with 4.0 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°). Filter-bound radioactivity was subsequently counted by liquid scintillation spectrometry at 40% counter efficiency. Samples were assayed in triplicate, with triplicate values varying by <10%. All experiments were repeated three to four times with <15% variation among the different experiments.

Adenylate cyclase. AC activity was routinely assayed as described by Salomon (52) with some modifications. Briefly, 100-μl reaction mixtures contained 30 mM Tris-HCl (pH 7.4), 5.0 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM cAMP, 1.0 mM isobutylmethylxanthine, 1.0 mM dithiothreitol, 5.0 mM creatine phosphate, 0.4 mg/ml creatine kinase, 100 mM NaCl, 0.1 mM GTP, and approximately 0.6 μCi of [α-^{32}P]ATP. Where indicated, 10 μM PGE_1 was added and all reactions were initiated by the addition of about 15 μg of membrane protein to the assay tubes. Incubations were carried out for 20 min at 37° and terminated by addition of 100 μl of stopping solution (2% sodium dodecyl sulfate, 40 mM ATP, 1.4 mM cAMP, pH 7.0).

Data analysis. Saturation isotherms were analyzed using the Scatchard transformation (53). Estimates of K_D and B_{max} were obtained using unweighted linear regression analysis of the transformed data. K_i values were calculated using the Cheng-Prusoff equation (54). B_{max} values were routinely calculated as both fmol/mg of protein and as fmol/10^6 cells. Differences in B_{max} values resulting from dBcAMP treatments were identical when the data were expressed either way. Only fmol/mg of protein values are shown.

Ligands. [^{3}H]DADLE (46.9 Ci/mmol), [^{3}H]PAC (40.0 Ci/mmol), [^{3}H]RAUW (75.0 Ci/mmol), and [^{3}H]QNB (30–40 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [^{3}H]DPN (41 Ci/mmol), [α-^{32}P]ATP (800 Ci/mmol), and [^{3}H]cAMP (33.5 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL).

Drugs and reagents. Cell culture media and growth supplements were purchased from Whitaker M.A. Bioproducts (Walkersville, MD). Morphine sulfate was obtained from Eli Lilly (Indianapolis, IN). Unlabeled DADLE was obtained from Peninsula Laboratories (Belmont, CA), Bio-Rad protein assay from Bio-Rad (Rockville Center, NY), ATP free of GTP contamination and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Morphology and growth properties. NG108-15 and NCB-20 cells exposed to 1.0 mM dBcAMP for 4 days expressed the morphological characteristics of differentiated cells previously reported (12) (Fig. 1). Untreated NG108-15 hybrid cells were polygonal, usually possessed short processes, and appeared to form clusters (Fig. 1A), whereas treatment with 1.0 mM dBcAMP caused the cells to aggregate and to emit a network of extensive, long, neurite-like processes that connected the cell aggregates (Fig. 1B). Untreated NCB-20 cells also appeared to grow in even monolayers of polygonal cells with short branched processes emerging from the cell bodies (Fig. 1C). dBcAMP treatment caused the cells to become rounder, further aggregate, and emit numerous processes (Fig. 1D). Treatment of both cell lines with dBcAMP for longer time periods (up to 7 days) did not strikingly alter the morphology of the cells, although the processes began to thicken and cytoplasmic droplets appeared on the surface of processes and cell bodies. dBcAMP treatment also reduced the rate of cell division in both NCB-20 and NG108-15 cell lines (Fig. 2). After 4 days of exposure to 1.0 mM dBcAMP, growth of both cells was inhibited by about 50%.

Down-regulation of opiate, muscarinic, and α_2-adrenergic receptors. Exposure of NCB-20 hybrid cells to 1.0 mM dBcAMP for 4 days greatly decreased the density of opiate receptor sites (Fig. 3A). Saturation isotherms of opiate agonist ([^{3}H]DADLE)-specific binding showed that many fewer receptor sites were labeled on differentiated NCB-20 cell membranes. The decreased binding in dBcAMP-treated cells was due to a reduction in specific ligand bound with no change in nonspecific binding. Scatchard analyses of the data (Fig. 3A, Table 1) showed a substantial reduction (82%) in opiate receptor site density from a B_{max} (mean ± SD) of 220 ± 48 fmol/mg of protein for undifferentiated cells, to 47 ± 11 fmol/mg of protein for dBcAMP-treated NCB-20 cultures.

In striking contrast, control and dBcAMP-treated NG108-15 cells showed no differences in the density of opiate receptor sites present (Fig. 3B). B_{max} values were 574 ± 23 fmol/mg of protein (control) and 510 ± 17 fmol/mg of protein (treated). Similar results were obtained with [^{3}H]DPN binding studies to differentiated NCB-20 and NG108-15 cells (Table 1). Ligand affinities of [^{3}H]DADLE and [^{3}H]DPN binding to NCB-20 membranes were unaltered by dBcAMP treatment (Table 1).

It is of interest to note that the density of opiate receptor sites on undifferentiated NG108-15 cells was about 2 times higher than the density of sites on undifferentiated NCB-20 cells (Fig. 1, Table 1). These data are not in agreement with those of McLawhon *et al.* (31), where NCB-20 cells were found to contain 4 times as many opiate receptor sites as NG108-15 cells. Our data agree with those of Law *et al.* (28, 29, 55), where B_{max} values for opiate-binding ligands were in the same range as those reported here. dBcAMP itself had no effect on the binding activity of [^{3}H]DADLE to cell membranes (data not shown).

In addition to opiate receptors, other N_i-coupled receptor sites on NCB-20 cells were also down-regulated by dBcAMP treatment (Table 1). Control and differentiated NCB-20 cells possessed muscarinic receptor sites with similar affinities for [^{3}H]QNB. The B_{max} values were, however, reduced by 50% in membranes from dBcAMP-treated cells. In contrast, no significant differences were detected in [^{3}H]QNB binding to control and differentiated NG108-15 cells (Table 1). The decreased sensitivity of NCB-20 muscarinic receptors to down-regulation by dBcAMP, relative to NCB-20 opiate receptor sites, may reflect the presence of different subpopulations of muscarinic receptor sites labeled by [^{3}H]QNB (M_1 and M_2 receptors) on NCB-20 cells, which are differentially sensitive to dBcAMP treatment. Agonist-induced down-regulation of muscarinic cholinergic receptors on N1E-115 mouse neuroblastoma cells was also maximally reduced by only 50% (56).

α_2-Adrenergic receptor sites on NCB-20 cells were also down-regulated by dBcAMP treatment. Again, in contrast, no down-regulation of α_2-receptors by dBcAMP treatment of NG108-15 cells was observed (Table 1). B_{max} values in NG108-15 cells were in good agreement with previous values (23, 57). Ligand

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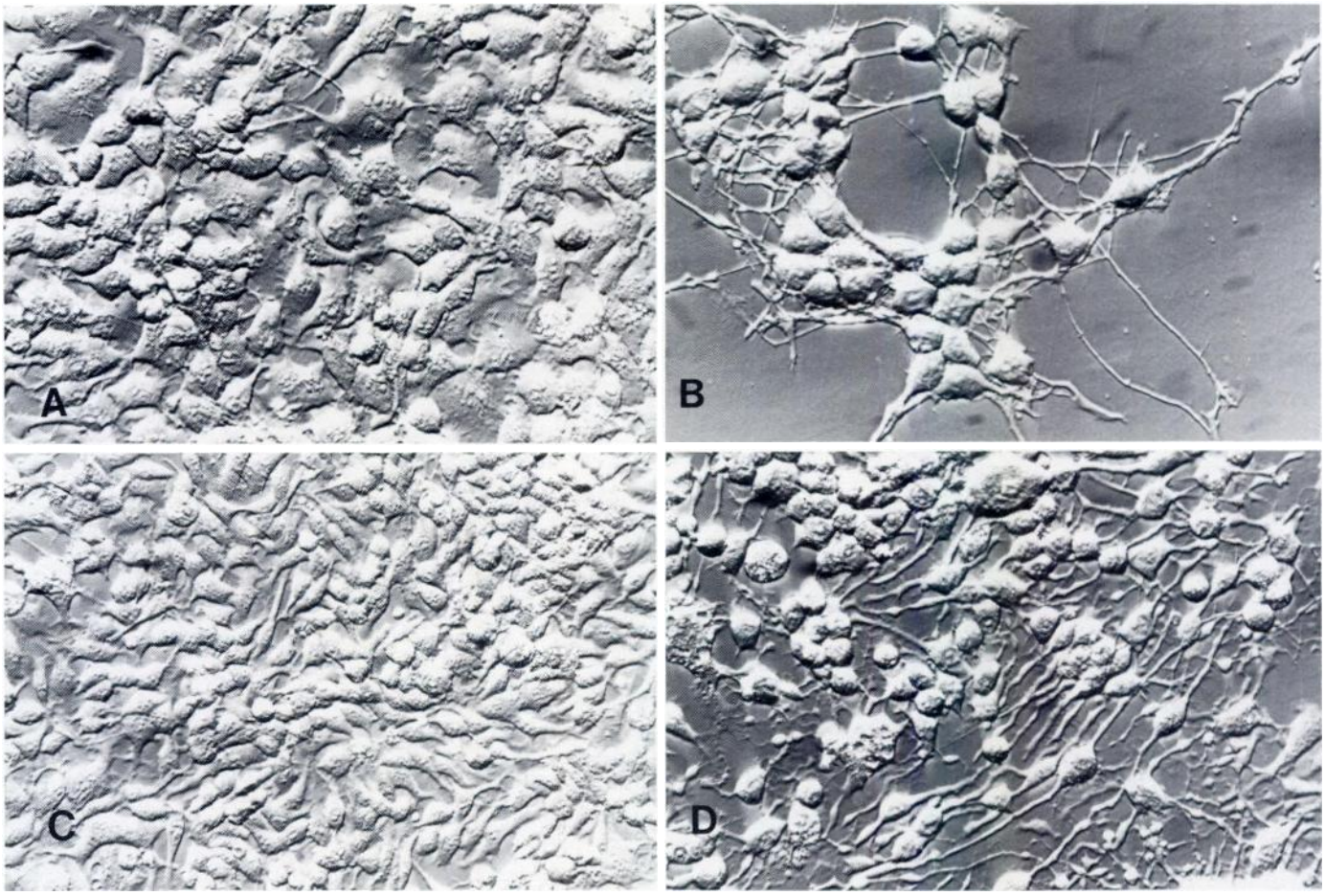

Fig. 1. Morphological comparison of untreated and dBcAMP-treated NCB-20 and NG108-15 cells. Cells were grown as described under Materials and Methods with no drugs (A and C) or with 1.0 mM dBcAMP (B and D) for 4 days. Phase contrast micrographs of untreated and treated NG108-15 (A and B) and NCB-20 (C and D) cells were taken at approximately × 300 magnification.

affinities were not significantly different in control and dBcAMP-treated NCB-20 and NG108-15 cells.

The α_2-antagonist ligand, [^{3}H]RAUW, confirmed that α_2-receptor site down-regulation was observed only in NCB-20 cells and not in NG108-15 cells (Table 1). Receptor site density in NCB-20 cells was decreased by 53% following dBcAMP treatment. In contrast, no down-regulation of α_2-receptors by dBcAMP treatment was seen in NG108-15 membranes.

Taken together, these data show striking differences in the regulation of opiate, muscarinic cholinergic, and α_2-adrenergic receptor sites by dBcAMP treatment in NCB-20 cells compared to NG108-15 cells. Receptor down-regulation was induced in NCB-20 cells but not in the related NG108-15 cell line, by treatment of the cultures with dBcAMP.

Dose response and time course of dBcAMP effect on opiate and muscarinic receptors. The dose-response relationship of the dBcAMP effect on NCB-20 and NG108-15 cultures was examined (Fig. 4). In NCB-20 cells, the magnitude of the down-regulation response was clearly dependent on the concentration of dBcAMP present in the growth medium. dBcAMP was very potent in inducing opiate receptor site down-regulation, with a 70% receptor loss at 30 μM dBcAMP. It was less potent in inducing muscarinic receptor site down-regulation, with a 50% loss in [^{3}H]QNB binding occurring at approximately 0.6 mM dBcAMP. The difference in the sensitivity of opiate and muscarinic receptor sites to down-regulation by dBcAMP may also reflect the existence of distinct subpopulations of muscarinic receptor sites on NCB-20 cells, only one of which is sensitive to dBcAMP treatment. In NG108-15 cells, no down-regulation of either opiate or muscarinic receptor sites was seen using up to 3.0 mM dBcAMP (Fig. 4B). At 5.0 mM dBcAMP, only a small reduction (20–30%) in [^{3}H]DADLE binding was observed, which is in striking contrast to the sensitivity of opiate and muscarinic receptor sites on differentiated NCB-20 cells.

The time course of opiate and muscarinic receptor site down-regulation in NCB-20 cells was examined by exposing cultures to 1.0 mM dBcAMP for different time periods (Fig. 5A). In this experiment, all cultures were seeded at an initial cell density of 5×10^5 cells/dish (day 0) and dBcAMP (1.0 mM) was added sequentially to each set used for the different time points over the subsequent 4 days. All cells were then harvested on day 5. After a 1-day treatment, [^{3}H]DADLE and [^{3}H]DPN binding was reduced by 64%. After 3 days, 90% of the binding activity was lost. The time course of the loss of [^{3}H]QNB binding to NCB-20 cells was similar to that of the opiate ligands except that binding activity was inhibited to a maximum of only 50% (see also Table 1). In contrast, NG108-15 cells were refractory to the effects of 1.0 mM dBcAMP, and even after 5 days of treatment there was no significant decrement in specific bind-

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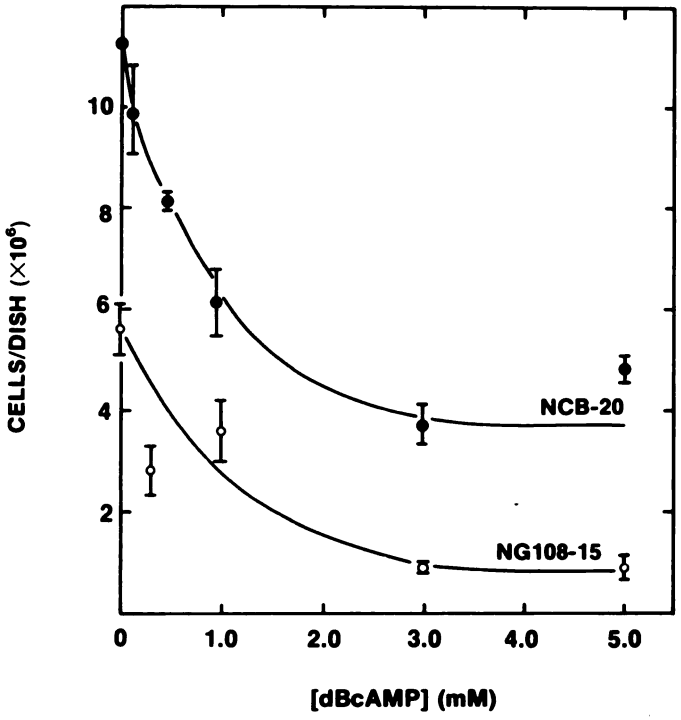

Fig. 2. Dose-dependent effect of dBcAMP on growth of NCB-20 and NG108-15 cultures. NCB-20 and NG108-15 cells, seeded at an initial cell density of 5 × 10^5 cells/dish, were treated with dBcAMP (0.3–5.0 mM) after 1 day of growth. Control cultures (0 mM) received an equivalent volume of media in the absence of dBcAMP. Cultures were allowed to grow for an additional 4 days. Cell titers were determined using a hemocytometer. Values represent the mean ± standard deviation of duplicate determinations from two to four experiments.

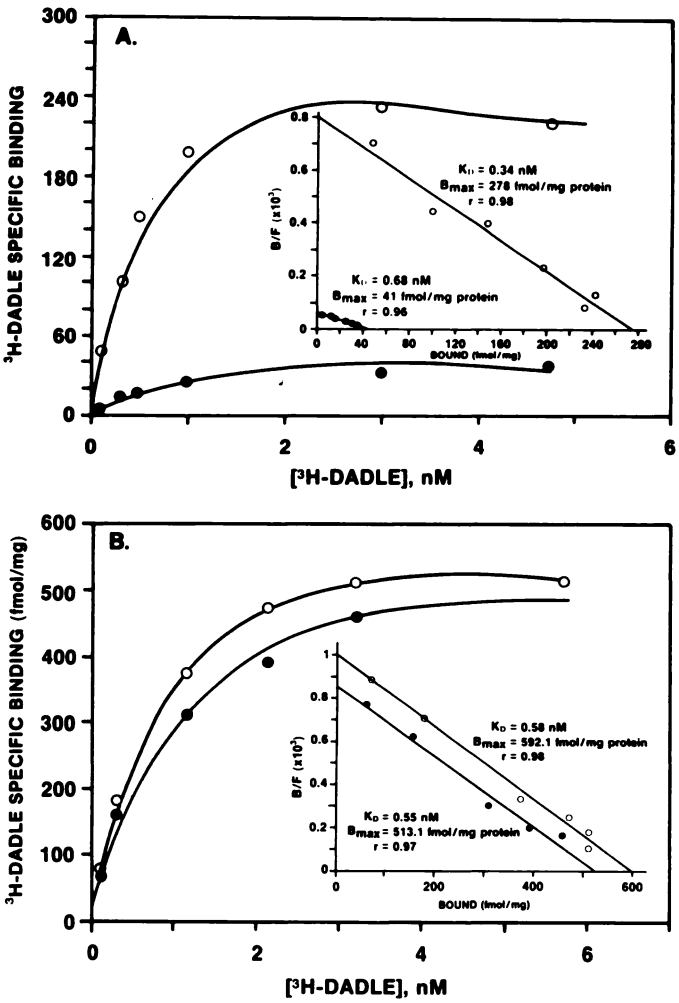

Fig. 3. Down-regulation of opiate receptor sites in NCB-20 but not NG108-15 cells. NCB-20 (A) and NG108-15 (B) cultures were untreated (○) or treated (●) with dBcAMP (1.0 mM, 4 days). Binding of [^{3}H]DADLE (0.1–5.0 nM) to membranes (0.2 mg of protein/tube) prepared from each set of cultures was measured at 25° for 90 min as described in Materials and Methods. Nonspecific binding was determined in the presence of 10 μM morphine. Points shown are from a single experiment assayed in triplicate. Similar results were obtained each of the three times this experiment was performed. *Insets*: Scatchard plots of ligand saturation data.

ing of opiate and muscarinic cholinergic ligands to NG108-15 membranes (Fig. 5B). These data suggest that the down-regulation of receptors on NCB-20 cells precedes the morphological differentiation of the cells, and, therefore, the two events may be unrelated. Additional evidence for dissociation of the two events is derived from the ability of dBcAMP to induce NG108-15 cell differentiation but not down-regulation of receptors on NG108-15 cell membranes.

Cell specificity of receptor down-regulation. The neuroblastoma parent, N18TG2, common to both hybrid cells, NCB-20 and NG108-15, and the rat glioma mutant, C6BU-1, parent of the NG108-15 cell hybrid, were examined for the ability of their receptors to be down-regulated by 1.0 mM dBcAMP. The binding of opiate ([^{3}H]DPN, [^{3}H]DADLE), muscarinic cholinergic ([^{3}H]QNB), and α_2-adrenergic ligands ([^{3}H]PAC, [^{3}H]RAUW) to the two neuroblastoma hybrids and to the parental cell lines was compared in a series of experiments (Table 2). Except for [^{3}H]QNB, specific binding of all ligands to N18TG2 membranes was significantly less than specific binding to both NCB-20 and NG108-15 cell membranes. However, no significant decrease in binding was observed in dBcAMP-treated N18TG2 membranes with any ligand tested. In fact, an increase in [^{3}H]PAC- and [^{3}H]RAUW-specific binding with dBcAMP treatment was observed.

In the C6BU-1 cell line, no appreciable specific binding was detected with any of the above ligands except [^{3}H]DPN (60% of total binding displaced by morphine). Treatment with dBcAMP did not inhibit [^{3}H]DPN binding to C6BU-1 membranes, and may have enhanced it. Parallel single point binding experiments performed on NCB-20 cell membranes showed a pronounced reduction in [^{3}H]DPN- and [^{3}H]DADLE-specific binding (85%), [^{3}H]QNB-specific binding (50%), and [^{3}H]PAC (25%)- and [^{3}H]RAUW (55%)-specific binding to dBcAMP-treated NCB-20 cultures. In contrast, under the same experimental conditions, no reduction in specific binding of all ligands examined to NG108-15 cells was observed following exposure to dBcAMP. These data show a cell-specific down-regulation of opiate, muscarinic, and α_2-adrenergic receptors by dBcAMP treatment only in the NCB-20 hybrid cell. The NG108-15 hybrid and its parents, N18TG2 and C6BU-1, were refractory to down-regulation by dBcAMP treatment.

Pharmacological specificity of undifferentiated and differentiated NCB-20 and NG108-15 cells. The inhibition of opiate, muscarinic, and α_2-adrenergic ligand binding by specific competitors was examined in undifferentiated and differentiated NCB-20 and NG108-15 cell cultures (Table 3). K_i values for unlabeled DADLE, morphine, and naloxone at both

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TABLE 1

Down-regulation of opiate, muscarinic cholinergic, and α_2-adrenergic receptors on NCB-20 but not NG108-15 cells

Cultures of NCB-20 and NG108-15 cells were treated with dBcAMP (1.0 mM, 4 days). Membranes (0.2 mg of protein/tube) were assayed for specific binding of [^{3}H]DADLE (0.1–5 nM), [^{3}H]DPN (30 pM–5 nM), [^{3}H]QNB (2–60 pM), [^{3}H]PAC (0.2–20 nM), and [^{3}H]RAUW (0.2–20 nM) as described in Materials and Methods. Nonspecific binding was measured in the presence of 10 μM morphine for opiate ligands (DADLE and DPN), 1.0 μM atropine for the muscarinic ligand QNB, and 0.1 mM (–)-norepinephrine for the α_2-adrenergic ligands (PAC and RAUW). Values represent means ± standard deviations of (n) experiments performed in triplicate.

Cell line	Ligand	B_{max}			K_D		(n)
		Control	dBcAMP-treated	% Control	Control	dBcAMP-treated	
		fmol/mg			nM		
NCB-20	DPN	272 ± 26	42 ± 14	15	0.28 ± 0.05	0.49 ± 0.14	(8)
	DADLE	220 ± 48	47 ± 11	21	0.56 ± 0.21	0.63 ± 0.15	(8)
	QNB	45 ± 4	22 ± 3	49	0.04 ± 0.01	0.03 ± 0.01	(6)
	PAC	76 ± 6	55 ± 2	72	1.5 ± 0.3	1.4 ± 0.1	(4)
	RAUW	198 ± 65	104 ± 6	52	1.8 ± 0.4	2.3 ± 0.4	(2)
NG108-15	DPN	590 ± 61	534 ± 31	91	0.32 ± 0.04	0.32 ± 0.05	(5)
	DADLE	574 ± 23	510 ± 17	89	0.61 ± 0.1	0.7 ± 0.1	(5)
	QNB	45 ± 10	45 ± 10	100	0.03 ± 0.003	0.03 ± 0.002	(3)
	PAC	106 ± 20	97 ± 17	92	1.7 ± 0.5	1.7 ± 0.6	(3)
	RAUW	165 ± 13	183 ± 49	111	2.1 ± 0.2	2.0 ± 0.4	(3)

[^{3}H]DADLE and [^{3}H]DPN sites were very similar using membranes from control and dBcAMP-treated NCB-20 cells, suggesting that the receptors remaining on differentiated NCB-20 cells following down-regulation were pharmacologically unaltered. Similar comparisons were made using NG108-15 cell membranes. Again, no significant differences in opiate K_i values were observed between untreated and dBcAMP-treated NG108-15 cultures.

Inhibition of [^{3}H]QNB-specific binding by atropine and carbachol yielded K_i values that were similar for control and dBcAMP-treated NCB-20 cells, and for control and dBcAMP-treated NG108-15 cultures. K_i values derived from competition curves of [^{3}H]PAC binding by phentolamine and norepinephrine were also similar in untreated and dBcAMP-treated NCB-20 cultures (Table 3). Thus, the pharmacological specificity of the receptor sites (opiate, muscarinic, α_2-adrenergic) was unchanged following differentiation in both cell lines and down-regulation in NCB-20 cells. Except for the K_i value of carbachol, which was approximately 10-fold lower in NG108-15 compared to NCB-20 cells, K_i values for all drugs at the three receptors were very similar in the two cell lines.

Effect of dBcAMP treatment on receptor function. The coupling of opiate receptors to AC in differentiated and undifferentiated NCB-20 and NG108-15 cell cultures was examined. Membranes were prepared from untreated and dBcAMP-treated (1.0 mM, 4 days) cultures and assayed for the ability of DADLE to inhibit PGE_1-stimulated AC activity (Fig. 6A). Basal enzyme activities in membranes from untreated and dBcAMP-treated cultures were approximately the same: 305–350 pmol of cAMP/20 min/mg of protein in NCB-20 cells, and 780–990 pmol of cAMP/20 min/mg of protein in NG108-15 cells. Stimulation by PGE_1 (10 μM) was 7–8-fold in both cell lines and was not affected in either cell line by dBcAMP treatment.

The maximal inhibitory response elicited by DADLE was significantly different in untreated and dBcAMP-treated NCB-20 cells (Fig. 6A). In untreated cells, DADLE (1 μM) inhibited PGE_1-stimulated AC to a maximum of 30%, whereas in dBcAMP-treated NCB-20 cells, a maximum inhibition of only 17% was observed. This 50% loss in opiate agonist response coincided with a loss of 90% of opiate receptor-binding sites, which suggests that there is a large opiate receptor reserve on NCB-20 cells. The potency of DADLE to inhibit PGE_1-stimulated AC, however, was not significantly different in control and dBcAMP-treated NCB-20 cells, with EC_{50} values of 47 nM and 28 nM, respectively. If the existence of a large receptor reserve were to account for the much smaller loss in receptor function compared to binding sites, then a right-shift in the DADLE dose-response curve would be predicted. The inherent error involved in the measurement of a small inhibitory response may account for the lack of an observable shift in the potency of DADLE in the two membrane preparations.

In contrast to NCB-20 cells, DADLE (1 μM) inhibited control and dBcAMP-treated NG108-15 AC to the same maximum extent of 56% (Fig. 6B). The EC_{50} values for DADLE were 17 nM and 11 nM, respectively. Similar values for maximal inhibition and potency of DADLE have been reported in NG108-15 cells by others (55). Thus, in the NG108-15 cells, where no opiate receptor site down-regulation by dBcAMP was observed, the receptor-effector coupling mechanism was unaltered. In contrast, in differentiated NCB-20 cells, where opiate receptor sites were down-regulated, a parallel functional desensitization was also observed.

Discussion

In this study we have observed that three receptor classes which are negatively linked to the AC effector system (opiate, muscarinic, and α_2-adrenergic), and which are present on the hybrid cell, NCB-20 (neuroblastoma N18TG2 × Chinese hamster brain glial cell), were down-regulated by treatment of NCB-20 cells with dBcAMP. In all cases, receptor site densities but not ligand affinities for each receptor were altered by dBcAMP treatment. The changes in receptor site density were the same whether density was expressed as fmol/mg of protein or fmol/10^6 cells (not shown). Opiate receptor site density was reduced by approximately 90%, whereas density of α_2-adrenergic and muscarinic receptor sites was reduced to a lesser extent (30–50%). In contrast, the densities and ligand affinities of the same three receptors on the closely related hybrid cell, NG108-15 (neuroblastoma N18TG2 × glioma C6BU-1), were unaffected by dBcAMP treatment. Thus, a striking cell-specific difference in receptor regulation between these two related hybrids was observed in response to dBcAMP.

dBcAMP has previously been used to differentiate several

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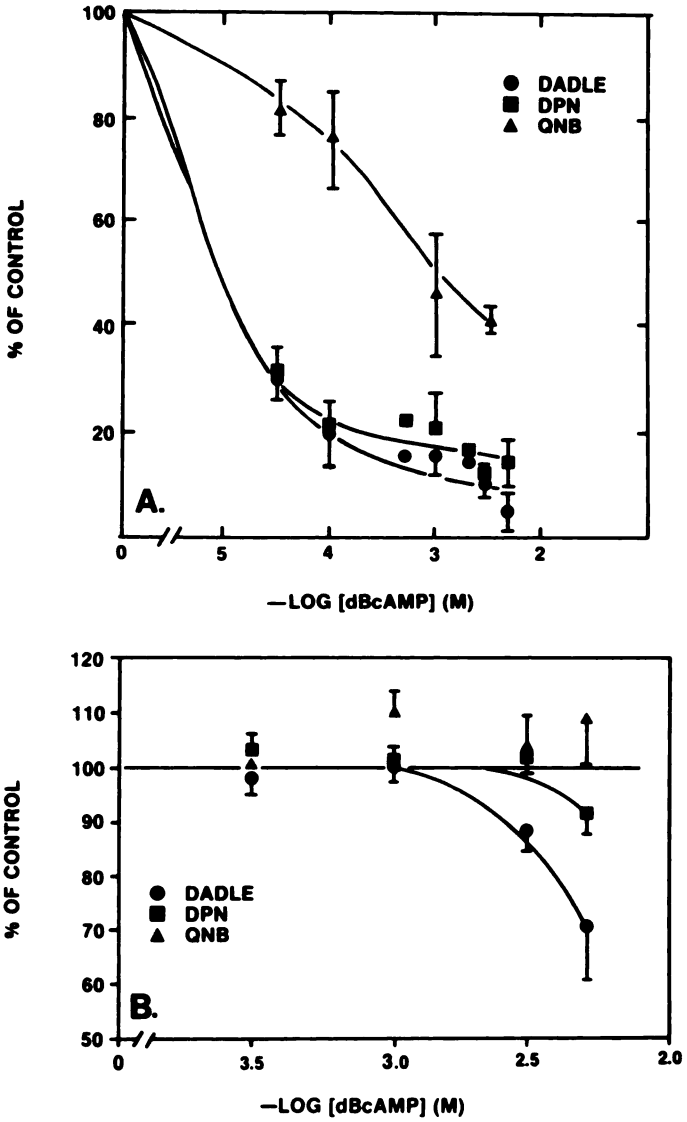

Fig. 4. Dose response curves for effect of dBcAMP on opiate and muscarinic receptors in NCB-20 and NG108-15 cells. Cultures of NCB-20 (A) and NG108-15 (B) cells were treated for 4 days with various concentrations of dBcAMP (0.3–5.0 mM). Binding of the opiate ligands, [^{3}H]DADLE (3.0 nM) and [^{3}H]DPN (1.0 nM), and the muscarinic cholinergic ligand [^{3}H]QNB (30.0 pM) to membranes (0.2 mg of protein/tube) prepared from each set of cultures was measured at 25° for [^{3}H]DADLE and [^{3}H]DPN, and 37° for [^{3}H]QNB, as described under Materials and Methods. All incubations were carried out for 90 min. Nonspecific binding was determined in the presence of 10 μM morphine for opiate ligands or 1.0 μM atropine for the muscarinic ligand. Results represent means ± standard deviations of triplicate determinations from two to four experiments. Specific opiate binding of [^{3}H]DADLE and [^{3}H]DPN to NCB-20 membranes in control (untreated) cultures was 210 ± 20 fmol/mg of protein and 240 ± 30 fmol/mg of protein, respectively. Specific [^{3}H]QNB binding in control cultures was 28 ± 2 fmol/mg of protein. Specific iopiate binding of [^{3}H]DADLE and [^{3}H]DPN to NG108-15 membranes in control cultures was 516 ± 44 fmol/mg of protein and 555 ± 132 fmol/mg of protein, respectively. Specific [^{3}H]QNB binding in control cultures was 22 ± 4 fmol/mg of protein.

tumor cells of neuronal or glial origin, including NG108-15 and NCB-20 cells (1). Differentiated NG108-15 cells are larger than untreated cells, with numerous long neurites extending from aggregated cell bodies. The neurites contain increased amounts of mitochondria, endoplasmic reticulum, and electron-transparent and large dense core particles (2, 3, 12). Differentiated

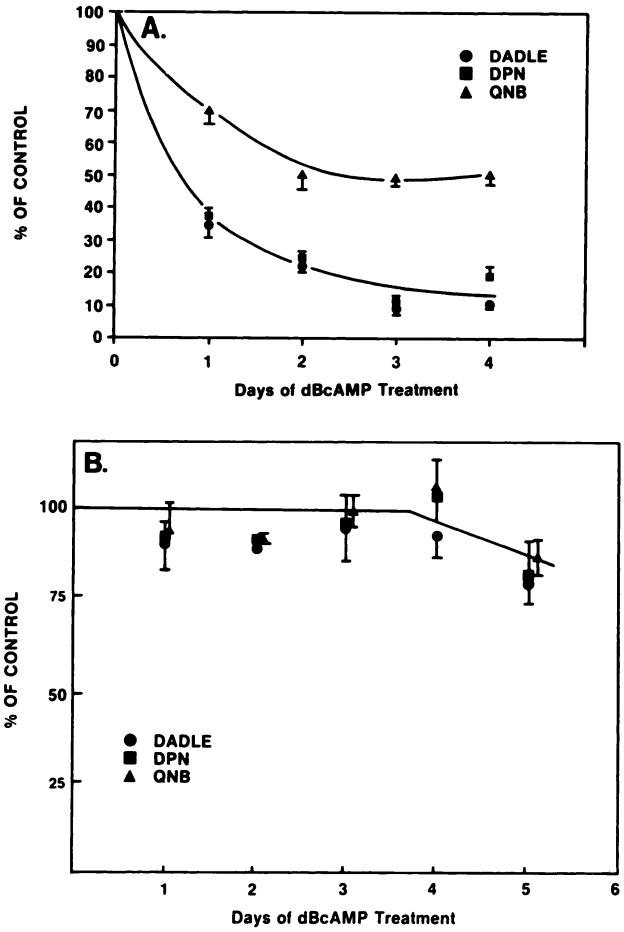

Fig. 5. Time course of the effect of dBcAMP on opiate and muscarinic receptors in NCB-20 and NG108-15 cells. Cultures of NCB-20 (A) and NG108-15 (B) cells were treated for 0–4 days with 1.0 mM dBcAMP (all cultures were 5 days old on day of assay). Membranes (0.2 mg of protein/tube) from each set of cultures were assayed for [^{3}H]DADLE (3.0 nM), [^{3}H]DPN (1.0 nM), and [^{3}H]QNB (30.0 pM) binding as described under Materials and Methods. Incubations were terminated after 90 min at 25° for opiate ligands and 37° for [^{3}H]QNB. Nonspecific binding was measured in the presence of 10 μM morphine for the opiates or 1.0 μM atropine for [^{3}H]QNB. Values represent the mean ± standard deviation of triplicate determination from two to four experiments. Untreated NCB-20 (0 day) cultures yielded specific binding values of 220 ± 10 fmol/mg of protein and 240 ± 20 fmol/mg of protein for [^{3}H]DADLE and [^{3}H]DPN, respectively. Specific [^{3}H]QNB binding in untreated cultures was 27 ± 2 fmol/mg of protein. Untreated NG108-15 (0 day) cultures yielded specific binding values of 520 ± 40 fmol/mg of protein and 560 ± 130 fmol/mg of protein for [^{3}H]DADLE and [^{3}H]DPN, respectively. Specific [^{3}H]QNB binding in control cultures was 24 ± 3 fmol/mg of protein.

NG108-15 cells contain higher cellular concentrations of Met^5- and Leu^5-enkephalin (12) than do undifferentiated cells and, more recently, have also been shown to possess increased voltage-sensitive calcium channel activity (58). Differentiation of NCB-20 cells shows many similar morphologic and biochemical changes: the cells appear rounder, are more clustered, and also emit numerous branched processes relative to undifferentiated cells; differentiated NCB-20 cells also show increased enkephalin concentrations.[2]

The parental neuroblastoma and glioma cell lines (N18TG2 and C6BU-1), when induced to differentiate, do not exhibit the highly differentiated ultrastructure observed with the hybrid

[2] K. Braas and D. C. U'Prichard, unpublished observation.

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TABLE 2

Effect of dBcAMP on opiate, muscarinic cholinergic, and α_2-adrenergic receptors on different cell lines

Cultures of NCB-20, NG108-15, N18TG2, and C6BU-1 cells were treated with dBcAMP (1.0 mM, 4 days). Membranes (0.2 mg of protein/tube) were assayed for specific binding of [^{3}H]DADLE (3.0 nM), [^{3}H]DPN (1.0 nM), [^{3}H]QNB (30.0 pM), [^{3}H]PAC (4.0 nM), and [^{3}H]RAUW (1.4 nM) as described in Materials and Methods. Nonspecific binding was measured in the presence of 10 μM morphine for opiate ligands (DADLE and DPN), 1.0 μM atropine for the muscarinic ligand QNB, and 0.1 mM (−)-norepinephrine for the α_2-adrenergic ligands (PAC and RAUW). Values represent means ± standard deviations of triplicate determinations performed between 3 and 10 times for different ligands.

Cell line	Ligand	Specific binding		
		Control	dBcAMP-treated	% Control
		fmol/mg		
NCB-20	DPN	230 ± 64	40 ± 11	17
	DADLE	204 ± 60	31 ± 5	15
	QNB	16 ± 4	8 ± 2	50
	PAC	54 ± 5	41 ± 7	76
	RAUW	88 ± 5	40 ± 2	43
NG108-15	DPN	440 ± 160	470 ± 140	106
	DADLE	420 ± 130	420 ± 108	100
	QNB	21 ± 7	23 ± 7	110
	PAC	64 ± 4	64 ± 11	100
	RAUW	68 ± 15	83 ± 19	122
N18TG2	DPN	190 ± 66	220 ± 47	118
	DADLE	65 ± 42	55 ± 18	84
	QNB	27 ± 4	17 ± 5	63
	PAC	11 ± 3	26 ± 11	250
	RAUW	17 ± 2	35 ± 3	203
C6BU-1	DPN	72 ± 26	150 ± 47	210
	DADLE	ND[a]		
	QNB	ND		
	PAC	ND		
	RAUW	ND		

[a] ND, not detectable.

cells (2), although both lines, particularly the C6BU-1 cells, emitted numerous long processes.

The observed down-regulation of opiate, muscarinic, and α_2-adrenergic receptor sites can be clearly dissociated from the differentiation process. First, the phenomenon is absent in the NG108-15 cells which have been induced to differentiate with 1.0 mM dBcAMP (Fig. 3, Table 1). In fact, treatment of NG108-15 cells with concentrations of dBcAMP 5–10-fold higher than that required to induce differentiation consistently failed to cause down-regulation of opiate, muscarinic, and α_2-adrenergic receptor sites (Fig. 4B). Prolonged treatment of NG108-15 cultures with 1.0 mM dBcAMP likewise failed to induce receptor down-regulation (Fig. 5B). Furthermore, N_i-coupled receptors on the N18TG2 parent were also not down-regulated despite their differentiated morphology (Table 2).

Second, receptors on differentiated NCB-20 cells were extensively down-regulated by dBcAMP treatment, with a time course that could be clearly dissociated from the time course of differentiation (Fig. 5A). Opiate and muscarinic receptor sites were down-regulated by 65% and 30%, respectively, within 1 day of treatment of NCB-20 cells with dBcAMP, and maximum down-regulation was achieved for both receptors after 2 days. From a morphological standpoint, however, NCB-20 cells continue to be progressively more differentiated by dBcAMP with time (1–4 days) in culture (data not shown). Furthermore, dBcAMP concentrations as low as 30 μM induced a 70% loss of opiate ligand binding after 4 days of treatment. At this low concentration of dBcAMP, NCB-20 cells do not appear highly differentiated and do not produce an increased number of neurites. These data, together, suggest that the process of differentiation in both hybrid cell lines is unrelated to heterologous down-regulation of opiate, muscarinic, and α_2-adrenergic receptor sites on NCB-20 cells.

The pharmacological characteristics of receptors remaining on dBcAMP-differentiated NCB-20 cells was examined and found to be the same as for receptors in undifferentiated cultures (Table 3). Not surprisingly, receptors on differentiated NG108-15 cells were also pharmacologically identical to undifferentiated NG108-15 cultures. The receptors on the two cell lines appear to be identical, although the muscarinic agonist carbachol has a 10-fold higher affinity in NG108-15 cells compared to NCB-20 cells.

Coupling of opiate receptors to AC was also examined in both cell lines before and after treatment with dBcAMP (Fig. 6). AC activity in membranes was stimulated 7–8-fold by PGE_1, in both cells, in control and dBcAMP-treated membranes. This would suggest that the PGE_1 receptor, an N_s-coupled receptor, is not affected by dBcAMP treatment in either NG108-15 or NCB-20 cells. PGE_1-stimulated activity was maximally inhibited by the opiate agonist DADLE (1 μM), to different degrees, 30% in NCB-20 membranes and 54% in NG108-15 membranes. dBcAMP treatment of NCB-20 cells resulted in a 2-fold decrease in this maximal level of opiate inhibition relative to untreated NCB-20 membranes. Thus, although

TABLE 3

Competition at opiate, muscarinic, and α_2-adrenergic receptor sites in control and dBcAMP-treated NCB-20 and NG108-15 cells

NCB-20 and NG108-15 cells were treated with dBcAMP (1.0 mM, 4 days) as described under Materials and Methods. Inhibition of [^{3}H]DADLE (3.0 nM), [^{3}H]DPN (1.0 nM), [^{3}H]QNB (30.0 pM), and [^{3}H]PAC (4.0 nM) specific binding was determined using six to eight concentrations of each unlabeled drug. K_i values were calculated using the Cheng-Prusoff equation (54). Results represent the means ± standard deviations of triplicate determinations from three to four experiments.

Ligand	Drug	NCB-20		NG108-15	
		Control K_i	+dBcAMP K_i	Control K_i	+dBcAMP K_i
		nM		*nM*	
[^{3}H]DPN	DADLE	0.7 ± 0.4	1.2 ± 0.7		
	Morphine	98.3 ± 34.6	128.7 ± 17.8	45.0 ± 0.5	43.5 ± 0.7
	Naloxone	80.6 ± 32.9	75.9 ± 43.7	26.0 ± 4.2	30.0 ± 1.4
[^{3}H]DADLE	DADLE	0.6 ± 0.2	0.9 ± 0.6		
	Morphine	61.5 ± 9.2	58.5 ± 17.7	54.6 ± 31.4	48.4 ± 32.9
	Naloxone	38.0 ± 4.2	34.5 ± 19.1	14.5 ± 4.9	11.5 ± 2.1
[^{3}H]QNB	Atropine	1.1 ± 0.5	0.9 ± 0.6	1.0 ± 0.5	1.2 ± 0.3
	Carbachol	3488.0 ± 443.0	3005.0 ± 305.0	225.5 ± 92.6	256.0 ± 94.8
[^{3}H]PAC	Phentolamine	22.5 ± 3.5	44.0 ± 12.7		
	Norepinephrine	23.5 ± 3.0	21.8 ± 3.1		

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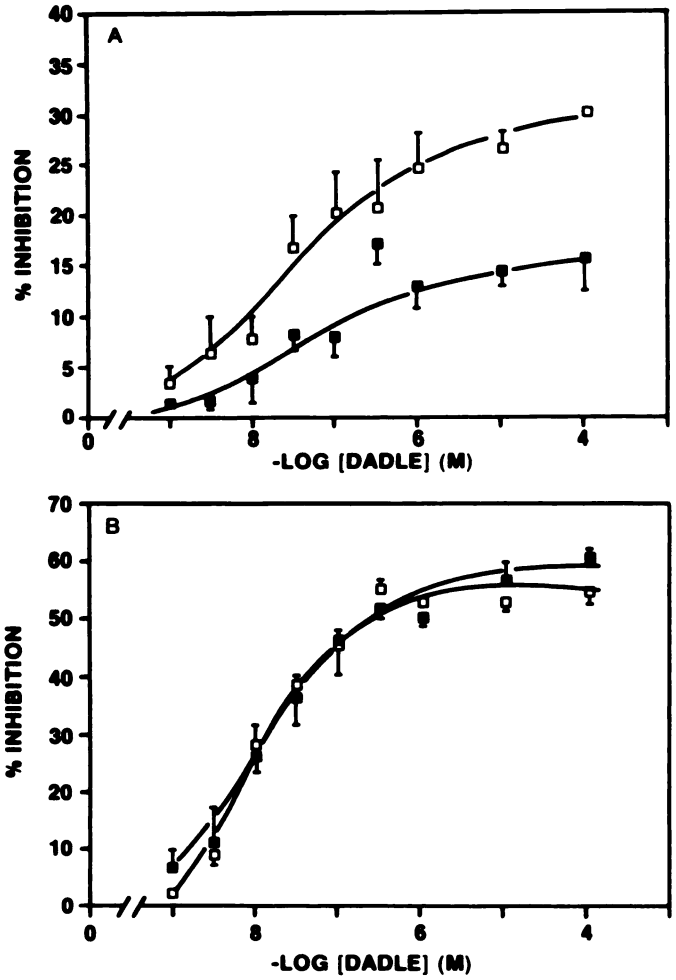

Fig. 6. Effect of dBcAMP treatment on opiate inhibition of AC activity in NCB-20 and NG108-15 cell membranes. Untreated and dBcAMP-treated NCB-20 (A) and NG108-15 (B) cell membranes (15 μg of protein/tube) were prepared and assayed for AC activity as described in Materials and Methods. Inhibition of PGE_1-stimulated (10 μM) AC activity was measured using various concentrations of DADLE (1 nM–100 μM). Basal activity was stimulated approximately 7-fold by PGE_1 in all cases. A. Basal AC activities in untreated (□) and dBcAMP-treated (■) NCB-20 membranes were 471 ± 213 and 415 ± 82 pmol of cAMP/20 min/mg of protein, respectively. IC_{50} values defined as the concentration of DADLE that yielded 50% inhibition of the stimulated activity for untreated and dBcAMP-treated cells were 29.2 nM and 54.6 nM, respectively. B. Basal AC activities in untreated (□) and dBcAMP-treated (■) NG108-15 membranes were 1031 ± 101 and 807 ± 37 pmol of cAMP/20 min/mg of protein, respectively. The IC_{50} values for DADLE inhibition of AC activity in untreated and treated membranes were 17 nM and 21 nM, respectively.

dBcAMP treatment reduced opiate receptor site density (B_{max}) by 90%, agonist-induced inhibition of AC activity was diminished by only 50% compared to undifferentiated cultures. These data suggest the presence of a large reserve of opiate receptor sites on NCB-20 cells. It is also apparent that down-regulation of NCB-20 opiate receptor sites accompanies an uncoupling or functional desensitization of the opiate receptor-AC effector system. Our data differ from the reported heterologous desensitization of the turkey erythrocyte system, where agonist or cyclic nucleotide exposure leads to a major loss of catecholamine and sodium fluoride responsiveness, perhaps due to phosphorylation of the receptor protein, but leads to no change in the number of receptors present on the cell (37, 42). In contrast, in a typical example of homologous desensitization, down-regulation of gonadotropin receptors as well as impaired AC stimulation by hormone was observed in a murine Leydig tumor cell (32).

In contrast to NCB-20 cells, maximum AC inhibition of both control and dBcAMP-treated NG108-15 cells was the same, 54% in both membrane preparations. Thus, functional desensitization was not observed, coincident with no loss of receptor-binding sites.

Since both NG108-15 and NCB-20 cells, when induced to differentiate, produce and store releasable endogenous agonist (enkephalins and acetylcholine), it might seem possible that the observed receptor down-regulation is agonist induced. This, however, is an unlikely explanation since the down-regulation is specific for NCB-20 cells, and neither cell produces the catecholamines which would down-regulate α_2-adrenergic receptors.

NG108-15 and NCB-20 cells share one parent in common, the N18TG2 neuroblastoma cell, previously shown to contain opiate receptors (55). The other parent of the NG108-15 cell, C6BU-1, derived from a rat glioma, lacked opiate receptors (59) but showed dramatic changes in morphology when induced to differentiate with dBcAMP. Since the two hybrid cells exhibited such a striking all-or-none sensitivity to dBcAMP in terms of receptor regulation, it was of interest to examine the available parents of the hybrid cultures to determine whether the differences between the hybrids were due to suppression of a possible gene complement responsible for receptor down-regulation in the NG108-15 hybrid cell, or to activation of a possible gene complement that induced receptor down-regulation in the NCB-20 cells.

Opiate, α_2-adrenergic, and muscarinic receptors were found on the N18TG2 parent common to both hybrid cells but not on the C6BU-1 glioma parent of the NG108-15 hybrid. It thus seems likely that the genes for these N_i-coupled receptors in both hybrid cells are derived from the N18TG2 parent. Opiate and α_2-adrenergic receptors on the N18TG2 cells were not down-regulated by dBcAMP treatment (Table 2), and muscarinic receptor-specific binding was only slightly reduced. [^{3}H] DPN-specific binding to C6BU-1 membranes, which may not represent an opiate receptor, was actually enhanced following treatment of cultures with dBcAMP. Thus, the "factor" which triggered receptor down-regulation in the NCB-20 cell was not present in the N18TG2 parent at all and was, therefore, in terms of its expression, contributed directly by the fetal Chinese hamster brain parent cell; alternatively, if the down-regulation "factor" was present in the N18TG2 cell, it would have to be activated by a product synthesized by the Chinese hamster brain cell parent. The latter hypothesis dictates that such activation is not functional in the C6BU-1 parent and, therefore, receptor down-regulation could not be detected in the NG108-15 hybrid cells. Had receptors on the N18TG2 parent, in fact, been susceptible to down-regulation by dBcAMP, it would have been necessary to also invoke a suppressor gene contributed by the C6BU-1 parent which would, in turn, prevent receptor down-regulation in the NG108-15 cells. The presence of such a suppressor gene may be ruled out on the basis of our data. At the present time, experiments to distinguish between the two possibilities cited for NCB-20 cells are in progress. In any event, it is clear that the signal for receptor down-regulation in NCB-20 cells must come from the Chinese hamster brain cell parent.

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The Chinese hamster brain cell is a normal, primary cell, whereas the other parental cells are transformed clonal cell lines. It is interesting to speculate that the process of transformation may, in some way, repress the signal(s) involved in receptor site regulation, resulting in a loss of normal cellular function.

The interaction of genomes in hybrid cells and the effect of this interaction on membrane receptor properties is clearly an interesting area for further research. For instance, it is evident that, although the N18TG2 parental cell is not particularly enriched in opiate, muscarinic, and α_2-adrenergic receptors, and the C6BU-1 parental cell may be absent in these receptors, the product of their fusion, namely, the NG108-15 cell, contains an abundance of these receptor types. Hybrid cultures thus provide a model system with which to study the complexities of receptor gene regulation.

References

1. Hamprecht, B. Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *Int. Rev. Cytol.* **49**:99–170 (1977).
2. Daniels, M. P., and B. Hamprecht. The ultrastructure of neuroblastoma glioma somatic cell hybrids. *J. Cell Biol.* **63**:691–699 (1974).
3. Prasad, K. N. Differentiation of neuroblastoma cells in culture. *Biol. Rev.* **50**:129–265 (1975).
4. Hamprecht, B., J. Traber, and F. Lambrecht. Dopamine beta hydroxylase activity in cholinergic neuroblastoma × glioma hybrid cells: increase of activity of *N,O'*-dibutyryl adenosine 3′:5′-cyclic monophosphate. *FEBS Lett.* **42**:221–226 (1974).
5. Hamprecht, B., W. Kemper, and T. Amano. Electrical response of glioma cells to acetylcholine. *Brain Res.* **101**:129–135 (1976).
6. Nelson, P., W. Ruffner, and M. Nirenberg. Neuronal tumor cells with excitable membranes grown *in vitro*. *Proc. Natl. Acad. Sci. USA* **64**:1004–1010 (1969).
7. Minna, J., D. Glazer, and M. Nirenberg. Genetic dissection of neuronal properties using somatic cell hybrids. *Nature New Biol.* **235**:225–231 (1972).
8. Chalazonitis, A., and L. A. Greene. Enhancement in excitability properties of mouse neuroblastoma cells cultured in the presence of dibutyryl cyclic AMP. *Brain Res.* **72**:340–345 (1974).
9. Kimhi, Y., C. Palfrey, I. Spector, Y. Barak, and U. Z. Littauer. Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. *Proc. Natl. Acad. Sci. USA* **73**:462–466 (1976).
10. Nelson, P. G., C. Christian, and M. Nirenberg. Synapse formation between clonal neuroblastoma × glioma hybrid cells and striated muscle cells. *Proc. Natl. Acad. Sci. USA* **73**:123–127 (1976).
11. Higashida, H., T. Kato, K. Kano-Tanaka, M. Okuya, and T. Tanaka. Proliferation and synapse formation of neuroblastoma × glioma hybrid cells: effect of glia maturation factor. *Brain Res.* **214**:287–299 (1981).
12. Braas, K., S. Childers, and D. C. U'Prichard. Induction of differentiation increases Met^5-enkephalin and Leu^5-enkephalin content in NG108-15 hybrid cells: an immunocytochemical and biochemical analysis. *J. Neurosci.* **3**:1713–1727 (1983).
13. MacDermot, H., H. Higashida, S. P. Matuzawa, J. Minna, and M. Nirenberg. Adenylate cyclase and acetylcholine release regulated by separate serotonin receptors of somatic cell hybrids. *Proc. Natl. Acad. Sci. USA* **76**:1135–1139 (1979).
14. Christian, C. N., P. G. Nelson, P. Bullock, D. Mullinax, and M. Nirenberg. Pharmacological responses of cells of a neuroblastoma × glioma hybrid clone and modulation of synapses between hybrid cells and mouse myotubes. *Brain Res.* **147**:261–276 (1978).
15. Klee, W. A. and M. Nirenberg. A neuroblastoma × glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. USA* **71**:3474–3477 (1974).
16. Sharma, S. K., M. Nirenberg and W. A. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* **72**:590–594 (1975).
17. Chang, K. J., R. J. Miller, and P. Cuatrecasas. Interaction of enkephalin with opiate receptors in intact cultured cells. *Mol. Pharmacol.* **14**:961–970 (1978).
18. Traber, J., K. Fischer, S. Latzin, and B. Hamprecht. Morphine antagonizes action of prostaglandin in neuroblastoma × glioma hybrid cells. *Nature (Lond.)* **253**:120–122 (1975).
19. Traber, J., R. Gullis, and B. Hamprecht. Influence of opiates on the levels of adenosine 3′,5′-cyclic monophosphate in neuroblastoma × glioma hybrid cells. *Life Sci.* **16**:1863–1868 (1975).
20. Brandt, M., R. J. Gullis, K. Fischer, C. Buchen, and B. Hamprecht. Enkephalin regulates the levels of cyclic nucleotides in neuroblastoma × glioma hybrid cells. *Nature (Lond.)* **262**:311–313 (1976).
21. Nathanson, N. M., W. L. Klein, and M. Nirenberg. Regulation of adenylate cyclase activity mediated by muscarinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **75**:1788–1791 (1978).
22. Sabol, S., and M. Nirenberg. Regulation of adenylate cyclase of neuroblastoma and glioma hybrid cells by alpha adrenergic receptors. I. Inhibition of adenylate cyclase mediated by alpha receptors. *J. Biol. Chem.* **254**:1913–1920 (1979).
23. Kahn, D. J., J. C. Mitrius, and D. C. U'Prichard. Alpha$_2$-adrenergic receptors in neuroblastoma × glioma hybrid cells. Characterization with agonist and antagonist radioligands and relationship to adenylate cyclase. *Mol. Pharmacol.* **21**:17–26 (1982).
24. Sharma, S. K., W. A. Klee, and M. Nirenberg. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA* **72**:3092–3096 (1975).
25. Sharma, S. K., W. A. Klee, and M. Nirenberg. Opiate-dependent modulation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **74**:3365–3369 (1977).
26. Klee, W. A., and R. A. Streaty. Opioid-dependent dual regulation of adenylate cyclase in a cell-free system. *Adv. Cyclic Nucleotide Res.* **14**:629–635 (1981).
27. Sabol, S., and M. Nirenberg. Regulation of adenylate cyclase of neuroblastoma × glioma hybrid cells by alpha adrenergic receptors. II. Long-lived increase of adenylate cyclase activity mediated by alpha receptors. *J. Biol. Chem.* **254**:1921–1926 (1979).
28. Law, P. Y., D. S. Horn, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment. *Mol. Pharmacol.* **22**:1–4 (1982).
29. Law, P. Y., D. S. Horn, and H. H. Loh. Down-regulation of opiate receptor in neuroblastoma × glioma NG108-15 hybrid cells. *J. Biol. Chem.* **259**:4096–4104 (1984).
30. Green, D. A., and R. B. Clark. Specific muscarinic-cholinergic desensitization in the neuroblastoma-glioma hybrid NG108-15. *J. Neurochem.* **39**:1125–1131 (1982).
31. McLawhon, R. W., R. E. West, R. J. Miller, and G. Dawson. Distinct high-affinity binding sites for benzomorphan drugs and enkephalin in a neuroblastoma-brain hybrid cell line. *Proc. Natl. Acad. Sci. USA* **78**:4309–4313 (1981).
32. Rebois, R. V., and P. H. Fishman. Down-regulation of gonadotropin receptors in a murine Leydig tumor cell line. *J. Biol. Chem.* **259**:3096–3100 (1983).
33. Harden, T. K. Agonist-induced desensitization of the beta-adrenergic receptor-linked adenylate cyclase. *Physiol. Rev.* **35**:5–32 (1983).
34. Lefkowitz, R. J., M. R. Wessels, and J. M. Stadel. Hormones, receptors and cyclic AMP: their role in target cell refractoriness. *Curr. Top. Cell. Regul.* **17**:205–228 (1980).
35. Perkins, J. P. Catecholamine-induced modificatin of the functional state of beta-adrenergic receptors. *Trends Pharmacol. Sci.* **2**:326–328 (1981).
36. Kassis, S. K., and P. H. Fishman. Functional alteration of the β-adrenergic receptor during desensitization of mammalian adenylate cyclase by β-agonists. *Proc. Natl. Acad. Sci. USA* **81**:6686–6690 (1984).
37. Lefkowitz, R. J., J. M. Stadel, and M. G. Caron. Adenylate cyclase-coupled β-adrenergic receptors: structures and mechanism of activation and desensitization. *Annu. Rev. Biochem.* **52**:159–186 (1983).
38. Hoyer, D., E. E. Reynolds, and P. B. Molinoff. Agonist-induced changes in the properties of β-adrenergic receptors on intact S49 lymphoma cells: time-dependent changes in the affinity of the receptor for agonists. *Mol. Pharmacol.* **25**:209–218 (1984).
39. Triggle, D. J. Desensitization. *Trends Pharmacol. Sci.* **1**:395–398 (1980).
40. Breant, B. S., S. Keppens, and H. deWulf. Heterologous desensitization of the cyclic AMP-independent glycogenolytic response in rat liver cells. *Biochem. J.* **200**:509–514 (1981).
41. Perkins, J. P. Desensitization of the response of adenylate cyclase to catecholamines. *Curr. Top. Membr. Transp.* **18**:85–108 (1983).
42. Strasser, R. H., R. A. Cerione, J. Codina, M. G. Caron, and R. J. Lefkowitz. Homologous desensitization of the β-adrenergic receptor. *Mol. Pharmacol.* **28**:237–245 (1985).
43. Harden, T. K., C. U. Cotton, G. L. Waldo, J. K. Lutton, and J. P. Perkins. Catecholamine-induced alteration in sedimentation behavior of membrane bound β-adrenergic receptors. *Science (Wash. D. C.)* **210**:441–443 (1980).
44. Chuang, D. M., W. J. Kinnier, L. Faber, and E. Costa. A biochemical study of receptor internalization during β-adrenergic receptor desensitization in frog erythrocytes. *Mol. Pharmacol.* **18**:348–355 (1980).
45. Strulovici, B., R. A. Cerione, B. F. Kilpatrick, M. G. Caron, and R. J. Lefkowitz. Direct demonstration of impaired functionality of a purified desensitized β-adrenergic receptor in a reconstituted system. *Science (Wash. D. C.)* **225**:837–840 (1984).
46. Stadel, J. M., P. Nambi, R. G. L. Shorr, D. F. Sawyer, M. G. Caron, and R. J. Lefkowitz. Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **80**:3173–3177 (1983).
47. Sibley, D. R., R. H. Strasser, M. G. Caron, and R. J. Lefkowitz. Homologous desensitization of adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *J. Biol. Chem.* **260**:3883–3886 (1985).
48. Noronha-Blob, L., D. C. U'Prichard, W. J. Kinnier, and M. Williams. Down-regulation of N_i-coupled receptors by dBcAMP in cultured neural hybrid NCB-20 cells: possible cell specificity. *Pharmacologist* **26**:164 (1984).
49. Blume, A. J., D. Lichtenstein, and G. Boone. Coupling of opiate receptors to

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adenylate cyclase: requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. USA* **76**:5626–5630 (1979).
50. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
51. Luthin, G. R., and B. B. Wolfe. Comparison of [^{3}H]pirenzepine and [^{3}H] quinuclidinylbenzilate binding to muscarinic cholinergic receptors in rat brain. *J. Pharmacol. Exp. Ther.* **228**:648–655 (1984).
52. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–55 (1979).
53. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
54. Cheng, Y. C., and W. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
55. Law, P. Y., J. Koehler, and H. H. Loh. Comparison of opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 and neuroblastoma × glioma NG108-15 hybrid cell lines. *Mol. Pharmacol.* **21**:483–491 (1981).
56. Feigenbaum, P., and E. El-Fakahany. Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist: possible involvement in desensitization of receptor function. *J. Pharmacol. Exp. Ther.* **233**:134–139 (1985).
57. Mitrius, J. C., and D. C. U'Prichard. Regulation of α_2-adrenoceptors by nucleotides, ions and agonists: comparison in cells of neural and non-neural origin. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:57–73 (1985).
58. Richard, C. L., D. C. U'Prichard, and L. Noronha-Blob. Voltage sensitive calcium channels in cultured neuronal hybrid cells. *Fed. Proc.* **45**:513, abstr. 2089 (1986).
59. Miller, R., K. Chang, J. Leighton, and P. Cuatrecasas. Interaction of iodinated enkephalin analogues with opiate receptors. *Life Sci* **22**:379–388 (1978).

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