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Heterologous Up-Regulation of N_i-Coupled Receptors in Cultured Neural Hybrid Cells by a Transferable Factor, Whose Expression Is Inhibited in a Cyclic AMP-Dependent, Cell-Specific Manner

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SUMMARY

Opiate, muscarinic, and α_2-adrenergic receptors on NCB-20 and NG108-15 neuroblastoma hybrid cells were up-regulated by treatment of the cells with media (CM) conditioned by previous incubation with either cell type. NG cells treated with CM from both NCB and NG cells (NCB-CM or NG-CM) showed a 2-fold increase in opiate receptor density relative to untreated cells, with no change in ligand affinities. Opiate receptor density on NG cells was also enhanced ~2-fold by CM derived from dibutyryl cyclic AMP (dBc)-treated NG cells (NG-dBc-CM) but not by CM from dBc-treated NCB cells, (NCB-dBc-CM). The data suggest that a transferable factor that up-regulates NG opiate receptors is produced by untreated NCB and NG cells, and is either suppressed or inactivated in dBc-treated NCB cells but not in dBc-treated NG cells. Muscarinic and α_2-adrenergic receptor site densities on NG cells were also up-regulated ~2-fold by NCB-CM but not by NCB-dBc-CM. Thus, the factor induced a heterologous up-regulation of three classes of N_i-coupled receptor sites on NG cells. The up-regulating factor, which accumulates in the media with time in culture, also acts directly upon cells that are synthesizing/secreting the factor (auto-up-regulation). Thus, opiate receptor density increased in untreated NCB and NG cells, as well as in dBc-treated NG cells, as a function of cell growth, but did not increase on dBc-treated NCB cells. Coupling of NG opiate receptors to adenylate cyclase (AC) was not altered by CM. Prostaglandin E_1-stimulated AC was maximally inhibited by (~40%) by 1 μM DADLE with the same efficiency and potency in untreated as in CM-treated NG cell membranes. Furthermore, NCB-dBc-CM which does not induce NG opiate receptor up-regulation and NCB-CM, which does induce it, had no effect on inhibition of AC by DADLE. The up-regulating factor is a relatively small molecule (molecular weight = 3000–6000), whose synthesis and/or secretion is suppressed by dBc in NCB but not in the related NG hybrid. This unique cell specificity may be exploited to study the mechanism of opiate, muscarinic, and α_2-adrenergic receptor expression and turnover in cultured neural hybrid cells.

Previously, we showed 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 NCB-20 (NCB) hybrid cell (neuroblastoma N18TG2 × Chinese hamster brain glial cell), were down-regulated by treatment of NCB cells with dBc (1). In all cases, receptor site density but not ligand affinities for each receptor were reduced. In contrast, the same three receptors on the closely related hybrid cell, NG108-15 (NG; neuroblastoma N18TG2 × glioma C6BU-1), were unaffected by dBc treatment. Thus, a striking cell-specific difference in receptor regulation was observed between these two related hybrids in response to dBc. Furthermore, opiate, α_2-adrenergic, and muscarinic receptors on the N18TG2 parent, which is common to both hybrid cells, were not down-regulated by dBc. Thus, the "factor" which triggered receptor down-regulation in the NCB cell was either not present in the shared N18TG2 parent at all, or, alternatively, if the "factor" was present in the N18TG2 cell, it would have to be regulated by a gene product from the other parent of the NCB hybrid, i.e., the Chinese hamster brain cell parent.

This study was undertaken to determine if the NG cell receptors shown previously to be resistant to direct dBc exposure could be down-regulated by transfer of the putative down-regulation factor excreted by dBc-treated NCB cells. NG cells were therefore grown in CM derived from untreated and dBc-treated NCB cells. We found that, not only were opiate receptors on NG cells *not* down-regulated by any factor, but they were in fact *up-regulated* by CM from untreated NCB cells. Furthermore, muscarinic and α_2-adrenergic receptor densities were also up-regulated (~2-fold) on NG cells. In contrast, CM

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ABBREVIATIONS: AC, adenylate cyclase; dBc, N^6, 2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; CM, conditioned medium; [^{3}H]DADLE, [^{3}H](D-Ala^2, D-Leu^5)enkephalin; [^{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; MWCO, molecular weight cut-off; N_i, inhibitory guanine nucleotide-binding regulatory protein; N_s, stimulatory guanine nucleotide-binding protein.

from dBc-treated NCB cells failed to up-regulate all three receptor classes on NG cells. Thus, it appeared that an up-regulating factor, which is produced and excreted into the media by untreated NCB cells, is suppressed or inactivated in dBc-treated NCB cells. This up-regulating factor is also produced by NG cells but, unlike NCB cells, dBc treatment does not suppress its synthesis/secretion. The up-regulating factor is also active on NCB cell opiate receptors. This unique cell specificity of the expression of the up-regulating factor is investigated in this paper. A preliminary communication of some of these findings has been previously published (2).

Materials and Methods

Cell growth and differentiation. NG108-15 and NCB-20 neuroblastoma cell hybrids, obtained at passage 13–15 from Drs. M. Nirenberg (National Institutes of Health, Bethesda, MD) and M. Rasenick (University of Illinois), respectively, were grown as previously described (1, 3). Briefly, cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1.0 mM glutamine, 100 μM hypoxanthine, 10 μM aminopterin, 20 μM thymidine, and antibiotics in a humidified atmosphere of 10% CO_2 in air at 37°. Stock cultures were subcultured in 150-cm^2 flasks (Corning Medical, Medfield, MA) every 5 days and the medium was changed every 2–3 days. Cells between passages 17 and 30 were routinely used.

dBc (1.0 mM) treatments were performed on 1-day-old culture (8×10^5 cells/dish) and continued for 4 days. Since dBc inhibits NCB and NG cell growth by 40–50% (1), twice the number of dBc-treated dishes were employed compared to untreated cultures.

Preparation of CM and treatment of cultures with CM. CM was obtained from 5-day-old cultures of NG or NCB cells. Initially, about 8×10^5 cells/dish were seeded in growth medium, swirled, and then left undisturbed to ensure uniform cell distribution. After 24 hr, dBc (1.0 mM) was added to one set of dishes, while parallel cultures received an equivalent volume of media. After another 4 days, media from untreated or dBc-treated cultures were removed under sterile conditions, pooled, centrifuged ($2000 \times g$, 5 min) to remove cell debris, and stored at 4° until used. Except where indicated, CM was routinely used within 8 hr of preparation. CM derived from untreated and dBc-treated NCB cells are designated NCB-CM and NCB-dBc-CM, respectively. Similarly, CM obtained from NG cells are designated NG-CM and NG-dBc-CM, respectively.

Cultures were exposed to CM using the following protocol. NG or NCB cells were seeded in DMEM (8×10^5 cells/dish). After 24 hr, medium was removed, and monolayers were overlayed with 15 ml of CM or dBc-CM. Parallel cultures were grown in 15 ml of regular growth medium without CM, in the absence or presence of 1.0 mM dBc. Fresh medium (2.0 ml) was added to all cultures on each of 2 subsequent days. After 3 days of the above treatments, membranes were prepared for opiate, muscarinic, and α_2-adrenergic receptor binding or for measurement of AC activity (1). Because cell growth in CM-treated cultures is inhibited (~50%), the number of dishes used per experiment were increased proportionately.

Preparation of membranes. Briefly, untreated, dBc-treated, or CM-treated cultures were homogenized in 50 mM Tris-HCl buffer, (pH 7.4, 0°), using a Brinkmann Polytron PT 10/35, and centrifuged three times (10 min at $46,000 \times g$) with intermediate resuspension in the same buffer. For AC assays (4), cells were homogenized in buffer containing 5.0 mM Tris-HCl (pH 7.4), 0.32 M sucrose, and 1.0 mM $MgCl_2$, and centrifuged ($600 \times g$) to remove nuclei and unbroken cells. The supernatant was centrifuged (10 min, $40,000 \times g$) to obtain a particulate membrane fraction, adjusted to a protein concentration of approximately 3 mg/ml, and stored frozen in liquid nitrogen. Protein concentrations were determined by the Bio-Rad method (5); cell titers were calculated with a hemocytometer.

Receptor binding assays. Binding of the opiate ligands, [^{3}H]DADLE and [^{3}H]DPN, of the muscarinic cholinergic antagonist, [^{3}H]QNB, and of the α_2-adrenergic ligands, [^{3}H]PAC and [^{3}H]RAUW, was determined as previously described (1). In saturation experiments, the ranges of ligand concentrations used were as follows: [^{3}H]DADLE, 0.05–3.0 nM; [^{3}H]DPN, 0.03–5 nM; [^{3}H]QNB, 1.0–50 pM; [^{3}H]PAC, 0.05–3 nM; and [^{3}H]RAUW, 0.03–6 nM. For all ligands, except [^{3}H]QNB (2.5 ml), an assay volume of 0.5 ml was used. Opiate and muscarinic ligands were incubated in 50 mM Tris (pH 7.4) for 90 min at 24° and 37°, respectively, while α_2-adrenergic binding in 50 mM Tris was carried out at 24° for 40 min. Nonspecific binding was determined in the presence of 10 μM morphine sulfate (opiate), 1 μM atropine sulfate (muscarinic), and 0.1 mM norepinephrine (α_2-adrenergic). After incubation, assay contents were filtered under vacuum over Whatman GF/B filters using a Brandel modified cell harvester, rinsed three times with 4.0 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°), and counted by liquid scintillation spectrometry at 40% counter efficiency. Samples were assayed in triplicate with triplicate values within each experiment varying by <10%.

Adenylate Cyclase. AC activity was assayed as described by Solomon (6) 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 included in the assay, and all reactions were initiated with ~15 μg of membrane protein. Reactions were terminated after 20 min at 37° 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 (7). Estimates of K_D and B_{max} were obtained using unweighted linear regression analysis of the transformed data. Values are expressed as means ± standard deviations for individual K_D and B_{max} values from several experiments. 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 CM treatments were identical when the data were expressed either way.

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

Drugs and reagents. Culture media and supplements were purchased from Whitaker M. A. Bioproducts (Walkersville, MD), morphine sulfate from Eli Lilly (Indianapolis, IN), unlabeled DADLE from Pennisula Laboratories (Belmont, CA), and Biorad protein assay kits from Bio-Rad (Rockville Center, NY). ATP free of GTP contamination and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Up-regulation of opiate receptors on NG cells. Previous studies showed that opiate receptors on differentiated NCB cells, but not on differentiated NG cells, were down-regulated by dBc treatment (4 days, 1.0 mM) (1). Opiate receptor site densities (B_{max}), but not ligand affinities (K_D), were reduced by 90% in dBc-treated NCB cells, suggesting that dBc may trigger a signal to down-regulate opiate receptors on these cells. We postulated that if the mechanism of opiate receptor site down-regulation involved a soluble stable factor that was extruded into the growth medium by NCB cells, it may be possible to induce down-regulation on NG cells using NCB-CM as the culture medium. One-day-old NG cells were therefore exposed for 3 days to CM from untreated or dBc-treated NCB cells, while parallel cultures were grown without CM, and a fourth subset was treated with dBcAMP (1.0 mM) directly.

Saturation isotherms of [^{3}H]DADLE specific binding to

membranes prepared from each set of cultures showed that the densities of opiate receptor sites in the absence of CM on untreated and dBc-treated NG cells were not significantly different, as previously found (Fig. 1A, Table 1). In contrast, NG cultures treated with NCB-CM showed a 2-fold increase in opiate receptor site density relative to cultures grown without CM (Fig. 1A, Table 1). However, NG cultures grown in CM from dBc-treated NCB cells showed no up-regulation of opiate receptor sites (Fig. 1A). Ligand affinities were unchanged under all four cell growth conditions (Table 1). These data indicate the presence of a factor synthesized by NCB cells which is responsible for up-regulation of opiate receptor sites on NG cells. dBc may suppress the synthesis and/or extrusion of this putative up-regulating factor in NCB cells. Furthermore, the signal responsible for the previously observed dBc-induced down-regulation of opiate receptor sites on NCB cells (1) could not be transferred to NG cells via a soluble factor.

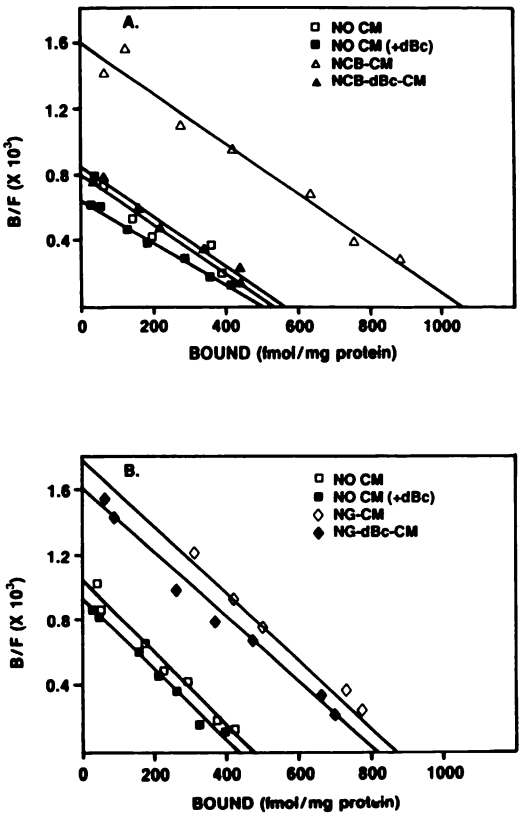

Fig. 1. Up-regulation of opiate receptor sites on NG108-15 cells. One-day-old cultures of NG cells were exposed (3 days) to CM from untreated NCB (△) or dBc-treated NCB (▲) cells (A), and to untreated NG (◇) or dBc-treated NG (◆) cells (B). Parallel cultures were grown in normal media (without CM) in the absence (□) or presence of dBc (1.0 mM) (■). Binding of [^{3}H]DADLE (0.05–3.0 nM) to membranes (~0.2 mg of protein/tube) prepared from each set of cultures was measured at 25° for 90 min. 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 five times this experiment was performed.

Experiments were next carried out to determine if opiate receptor sites on NG cells could also be up-regulated by CM derived from NG, as they were by NCB-CM. NG cultures were grown (3 days) in CM obtained from untreated or dBc-treated NG cultures, while parallel cultures were grown in regular growth media in the absence or presence of dBc. Again, opiate receptor site density on NG cells was increased about 2-fold by CM, relative to cells grown without CM (Fig. 1B, Table 1). Similar results, i.e., an approximate 2-fold up-regulation of opiate receptor site density, were obtained with NG cells grown in NG-dBc-CM (Fig. 1B). Similar results were also obtained with the opiate antagonist [^{3}H]DPN (Table 2). Again, opiate receptor site density increased (2-fold) on NG cells when grown in NCB-CM but not in CM from dBc-treated NCB; and, as previously reported (1), NG cells treated directly with dBc showed no difference in opiate receptor density compared to untreated cultures (Table 2). Once again, ligand affinities of [^{3}H]DADLE and [^{3}H]DPN were not significantly altered, regardless of the conditions under which NG cells were cultured (Tables 1 and 2). The similarity of the observations noted with the agonist and antagonist opiate ligands confirms the likelihood that the binding site changes reflect true changes in opiate receptor density.

These data, together, suggest that the up-regulating factor postulated above, which is responsible for the increase in opiate receptor density on NG cells, is present in CM obtained from either NCB or NG cells. dBc suppresses in NCB, but not in NG cells, the synthesis and/or extrusion of this putative up-regulating factor into the media. An alternative possibility is that dBc directly antagonizes the action(s) of the up-regulating factor at the receptor level rather than affect its synthesis and/

TABLE 1

Effect of CM on [^{3}H]DADLE binding to NCB-20 and NG108-15 membranes

NG and NCB cells were treated with CM from untreated (control) or dBc-treated NCB or NG cells. Other cultures were grown in normal media (no CM) with and without dBc (1.0 mM). After 3 days, membranes from each set of cultures (0.2 mg of protein/tube) were assayed for [^{3}H]DADLE specific binding (0.05–3.0 nM). Nonspecific binding was measured in the presence of 10 μM morphine. Values represent mean ± standard deviation of 2–4 experiments (on NCB cells) and 5–11 experiments (on NG cells), each assayed in triplicate.

Source of CM	NG108-15			NCB-20		
	K_D	B_{max}		K_D	B_{max}	
		fmol/mg Protein	fmol/10^6 Cells		fmol/mg Protein	fmol/10^6 Cells
	nM			nM		
No CM (control)	0.56 ± 0.1	570 ± 150	90 ± 19	0.54 ± 0.07	210 ± 53	20 ± 4.4
No CM (+dBc)	0.64 ± 0.2	650 ± 180	91 ± 15	0.61 ± 0.08	37 ± 6[a]	4.2 ± 0.42[a]
NCB-CM (control)	0.59 ± 0.06	1200 ± 270[b]	190 ± 54[b]	0.47 ± 0.07	310 ± 140[b]	30 ± 5.4[b]
NCB-dBc-CM	0.59 ± 0.06	640 ± 140[a]	110 ± 19[a]	0.65 ± 0.22	47 ± 10[a]	8.0 ± 3.4[a]
NG-CM (control)	0.57 ± 0.09	1100 ± 220[b]	150 ± 16[b]	0.39 ± 0.07	420 ± 25[b]	43 ± 11[b]
NG-dBc-CM	0.74 ± 0.09	990 ± 240	160 ± 25	0.75 ± 0.23	95 ± 27[a]	13 ± 3[a]

[a] B_{max} values are statistically significant ($p < 0.001$) from respective controls.
[b] B_{max} values are statistically significant from no CM (control) values.

TABLE 2

Up-regulation of opiate, muscarinic, and α_2-adrenergic receptors on NG108-15 cells

NG cells were treated with CM from untreated or dBc-treated NCB cells (NCB-CM, NCB-dBc-CM). Parallel NG cultures were grown in normal media without CM in the absence or presence of dBc (1.0 mM) for 3 days. Membranes (0.2 mg of protein/tube) from each set of cultures were assayed for specific binding of [^{3}H]DPN (0.1–5 nM), [^{3}H]QNB (2.0–60 pM), [^{3}H]PAC (0.2–6 nM), and [^{3}H]RAUW (0.2–6 nM) as described under Materials and Methods. Nonspecific binding was measured in the presence of 10 μM morphine for the opiate ligand (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 three to five experiments each assayed in triplicate.

Source of CM	[^{3}H]DPN			[^{3}H]QNB		
	K_D	B_{max}		K_D	B_{max}	
		fmol/mg Protein	fmol/10^6 Cells		fmol/mg Protein	fmol/10^6 Cells
	nM			nM		
No CM (control)	0.48 ± 0.2	610 ± 65	110 ± 8	0.03 ± 0.02	45 ± 9	8 ± 0.1
No CM (+dBc)	0.32 ± 0.1	530 ± 31	120 ± 7	0.03 ± 0.01	45 ± 10	7 ± 0.1
NCB-CM	0.49 ± 0.2	1200 ± 160[a]	220 ± 49[a]	0.05 ± 0.02	87 ± 11[a]	14 ± 0.5[a]
NCB-dBc-CM	0.53 ± 0.2	640 ± 170	120 ± 22	0.04 ± 0.01	41 ± 9	8 ± 0.7
Source of media	[^{3}H]PAC			[^{3}H]RAUW		
	K_D	B_{max}		K_D	B_{max}	
		fmol/mg Protein	fmol/10^6 Cells		fmol/mg Protein	fmol/10^6 cells
	nM			nM		
No CM (Control)	1.8 ± 0.9	71 ± 15	17 ± 8	1.5 ± 0.1	250 ± 42	44 ± 10
No CM (+dBc)	1.7 ± 0.5	79 ± 17	18 ± 6	2.0 ± 0.4	230 ± 30	46 ± 5
NCB-CM	2.1 ± 0.9	150 ± 43[a]	33 ± 18[a]	1.6 ± 0.1	490 ± 120[a]	87 ± 27[a]
NCB-dBc-CM	2.6 ± 1.1	83 ± 28	21 ± 12	1.4 ± 0.2	230 ± 6	48 ± 3

[a] B_{max} values are significantly different from control at: $p < 0.01$ ([^{3}H]DPN, [^{3}H]QNB), $p < 0.02$ ([^{3}H]RAUW), and $p < 0.05$ ([^{3}H]PAC).

or secretion. To rule out this possibility, 1.0 mM dBc was added to previously prepared NCB-CM, and NG cells were grown as before in CM or in CM + dBc. A 2-fold up-regulation of opiate receptor density was observed in both cases relative to cultures not exposed to CM (data not shown), indicating that direct antagonism by dBc at the receptor level is not the explanation for the absence of opiate receptor up-regulation by NCB-dBc-CM.

Up-regulation of opiate receptors on NCB-20 cells. Experiments were carried out next to determine if, like NG cells, opiate receptors on NCB cells could also be up-regulated by factor(s) present in CM. This was of particular interest, since a cell-specific difference in opiate, muscarinic, and α_2-adrenergic receptor down-regulation had been previously observed (1). CM was prepared from untreated and dBc-treated NG cells, as well as from untreated and dBc-treated NCB cells. Four sets of NCB cultures were grown in the above CM (NG-CM, NG-dBc-CM, NCB-CM, and NCB-dBc-CM). Parallel NCB cultures were grown without CM or treated directly with dBc. After 3 days, ligand saturation binding isotherms were obtained for the six sets of NCB cultures exposed to the various media. As with NG cells, NCB opiate receptor site density was increased by CM derived either from NCB or NG cells (Table 1). As expected (1), direct treatment of NCB cells with dBc reduced opiate receptor sites by 90% (Table 1). Not surprisingly, therefore, NCB cells grown in CM obtained from dBc-treated NCB or NG cells showed no up-regulation and, in fact, opiate receptors were down-regulated, presumably due to the presence of residual dBc in CM.

Data from several [^{3}H]DADLE binding experiments, expressed on the basis of fmol/mg of protein as well as fmol/10^6 cells, are summarized in Table 1. As previously reported, NG cells possessed about a 3-fold higher density of opiate receptor sites compared to NCB cells, whereas the affinity of [^{3}H]DADLE to both cell lines was identical (Ref. 1; Table 1). NG cells grown in CM from either cell type (NCB or NG) showed opiate receptor site up-regulation. In contrast, up-regulation depended on the cell source if CM from dBc-treated cultures were used. Thus, opiate receptor site up-regulation was observed if NG cells were cultured in NG-dBc-CM but not in NCB-dBc-CM, and these results were the same when expressed on a fmol/cell basis (Table 1).

Opiate receptor density on NCB cells was also enhanced by CM from either cell type. However, CM derived from dBc-treated cultures produced results that were not as clear because, unlike NG cells, opiate receptors on NCB cells are down-regulated by dBc directly (1). Therefore, not surprisingly, CM from dBc-treated NCB and NG cells did not up-regulate NCB opiate receptors but, in fact, down-regulated them (Table 1). Interestingly, opiate receptor site density on NCB cells grown in NG-dBc-CM is 2-fold higher (B_{max} = 95 ± 27 fmol/mg of protein) compared to cells grown in NCB-dBc-CM (B_{max} = 47 ± 10 fmol/mg of protein) or to direct dBc treatment of NCB cells (Table 1). This finding is consistent with our previous findings (1), and with the hypothesis that the up-regulating factor is suppressed by dBc in NCB cells only. Thus, a partial up-regulation stimulus is observed when NCB cells are grown in NG-dBc-CM, countering the direct effects of dBc itself on NCB cells. In contrast, there is no up-regulation stimulus when NCB cells are grown in NCB-dBc-CM, presumably because the up-regulating factor is either absent or nonfunctional in NCB-dBc-CM.

Development of opiate receptors during cell growth. The studies described above showed that opiate receptors on either NCB or NG cells were up-regulated by CM derived from each respective cell (auto-up-regulation). Thus far, only CM from 5-day-old cultures had been employed. We reasoned that if a putative up-regulating factor was being accumulated in the media during that time period, the factor might also act directly upon cells that were secreting the factor, and cause an increase in opiate receptor density as a function of cell growth. To test this, NCB cells were grown in the presence or absence of dBc

(1.0 mM), for 1–5 days without a media change (Fig. 2A). Saturation binding isotherms of [^{3}H]DADLE specific binding to NCB membranes prepared on each day of culture showed, in the absence of dBc, a 3-fold increase in opiate receptor site density over a period of 1–7 days in culture (Fig. 2A). In contrast, dBc-treated NCB cells showed a 75% decline in the density of opiate receptors from 1 to 7 days in culture (Fig. 2A).

NG cells examined in the same manner yielded different results. Opiate receptor site density was enhanced from 2 to 7 days in culture nearly 5-fold, compared to a 3-fold increase in NCB cultures over the same time (Fig. 2B). However, unlike NCB cells, dBc-treated NG cells showed the same rate of increase of opiate receptors as untreated NG cells (Fig. 2B). The *insets* in Fig. 2 show growth curves of untreated and dBc-treated NCB and NG cells. dBc reduces cell growth in both cases by approximately 50% (1); therefore, changes in cell growth rate do not account for lack of development of opiate receptors in dBc-treated NCB cells. Thus, opiate receptors were auto-up-regulated in untreated NCB cells and in both untreated and dBc-treated NG cultures during growth, by a factor that was apparently stable at 37° since it could be transferred via

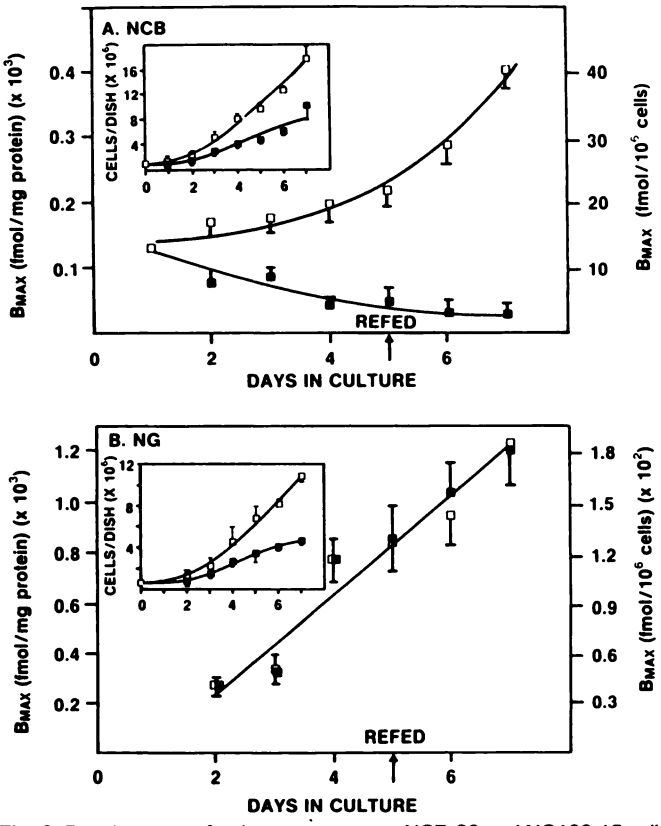

Fig. 2. Development of opiate receptors on NCB-20 and NG108-15 cells during cell growth. NCB (A) or NG (B) cells seeded at an initial cell density of 5 × 10^5 cells/dish were grown in the absence (□) or presence (■) of 1.0 mM dBc which was added 24 hr after seeding. All cultures were refed on day 5 with media ± dBc. Specific binding of [^{3}H]DADLE (0.05–3.0 nM) to NCB and NG cell membranes (0.2 mg of protein/tube) was measured on each day of culture. Incubations were done at 25° for 90 min. Nonspecific binding was measured in the presence of 10 μM morphine. Values represent means ± standard deviations of triplicate determinations from three experiments. *Insets:* Growth curves of NCB (A) and NG (B) cells in the presence (□) and absence (■) of dBc (1.0 mM). Cell counts were determined with a hemocytometer. Values represent means ± standard deviations.

CM to other cultures where it was capable of inducing further receptor up-regulation. If dBc was present during growth of NCB cultures, however, auto-up-regulation was prevented and down-regulation occurred over time. Whether the down-regulation of opiate receptors observed in dBc-treated NCB cells (Ref. 1, Fig. 2A) is due to the absence of up-regulation or to the presence of a different down-regulation factor is unclear from these experiments.

The K_D values of [^{3}H]DADLE specific binding to NCB and NG cell membranes were unaltered in 2- to 7-day-old cultures (data not shown); therefore, auto-up-regulation of opiate receptors on both cell lines is due only to changes in receptor density and not ligand affinity.

Up-regulation of muscarinic and α_2-adrenergic receptors in NG cells. Experiments were next performed to determine if other receptors present on NG cells could also be up-regulated by the factor present in CM. One-day-old NG cultures were overlayed with CM from untreated or dBc-treated NCB cells as before, and parallel cultures were grown without CM. After 3 days, isotherms of [^{3}H]QNB binding revealed that the density of muscarinic cholinergic receptors on NG cells was increased 2-fold when they were grown in CM from untreated but not dBc-treated NCB cells (Fig. 3A, Table 2). α_2-Adrenergic receptor density on NG cell membranes was also up-regulated 2-fold by NCB-CM but not by NCB-dBc-CM (Fig. 3, B and C; Table 2), as determined by [^{3}H]PAC (agonist) and [^{3}H]RAUW (antagonist) binding, and this increase was evident for both receptors when data were expressed as fmol/10^6 cells (Table 2). Once again, as with the opiate ligands, no change in affinities of [^{3}H]QNB, [^{3}H]PAC, and [^{3}H]RAUW for muscarinic and α_2-adrenergic receptors on NG cells was observed under any growth condition (Table 2).

The observation that the number of α_2-adrenergic receptor sites labeled by the antagonist [^{3}H]RAUW is greater than the number labeled by the agonist [^{3}H]PAC (Table 2) has been seen before in NG cells (8), and is due to the occurrence of the NG cell α_2-adrenergic receptor in two major affinity states which are differentially and somewhat selectively labeled by the agonist and antagonist ligands (9). Detailed saturation isotherms over very wide concentration ranges reveal that agonist and antagonist ligands label the same total population of NG α_2-adrenergic receptors (10). As with the opiate agonist and antagonist binding data (above), the similar direction of changes noted with [^{3}H]PAC and [^{3}H]RAUW suggests that the binding site changes reflect true changes in α_2-adrenergic receptor density.

Thus, the up-regulating factor produced by NCB cells but not by dBc-treated NCB cells increased muscarinic and α_2-adrenergic receptor density as well as opiate receptor density on NG cells, suggesting a heterologous up-regulation by the factor.

Dose response relationship between concentration of up-regulating factor and opiate receptor density. Up-regulation of opiate receptors on NCB and NG cells was previously induced with 5-day-old CM. From the growth curve experiments (Fig. 2), we predicted that up-regulation would be proportional to the "concentration" of up-regulating factor, i.e., to "age of the CM." To test this, CM was prepared daily from 1- to 4-day-old cultures of NCB or NG cells and stored at 4°. CM was also prepared from NCB and NG cultures treated with dBc for 1–4 days. One-day-old NG cells were overlayed with 1-

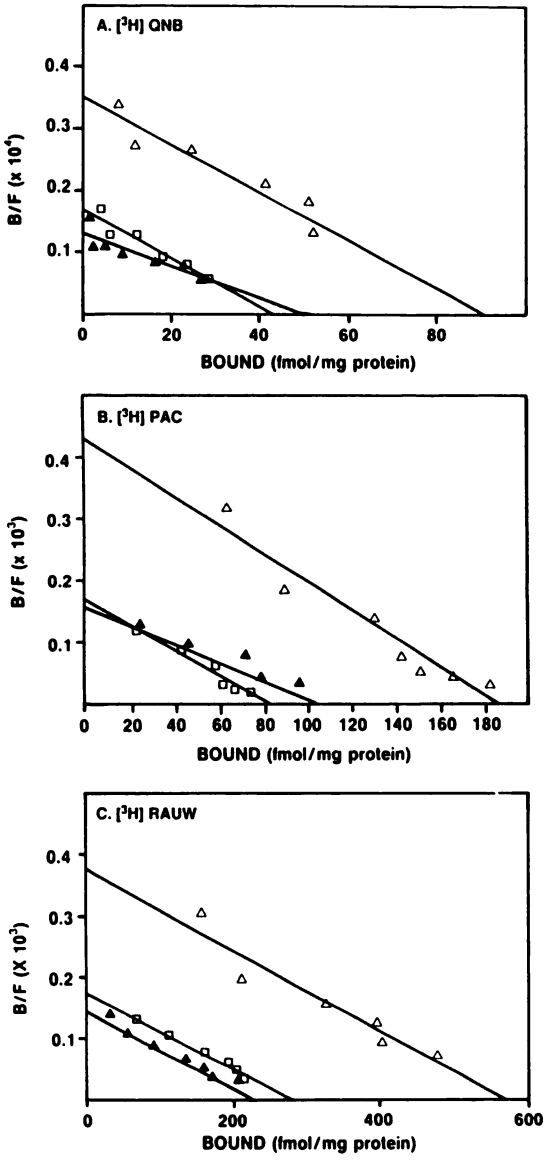

Fig. 3. Up-regulation of muscarinic cholinergic and α_2-adrenergic receptors in NG108-15 cells. One-day-old NG cultures were treated for 3 days with normal media without CM (□), or with CM from untreated (△) or dBc-treated (▲) NCB cultures. Binding of [^{3}H]QNB (1.0–50 pM) (A) to each set of NG cell membranes (0.2 mg of protein/tube) was carried out at 37° for 90 min using 1.0 μM atropine sulfate to define nonspecific binding. Binding of [^{3}H]PAC (0.05–3 nM) (B) and [^{3}H]RAUW (0.03–6.0 nM) (C) was performed at 24° for 40 min using 0.1 mM norepinephrine to define nonspecific binding. B_{max} values shown are from one representative experiment assayed in triplicate for each ligand. K_D values were unchanged under any growth condition: 39 ± 5 pM (QNB), 2.2 ± 0.4 nM (PAC), and 1.5 ± 0.1 nM (RAUW). Similar values were obtained in replicate experiments.

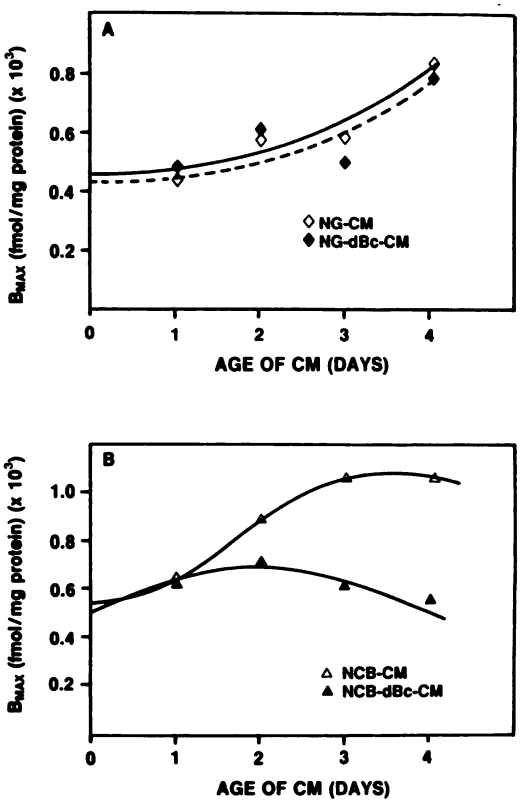

Fig. 4. Dose response curves for effect of age of CM on NG108-15 opiate receptors. CM were prepared daily from 1- to 4-day-old cultures of untreated (◇, △) or dBc-treated (◆, ▲) NG (A) or NCB (B) cells. One-day-old NG cells were overlayed with each CM preparation from 1-, 2-, 3-, or 4-day-old NG cells (◇), dBc-treated NG cells (◆), NCB cells (△), or dBc-treated NCB cells (▲). Parallel NG cultures were grown in the absence of CM (represented by day 0). Binding of [^{3}H]DADLE (0.05–3.0 nM) to NG membranes was measured after 3 days. B_{max} values from one representative experiment assayed in triplicate are shown. Similar results were obtained from two additional experiments.

to 4-day-old CM from untreated and dBc-treated NG cells (Fig. 4A). After 3 days, [^{3}H]DADLE saturation binding experiments were performed on NG membranes. Parallel NG cultures were grown for 3 days in normal media without CM (represented by day 0, Fig. 4A). Opiate receptor site density on NG cells increased in a dose-dependent manner, i.e., in direct proportion to the age of CM. Thus, media from 4-day-old cultures, which presumably contained a higher concentration of up-regulation factor, yielded higher B_{max} values than media which had been conditioned for shorter time periods, regardless of whether CM originated from untreated or dBc-treated NG cells (Fig. 4A). CM from NCB cells also produced a dose-dependent up-regulation of NG opiate receptors, but CM from dBc-treated NCB cells did not (Fig. 4B). Thus, B_{max} values were between 500 and 600 fmol/mg of protein, independent of the age of NCB-dBc-CM. In all cases, K_D values were unaltered (data not shown) and changes were reflected only in B_{max} values expressed either as fmol/mg of protein or as fmol/10^6 cells.

Time course of opiate receptor site up-regulation. The time course of opiate receptor up-regulation was examined using 5-day-old CM from NCB cells (Fig. 5). NG cultures (5 × 10^5 cells/dish) were overlayed each day with NCB-CM prepared and stored at 4° (see Materials and Methods). All cultures treated for 1–5 days were harvested on the sixth day. Saturation isotherms of [^{3}H]DADLE specific binding to NG membranes showed a progressive increase in opiate receptor site density with exposure time (Fig. 5). No up-regulation was detected after 1 day of treatment. B_{max} values increased by 28% and 56% after 2- and 3-day treatments, respectively, and longer periods produced no further significant enhancement. Once again, ligand affinity constants were the same in NG cells grown without CM (data not shown) or in CM for different time periods. Thus, opiate receptor site up-regulation appeared to

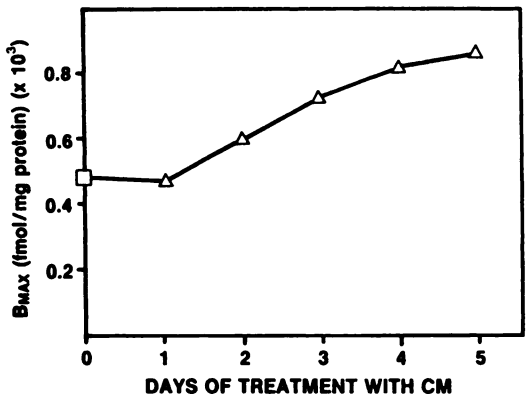

Fig. 5. Time course of CM effects on NG108-15 opiate receptor up-regulation. NG cultures (5×10^5 cells/dish) were treated with CM from NCB cells for 1–5 days (△) or were grown without CM (□). All cultures were harvested on the sixth day. [^{3}H]DADLE (0.05–3.0 nM) specific binding to NG membranes (0.2 mg of protein/tube) was measured (25°, 90 min) using 10 μM morphine to determine nonspecific binding. Values shown are from a representative experiment assayed in triplicate. Similar results were obtained with two additional experiments.

be dependent both on the concentration and the length of exposure to the excreted factor in CM.

Molecular weight estimation of up-regulating factor. The size of the up-regulating factor was estimated using dialysis bags with different MWCO sizes. CM from NCB cells was dialyzed against media for 24 hr with two media changes. One-day-old NG cultures were grown for 3 days in undialyzed or dialyzed CM (Table 3). Parallel cultures were grown in undialyzed and dialyzed normal media. Saturation isotherms of [^{3}H]DADLE specific binding to NG cell membranes prepared from each set of cultures were obtained. As before, opiate receptor sites on NG cells were up-regulated 2-fold by NCB-CM (B_{max} = 700 ± 160 fmol/mg of protein compared to 1300 ± 250 fmol/mg of protein). However, if CM was dialyzed using bags with MWCO sizes of 12–14 kDa and 6–8 kDa, up-regulation of opiate receptor sites was lost. In contrast, opiate receptor density was enhanced (2-fold) when NG cultures were grown in CM dialyzed in a 3.5-kDa bag. B_{max} values increased from 830 ± 190 fmol/mg of protein to 1600 ± 240 fmol/mg of protein. These data demonstrate that the up-regulating factor secreted by NCB cells passed through dialysis bags with a MWCO equal to or greater than 6–8 kDa, but was retained in bags with a MWCO of 3.5 kDa, suggesting that the up-regulating factor may be a relatively small molecule (molecular weight <6000). K_D values were not significantly changed under any of the growth conditions (Table 3).

Effect of up-regulating factor on opiate receptor function. The coupling of opiate receptors to AC was examined in NG cell membranes prepared from cultures grown in various CM (Fig. 6). One-day-old NG cells were treated (3 days) with CM from NCB or NG cells, or with CM from dBc-treated NCB cells, or were grown without CM. Membranes prepared from each set of cultures were assayed for the ability of DADLE to inhibit PGE_1-stimulated AC activity. Basal enzyme activities ranged from 500 to 900 pmol of cAMP/20 min/mg of protein, and PGE_1-stimulated basal AC approximately 7-fold in all membrane preparations. A maximum inhibition of about 40% was observed in all cases. The potency of DADLE to inhibit PGE_1-stimulated AC was also not different in NG membranes derived from untreated or CM-treated cultures (Fig. 6). Thus, although opiate receptor density is increased 2-fold in CM-treated NG cultures, a coincident enhancement of receptor function was not observed. Correspondingly, NG cells cultured in CM from dBc-treated NCB cells yielded a dose response curve with DADLE similar to that of cells grown in NCB-CM. These data suggest that an opiate receptor reserve is present on NG cells, as was previously suggested in this system (1).

Discussion

Previously, we observed that opiate, muscarinic, and α_2-adrenergic receptors present on the NCB-20 (neuroblastoma N18TG2 × Chinese hamster brain) hybrid cell were down-regulated by treatment with dBc. In contrast, all three AC (N_i)-coupled receptors on the closely related NG108-15 hybrid cell (neuroblastoma N18TG2 × glioma C6BU-1) were unaffected by dBc treatment. In all three cases, receptor site densities but not ligand affinities were reduced. This unique cell specificity of receptor down-regulation was seen once again in studies reported here, and can now be described more accurately as a cell-specific inhibition of a nonspecific up-regulation mechanism. We have shown that opiate, muscarinic, and α_2-adrenergic receptors on NCB, as well as on NG, cells were up-regulated by treatment of either cell with CM, which presumably contained an "up-regulation factor." Thus, the factor produced by one hybrid cell (e.g., NCB) up-regulates opiate receptors both on itself (NCB) and on the related hybrid (NG) to the same maximal extent (~2-fold). In contrast, CM derived from dBc-treated NCB cells did not up-regulate opiate, muscarinic, and α_2-adrenergic receptors on NG cells, whereas CM from dBc-treated NG cells did. A reasonable explanation of the data is that this up-regulating factor is produced and excreted into the media by untreated NG and NCB cells, but is suppressed or inactivated in dBc-treated NCB cells, and not in dBc-treated NG cells. The factor accumulates in media with time in culture,

TABLE 3

Effect of dialysis of CM on [^{3}H]DADLE binding to NG108-15 cell membranes

NCB-CM prepared as described under Materials and Methods and normal growth medium (control) were dialyzed for 24 hr against media using dialysis bags of different MWCO pore sizes. Other samples of medium and NCB-CM were left undialyzed. One-day-old NG cultures were grown in the various media for 3 days. Saturation isotherms of [^{3}H]DADLE (0.05–3.0 nM) specific binding to membranes prepared from each set of cultures were obtained using 10 μM morphine to define nonspecific binding. B_{max} and K_D values shown are the means ± standard deviations of two to three experiments, each performed in triplicate.

Condition	Dialysis pore size (MWCO, kDa)							
	0 (undialyzed)	12–14	6–8	3.5	0 (undialzyed)	12–14	6–8	3.5
	B_{max} (fmol/mg protein)				K_D (nM)			
Media (Control)	700 ± 160	430 ± 11	750 ± 200	830 ± 190	0.57 ± 0.01	0.66 ± 0.01	0.61 ± 0.1	0.59 ± 0.09
NCB-CM	1300 ± 250	370 ± 4	850 ± 150	1600 ± 240	0.62 ± 0.07	0.64 ± 0.09	0.59 ± 0.01	0.73 ± 0.05

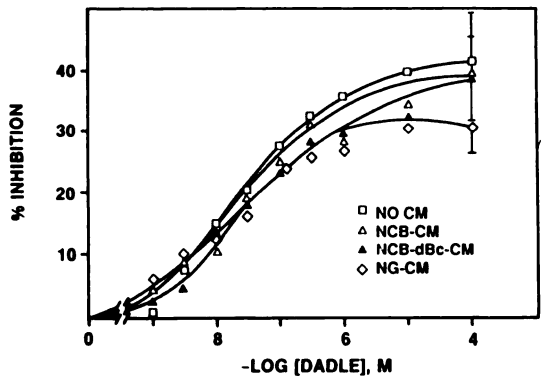

Fig. 6. Effect of CM on opiate inhibition of AC activity in NG108-15 membranes. NG membranes were prepared from cultures grown for 3 days in normal media with no CM (□), or in CM derived from NG cells (◇), NCB cells (△), or dBc-treated NCB cells (▲). Inhibition of PGE_1-stimulated (10 μM) AC activity was measured using various concentrations of DADLE (1 nM–100 μM). Basal activity was stimulated approximately 7-fold by PGE_1 in all cases. Basal (mean ± standard error) AC in membranes with no CM, NCB-CM, NCB-dBc-CM, and NG-CM were 490 ± 84, 800 ± 90, 470 ± 150, and 950 ± 69 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, were 46 nM, 51 nM, 26 nM, and 14 nM, respectively. Values shown (per cent inhibition) are the mean of five experiments, each assayed in triplicate. *Error bars* (standard deviation) are shown only at 100 μM DADLE; all other values also overlap and have been omitted for clarity.

resulting in an up-regulation response which is proportional to the "age of CM" (Fig. 4).

Since opiate receptors on NCB and NG cells were up-regulated by CM from the same cell, we examined specific opiate binding to NCB and NG cell membranes as a function of cell growth (Fig. 2). If the up-regulating factor were accumulating in the media and acting directly upon cells that were synthesizing/secreting the factor (a phenomenon termed, here, "auto-up-regulation"), then enhanced opiate receptor site density might be expected as cell density increased. This phenomenon was indeed observed in untreated and dBc-treated NG cells (Fig. 2) and in untreated NCB cells. However, dBc-treated NCB cells showed no increase in opiate receptor density even though cell density increased by one log (Fig. 2). This result is consistent with the absence of an opiate up-regulating factor in CM derived from dBc-treated NCB cells. In fact, not only did opiate receptors fail to develop *in vitro* on dBc-treated NCB cells, but receptor density was also dramatically down-regulated (by 75–90%) (Ref. 1; Fig. 2). Whether this down-regulation phenomenon is due simply to the absence of an up-regulating factor or to the existence of a separate down-regulating entity is unclear.

The direct effects of cell density on up-regulation are clearly ruled out since cultures grown in CM from dBc-treated NG or NCB cells achieved about the same cell density, but only the latter cultures showed no up-regulation (Fig. 2). This observation is of interest since the expression of many cellular responses have been shown to be cell density dependent or cell cycle dependent (11–15). The up-regulating factor appeared to selectively influence certain receptor classes rather than to cause a generalized increase in plasma membrane protein and/or surface area. Thus, basal and PGE_1-stimulated AC activity was the same, regardless of whether NG cells were grown in the absence or presence of CM, or whether the CM was derived from untreated or dBc-treated NCB cells, suggesting that the PGE_1 receptor (an N_s-coupled receptor) and AC were two plasma membrane markers which were unaltered in response to the autocrine factor contained in CM. However, in demonstrating in these experiments parallel regulatory phenomena with three AC (N_i)-coupled receptors, we cannot conclude from the present data that the regulation is specific to this receptor class.

The unique cell specificity of both down-regulation and inhibition of up-regulation of opiate, muscarinic, and α_2-adrenergic receptors on neuroblastoma cells suggests that a common relationship may exist between these two phenomena. A parallel situation exists where dBc itself down-regulates these AC-coupled receptor sites on NCB cells and also prevents the synthesis of a transmissible up-regulating factor in the same cell. Untreated NCB and NG cells and dBc-treated NG cells all exhibit the converse relationship, where opiate, muscarinic, and α_2-adrenergic receptors are not down-regulated, nor is the production of an up-regulating factor inhibited in these cells. One mechanism which could account for both phenomena may involve synthesis of a product (a down-regulating factor, "y"), which may in turn inhibit synthesis of an up-regulating factor, ("x"). Thus, synthesis of "y," which is triggered by dBc only in the NCB cell, prevents or reduces production of "x," resulting in heterologous down-regulation of all three receptors on dBc-treated NCB cells. Therefore, CM derived from dBc-treated NCB cells would not be expected to up-regulate opiate receptors on NG or NCB cells, either because it does not contain "x," or because it also contains the inhibitory factor "y," whose effect is to inhibit the expression of "x." An extension of this hypothesis requires that "x" be a diffusible, stable product that can freely traverse the plasma membrane.

Agonist-induced receptor desensitization has been associated with receptor phosphorylation in many cell systems (16–18). Although it is clear that the observed down-regulation of AC-coupled receptors on NCB cells is not agonist induced, but is mediated by cAMP, the mechanism of down-regulation may involve heterologous phosphorylation of the three receptors themselves, or a common protein associated with them, and the N_i protein, among others, may be one possible candidate. However, regardless of the phosphorylation site, it might be speculated that "y" is a cAMP-dependent protein kinase whose synthesis and/or function is triggered by dBc, but only in NCB cells, and the signal for its synthesis must have been inherited from the Chinese hamster brain cell parent of NCB cells (1). The nature of "x," the putative up-regulating factor, is also not known except that "x" is a molecule considerably smaller than many other factors which are released into CM by cultured cells. These include macromolecular factors such as neurite outgrowth-promoting factors (19–21), nerve growth factor (22), and others involved in cell proliferation (23) and differentiation of cholinergic neurons (24). Since "x" appears to be synthesized constitutively by neuroblastoma hybrid cells, its inhibition by dBc may be associated with repression of a gene product in dBc-treated NCB cells. This signal may reside either in the Chinese hamster fetal brain cell parent of the NCB hybrid or in the N18TG2 parent, which is shared by both hybrid cells. Experiments related to these and other aspects of receptor regulation are the subject of future investigations.

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