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Mutants of PC12 Cells with Altered Cyclic AMP Responses

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PC12 cells, derived from a rat pheochromocytoma, were mutagenized and selected in media containing agents known to elevate intracellular concentrations of cyclic AMP (cAMP). More than 40 clones were isolated by selection with cholera toxin or 2-chloroadenosine or both. The variants that were deficient in accumulating cAMP were obtained by using a protocol in which 1 μM 8-bromo-cAMP was included in addition to the agonist. Certain of these variants were partially characterized with respect to the site of altered cAMP metabolism. The profiles of adenylate cyclase activity responsiveness of certain variants to guanosine-5′-(β,γ-imido) triphosphate and to forskolin resembled those of UNC and cyc phenotypes of S49 lymphoma cells, which are functionally deficient in the GTP-sensitive coupling protein, N_s. Other variants were characterized by increased cyclic nucleotide phosphodiesterase activity at low substrate concentration. Diverse morphological traits were observed among the variants, but it was not possible to assign them to a particular cAMP phenotype. Two revertants of a PC12 mutant were isolated and observed to have regained a cellular cAMP response to 2-chloroadenosine and to forskolin. It is hoped that these PC12 mutants will have utility for defining cAMP-mediated functions, including any links to the action of nerve growth factor, in cells derived from the neural crest.

The use of variants of cloned cells clearly has advanced the understanding of the regulation of cyclic AMP (cAMP) concentration and illuminated the means by which it exerts its effects (13, 23). Phenomena involving cAMP in a particular cell type may not occur generally, however, as exemplified by the diverse relationships of cAMP concentration to proliferation of cultured cells (4).

The PC12 cell line, derived from a rat pheochromocytoma, has been studied extensively as a model for growth and differentiation of cells derived from the neural crest. In response to nerve growth factor, the tumor cells undergo striking morphological and functional changes and develop into cells resembling neurons (34). The cells synthesize cAMP in response to the activation of an adenosine receptor coupled to adenylate cyclase (17), but the effect of nerve growth factor on cAMP content is inconsistent and of small magnitude (34). The cyclic nucleotide has been proposed to serve as a permissive or synergistic factor rather than to function as an obligatory intermediate (16), but other evidence supports dissociation of effects of cAMP and of nerve growth factor (14, 20, 30).

Variants of PC12 cells deficient in cAMP accumulation or refractory to its actions should be useful in defining more precisely the role of cAMP in these processes, and additionally they might provide clues linking the cyclic nucleotide to other functions of cells derived from the neural crest. We have applied the rationale initially used in studies of the S49 lymphoma system to the PC12 line, and we have employed procedures closely similar to those of Bothwell et al. (3), who obtained PC12 variants with altered response to nerve growth factor. High intracellular concentrations of cAMP can be obtained in these cells by treating with 2-chloroadenosine, which acts on adenosine receptors in the plasma membrane, or by treating with cholera toxin, which irreversibly activates cyclase. In either case, the treated cells stop dividing, extend processes, and exhibit cytotoxicity with time. In this paper we describe the procedures that have been used to obtain more than 40 different clones and the cAMP phenotypes, which appear separable into at least two general categories with respect to forskolin stimulation. Revertants to a wild-type cyclic nucleotide phenotype have been isolated and are also described briefly.

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MATERIALS AND METHODS

Materials. Media and chemicals were obtained from the following sources: RPMI 1640 medium and serum, GIBCO Laboratories; 2-chloroadenosine, cholera toxin, cAMP, 8-bromo-cAMP (8-BrcAMP), and ethyl methanesulfonate, Sigma Chemical Co.; forskolin, Calbiochem-Behring; [^{3}H]ATP, Amersham Searle Corp.; [^{3}H]cAMP and guanosine-5′-(β,γ-imido)triphosphate (GppNHp), ICN Chemical and Radioisotope Division; [^{125}I]2′-*O*-succinyl cAMP tyrosine methyl ester, New England Nuclear Corp.

Cell culture and selection. PC12 cells obtained from Mark Bothwell, Princeton University, were maintained in RPMI 1640 medium supplemented with 10% horse serum and 5% newborn calf serum in 5% CO_2–95% air at 37°C. Before selection, PC12 cells were shown to express levels of plasma membrane nerve growth factor receptors and PC12-specific antigens characteristic of the cell line (2). Details of culture techniques and of mutagenizing the cells with ethyl methanesulfonate followed procedures described by Bothwell et al. (3). Briefly, cells were treated overnight with growth medium containing 200 μg of ethyl methanesulfonate per ml. Approximately 30% of cells survived, and these were grown for at least five passages before selection, which was carried out as follows. Cells were seeded at approximately 2×10^6 per Corning 75-cm^2 flask and cultured continuously in media containing 10 μM 2-chloroadenosine, 1.0 μg of cholera toxin per ml, or both 2-chloroadenosine and cholera toxin. Under each of these conditions, half of the cultures were exposed to selective medium containing 1 μM 8-BrcAMP in addition to the other materials. Within 7 days most cells had detached from the surface, and within 3 weeks small islands of cells could be seen microscopically at a frequency of several per flask. Clones were isolated, serially propagated, and doubly recloned in selective medium (including 1 μM 8-BrcAMP for

the three groups that initially received it). After mass culture had been achieved, cells were removed from selective medium. Variant D-2, however, was maintained in medium containing 1 μM 8-BrcAMP unless otherwise stated. Variants selected with 2-chloroadenosine were designated as D, those selected with cholera toxin were designated as T, and those selected with both agonists were designated as TD. Each series of variants was serially numbered, but for purposes of simplicity, particular clones described in this report have been renumbered as D-1, D-2, etc.

Cellular content of cAMP. Cells were grown to confluency in Corning 25-cm^2 flasks. Experiments were conducted on the day after replenishment of medium. The cells were then gently washed once with serum-free medium and preincubated 30 min at 37°C in 3 ml of serum-free medium. Forskolin or 2-chloroadenosine was added for 10 min, the medium was aspirated, and 1.0 ml of cold 5% trichloroacetic acid was added. Portions of the supernatant material were acetylated and analyzed by the radioimmunoassay procedure of Harper and Brooker (19) with [^{125}I]2′*O*-succinyl cAMP tyrosine methyl ester as the ligand. Values are minimally the average of biological triplicates.

Activities of adenylate cyclase and cyclic nucleotide phosphodiesterase. Confluent monolayers of cells from 75-cm^2 flasks (about 10^7 cells) were washed twice and scraped into 10 ml of phosphate-buffered saline (lacking calcium and magnesium) and centrifuged briefly. Pellets were suspended in a homogenizing buffer of 20 mM imidazole (pH 7.5), 6 mM $MgCl_2$, 3 mM dithiothreitol, and 1.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and homogenized by 10 strokes of a tight-fitting (type B) pestle in a Dounce homogenizer. The homogenates were used directly for adenylate cyclase activity determinations, or particulate fractions were obtained by centrifugation at 20,000 × *g* for 30 min, suspension in homogenizing buffer, and recentrifugation. Adenylate cyclase activity was determined by the procedure of Brostrom et al. (5). Cyclic nucleotide phosphodiesterase activity measurements were carried out by the method of Brostrom and Wolff (6). The incubation volume was 300 μl, containing 10^6 cpm of [^{3}H]cAMP and either 1 or 25 μM unlabeled cAMP. Protein was measured by the method of Lowry et al. (24).

RESULTS

Selection of variant cells. Mutagenizing PC12 cells with ethyl methanesulfonate resulted in greater than 70% cytotoxicity. The surviving population was subjected to one of three basic selection protocols, each of which contained cultures supplemented with 1 μM 8-BrcAMP and cultures not so supplemented (Tables 1 and 2; see above). The addition of 8-BrcAMP had no detectable effect on cell morphology, as monitored over a 3-week period. The analog was included to provide a minimal concentration in the event of a cAMP requirement for growth (28), which was not satisfied because of a dysfunctional adenylate cyclase. This possibility is considered further below. Cholera toxin and 2-chloroadenosine, at the concentrations used, resulted in cellular cAMP levels exceeding 200 pmol/mg of protein and were clearly cytotoxic to control PC12 cells within a period of 1 week. Selected clones were serially propagated for detailed characterization. After mass culture had been achieved, cells were removed from selection.

TABLE 1. Number of agonist-resistant variants appearing with or without 8-BrcAMP in the selection medium[a]

Agonist used in selection	No. of colonies/10^7 cells	
	With 8-BrcAMP	Without 8-BrcAMP
2-Chloroadenosine (10 μM)	8	4
Cholera toxin (1 μg/ml)	13	16
Cholera toxin and 2-chloroadenosine	3	0

[a] PC12 cells were mutagenized with ethyl methanesulfonate and selected as described in the text. Each value represents the number of colonies isolated after growth in medium containing a given agonist with or without 1 μM 8-BrcAMP.

TABLE 2. Comparison of the cAMP accumulation phenotypes between variants isolated in the presence and absence of exogenous 8-BrcAMP[a]

Agonist-resistant variants	No. of variants isolated with reduced cAMP accumulation in response to:	
	10 μM 2-chloroadenosine	10 μM forskolin
With 1 μM 8-BrcAMP	24 of 24	22 of 24
Without 8-BrcAMP	0 of 20	0 of 20

[a] PC12 cells were mutagenized with ethyl methanesulfonate and selected as described in the text. After isolation, variants were tested for the ability to accumulate cAMP after a 10-min challenge with the agonist as described in the text. Variants are considered to exhibit a reduced accumulation phenotype if variant cell extracts contained less than 10% of PC12 cAMP levels in the assay. Exact values for representative variants are given in Table 3.

Many variants exhibited morphological traits distinct from those of PC12 cells, and a wide range of particular features was observed (Fig. 1). Variant TD-1, for example, and many additional clones selected in the same protocol resembled PC12 cells. Alternatively, D-2 and others were flat and spontaneously extended long processes. Cells exposed to such a stringent mutagenesis event may contain multiple genomic defects; although cAMP is known to exert effects on the cytoskeleton, it is therefore not possible to associate cAMP phenotype with a particular morphological trait in these variants.

Cellular cAMP in response to agonists. The basis of selection was the acquired capacity of cells to maintain low levels of cAMP under conditions that would normally cause toxic levels or to resist such toxic levels. It was of interest to determine the cAMP responsiveness of individual variants to agonists acting at different sites within the receptor-cyclase complex. 2-Chloroadenosine, acting via plasma membrane receptors, and forskolin, acting in part directly on the cyclase catalytic subunit (9, 11, 27), are effective stimulators of mammalian adenylate cyclase. Table 3 shows that both ligands caused substantial increases in intracellular cAMP concentration in wild-type PC12 cells. PC12 basal values of 6 pmol of cAMP per mg of protein were elevated to 250 pmol/mg after treatment with 10 μM 2-chloroadenosine and to values greater than 500 pmol/mg after association with 10 μM forskolin under these conditions. The time to attain half-maximal values for both PC12 and D-2 cells treated with 2-chloroadenosine or forskolin was 2 min or less. Plateau values were attained by 8 min.

When the variants were characterized by their cAMP responsiveness to these ligands, two general phenotypes were apparent. The first included 20 distinct clones represented by T-1 (cholera toxin resistant; Table 3) and had been derived after selection in either 2-chloroadenosine or cholera toxin without exogenous 1 μM 8-BrcAMP. T-1 responded to both agonists in a manner similar to parental PC12 cells. The ability to proliferate in cholera toxin, but accumulate cAMP after challenge with forskolin or 2-chloroadenosine, is con-

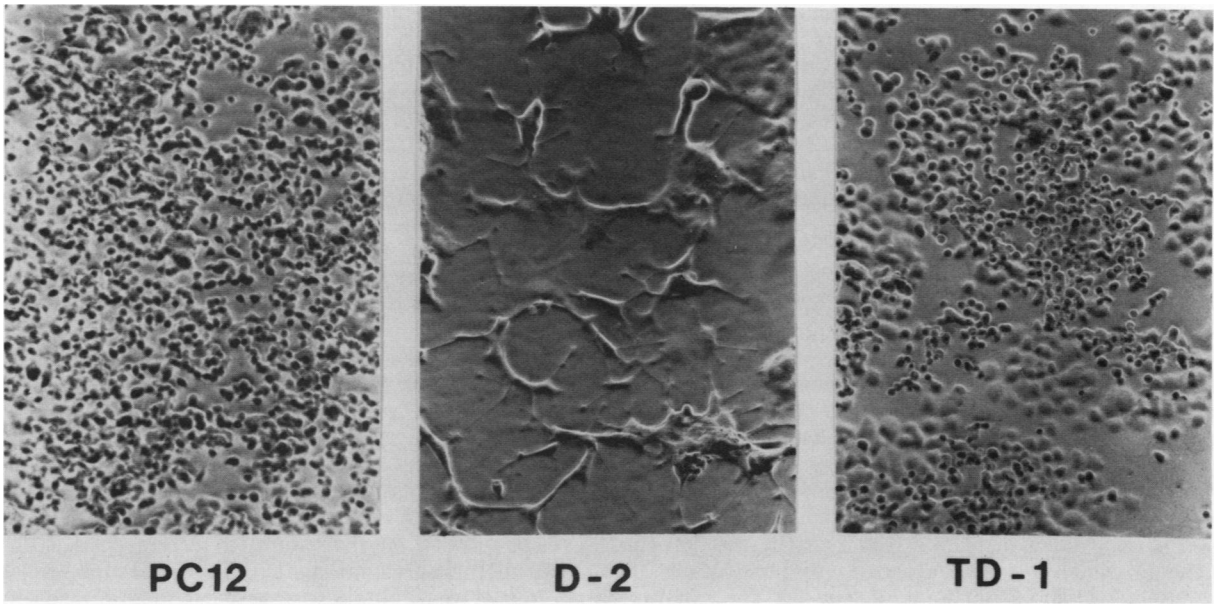

FIG. 1. Morphological variation of cells selected in 2-chloroadenosine (D-2) or cholera toxin and 2-chloroadenosine (TD-1). After selection, the cells were cultured until the stationary phase in medium containing serum, but no selective agents, and photographed under phase contrast.

sistent with there being a lesion either in the cholera toxin receptor or in cAMP-dependent protein kinase. Variants with defects in cAMP-dependent protein kinase are known to occur with high frequency in other systems after selection in dibutyryl cAMP (12). Since our interests were in PC12 variants with defects in accumulating cAMP, the cell lines typified by T-1 have not been considered further.

The second group of 24 clones included variants that exhibited relative nonresponsiveness to challenge by either 2-chloroadenosine or forskolin. Their response to 2-chloroadenosine was not significantly elevated above basal (unstimulated) values of approximately 5 pmol of cAMP per mg of protein. Furthermore, they accumulated less than 30 pmol of cAMP per mg of protein after exposure to 10 μM forskolin for 10 min. Wild-type PC12 cells usually accumulated in excess of 250 and 500 pmol of cAMP per mg of protein with 2-chloroadenosine and forskolin, respectively. Since forskolin and 2-chloroadenosine are presumed to stimulate cAMP accumulation by different means, it can be inferred that these variants possess a defect involving a function common to the mechanism of action of both ligands. These variants were considered to be candidates for possessing cyclic nucleotide metabolism lesions at a site beyond the adenosine receptor.

TABLE 3. Cellular accumulation of cAMP

Cell type	cAMP[a]		
	Basal	2-Chloroadenosine	Forskolin
PC12	6	282	580
T-1	5	450	720
D-1	2	3	15
D-2	3	6	20
TD-1	2	3	15
TD-3	1	3	27

[a] Picomoles of cAMP per milligram of protein 10 min after the addition of 2-chloroadenosine or forskolin to a final concentration of 10 μM. Details of the procedure are described in the text. The values given were obtained after peak accumulations of cAMP levels were obtained.

Variants D-1, D-2, and TD-1 were cloned three times and then maintained without selective media for more than 6 months. On retesting the cellular cAMP accumulation in response to 2-chloroadenosine and to forskolin, each variant retained the original phenotype. It is of interest that D-2 retained its cAMP phenotype after passage at high density in the absence of selection and without 8-BrcAMP.

Adenylate cyclase activity. Variants that appeared to be deficient in cAMP accumulation to both agonists in the assay on intact cells were examined for their ability to synthesize cAMP from [^{3}H]ATP in an assay with cellular homogenates. In view of the fact that forskolin stimulates other mammalian adenylate cyclases directly, it is not surprising that this ligand is an effective stimulator of PC12 cyclase (Fig. 2). Forskolin (100 μM) resulted in a greater than 12-fold increase in PC12 adenylate cyclase activity. Both the basal and forskolin-stimulated activities of variant TD-3 were similar

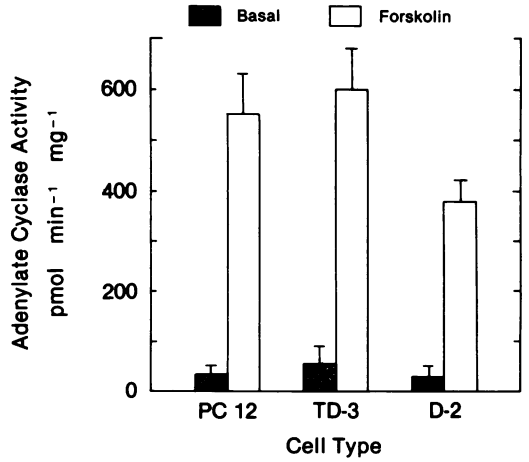

FIG. 2. Adenylate cyclase activity of cell homogenates in response to 100 μM forskolin. Error bars refer to standard deviations.

to those of PC12 cells. Clearly this variant possesses a functional adenylate cyclase, and, since it was selected for growth in the presence of cholera toxin and 2-chloroadenosine and failed to accumulate cAMP after challenge of intact cells with agonist, a postcyclase step must be considered to explain its low cAMP content (see below). Variant D-2 also exhibited a functional adenylate cyclase that was stimulated by forskolin, although to a lesser absolute value than the wild type. Revertants of this variant are described subsequently.

Hormone stimulation of adenylate cyclase activity in other mammalian systems has been shown to be mediated by a GTP-sensitive polypeptide that functionally couples the plasma membrane receptor to the cyclase catalytic subunit (23). This protein, which is variously designated as coupling factor, G/F, or N_s, binds GTP and the GTPase-resistant analog, GppNHp. GppNHp alone will activate adenylate cyclase in crude membrane preparations (9, 18). The ability to be stimulated by GppNHp is therefore indicative of the presence of both a functional adenylate cyclase and an N_s protein. Figure 3 shows that GppNHp treatment of PC12 cell homogenate resulted in at least a fivefold elevation of cyclase activity, or about 200 pmol/min per mg. The variants TD-1, TD-2, and D-1 can be characterized by their response profiles to GppNHp and forskolin (Fig. 3). Clearly, all of these variants possessed substantial cyclase activity, but the forskolin-mediated elevation was always less than half of that seen in PC12 cells. TD-1 and TD-2 can be distinguished from each other by their relative responses to GppNHp. The GTP analog stimulated the TD-1 adenylate cyclase to nearly the same proportion and extent as that seen in PC12 cells. This is similar to the uncoupled phenotype (UNC) reported in the S49 lymphoma system, in which variants occurred that were resistant to cell killing by agonists and that possessed a dysfunctional N_s protein (18). The N_s protein of that mutant still mediates adenylate cyclase activation if treated with GppNHp, but is uncoupled from plasma membrane hormone receptors. TD-2 exhibited cyclase activation in response to forskolin comparable to that of TD-1. GppNHp, however, was only slightly, if at all, effective in elevating cyclase activity. Variants typified by TD-2 resembled, in this respect, the cyc mutant of S49 lymphoma cells. In that instance cyc^- refers to a phenotype initially believed to be lacking cyclase, but now understood to lack an N component mediating cyclase stimulation and to retain a guanine nucleotide-dependent inhibitory component (20).

Variant D-1 presented another phenotype in which basal, GppNHp, and forskolin increases in cyclase activity were all significantly less than that observed with PC12 cells. However, although the absolute amount of increase was less, the proportion (fold increase) in activity with either GppNHp or forskolin was approximately the same as that in the wild type. That is, GppNHp and forskolin resulted in 6- and 12-fold increases in cyclase activity, respectively. This diminution is reflected in the maximal capacity of the system to respond to these ligands since the maximum value of activation at saturating concentrations of forskolin or GppNHp was also less than in PC12 (data not shown). Furthermore, comparison of the washed particulate fractions of PC12 and D-1 homogenates revealed a pattern of activity identical to that seen in the crude homogenate, suggesting that a soluble inhibitor of phosphodiesterase was not responsible for uniformly low D-1 cyclase activity.

Phosphodiesterase activity. An S49-derived variant selected for its resistance to cAMP-mediated cytotoxicity has been shown to contain an elevated cAMP-degrading capability (15). It therefore seemed possible that if PC12 cAMP levels could be similarly negatively regulated, variants that elevated phosphodiesterase activity might occur. Those variants might contain normal (or near normal) levels of adenylate cyclase activity, but their ability to degrade [^{3}H]cAMP to 5′-AMP might be greater than that of the wild type. Cells of the nervous system contain several phosphodiesterase activities that differ in physical and kinetic properties (8). For the purposes of preliminary screening we measured rates of conversion of labeled cAMP by using high (25 μM) and low (1 μM) concentrations of substrate to assess the relative activities of two commonly occurring types of phosphodiesterase. Variants typified by TD-3 clearly possessed increased phosphodiesterase activity at a low substrate concentration (Fig. 4). D-1 cells exhibited levels similar to those of PC12 cells. PC12-like levels of phosphodiesterase activity have also been observed in other variants, including D-2, TD-1, and TD-2. It is known that adaptive increases in phosphodiesterase activity occur in many cell types in response to elevated intracellular cAMP (7, 10). This phenotypic modulation would seem to be an unlikely explanation of the increased activity in TD-3 because the cells were grown in the absence of selective conditions for at least 50 doubling periods.

D-2 revertants. Clone D-2 was derived from PC12 cells after selection in 2-chloroadenosine. As demonstrated, cellular accumulation of cAMP was substantially less in response to 2-chloroadenosine or forskolin, although the forskolin-

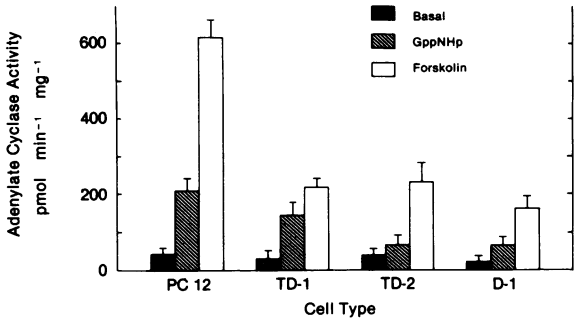

FIG. 3. Adenylate cyclase activity of cell homogenates in response to 100 μM GppNHp or 100 μM forskolin.

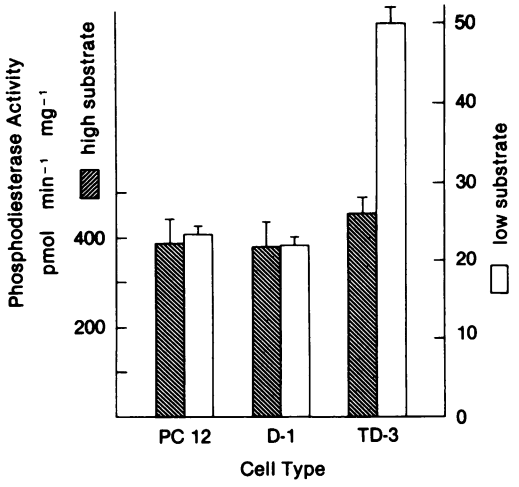

FIG. 4. Phosphodiesterase activity of cell homogenates with high (25 μM) or low (1 μM) concentrations of cAMP as substrate.

stimulated levels of adenylate cyclase activity of homogenates were significant, being about that of PC12 cells. Levels of phosphodiesterase activity were also like those of the wild type. The nature of the change in D-2 could be examined more effectively if revertants to a wild-type phenotype were available. In seeking such revertants we noted that at low density plating, the growth of D-2 was slower in the absence than in the presence of 1 μM 8-BrcAMP (data not shown), and this provided the means for identifying potential revertant populations of cells. D-2 was plated at a density of 10^4 cells per 25-cm^2 flask in the absence of 8-BrcAMP. Cell growth continued in the absence of the analog, but was less vigorous than in its presence. Large, homogeneous clones appeared, however, at a low frequency (ca. 10^{-4}), and these were clearly distinct from the background of much smaller islands of slowly growing cells. Although exact quantitation of growth rates was not performed, the apparent requirement for 8-BrcAMP by D-2 to replicate at low density provided a useful device for obtaining putative revertants to a wild-type cyclic nucleotide metabolism phenotype. Isolates were recloned to ensure a homogeneous population of cells. The cellular cAMP responses to 2-chloroadenosine and forskolin of the putative revertants were compared with those of both the parent line (D-2) and wild-type PC12 cells (Fig. 5). Cells which had been under selection were grown to confluency for at least 10 days in the absence of any selection. Again, forskolin and 2-chloroadenosine were effective stimulators of cAMP accumulation in PC12 cells. D-2, however, exhibited virtually no stimulation by 2-chloroadenosine and little with forskolin. D-2-derived revertants D-2r1 and D-2r2 displayed substantial responsiveness to both 2-chloroadenosine and forskolin, suggesting that genuine stable reversion had occurred. Although wild-type levels of responsiveness were not seen, the ability of these revertants to respond to challenge by both ligands suggests that a single, postreceptor defect is responsible for the D-2 phenotype. That is, the likelihood is very low that forskolin and 2-chloroadenosine responsiveness would be simultaneously restored at these frequencies if two distinct loci were involved. The revertants occurred at a frequency greater than that expected for spontaneous mutation, which implies that regulatory rather than structural alterations had occurred in the D-2 parent. Significantly, cells of both D-2r1 and D-2r2 morphologically more closely resembled D-2, their immediate parent, in being morphologically flat. This suggests that in this case the cAMP phenotype and morphology are unrelated.

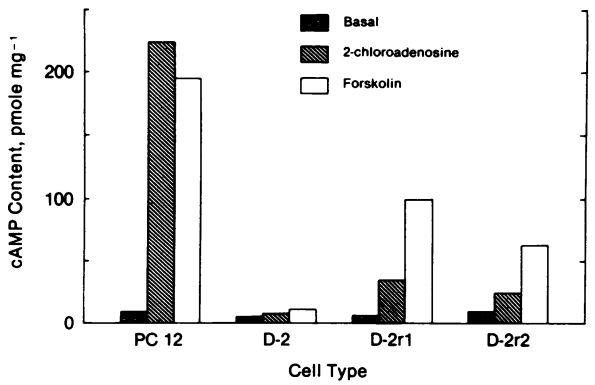

FIG. 5. cAMP content of variant D-2 and of two revertants 10 min after the addition of 2-chloroadenosine or forskolin to a final concentration of 10 μM.

DISCUSSION

Variants of PC12 pheochromocytoma cells have been selected for the purpose of exploring cAMP-mediated functions in cells derived from the neural crest. The variants exhibited resistance to cholera toxin and to 2-chloroadenosine, ligands known to cause accumulation of cAMP to high levels and to cause growth arrest or cytopathology of wild-type cells. Generally, elevated intracellular concentrations of cAMP have been associated with the inhibition of cell replication, but instances of an apparently positive regulatory role of cAMP in cell proliferation have been reported (4, 28). The demonstration that cAMP is essential for yeast to traverse the cell cycle (25) and the report of an increased mitotic rate of neuroblastoma cells treated with 8-BrcAMP (33) prompted us to supplement selective media in some cases with 1 μM 8-BrcAMP. Reports of exogenous cAMP acting at very low concentrations on DNA synthesis of calcium-depleted cells (4) and for prolonged periods on the tyrosine hydroxylase activity of neuroblastoma cells (33) provided additional plausibility for exploring in this way a possible cAMP requirement for growth.

The primary experimental objectives of this study have been our analysis of the 24 variants termed low responders because of a substantial defect in accumulating cAMP after a brief test exposure to 2-chloroadenosine or forskolin. All of these low responders were obtained with selective media containing 1 μM BrcAMP in addition to 2-chloroadenosine or cholera toxin (Tables 1 and 2). Twenty variants isolated in the absence of exogenous cyclic nucleotide responded with wild-type levels of cAMP accumulation.

It is desirable to distinguish stable alterations in cAMP metabolism due to genotypic change from the numerous types of phenotypic modulations known to be associated with increased levels of cAMP in other cell types. Short-term exposure to agonists or cAMP analogs is casually related, for example, to modifications of receptors (32) and phosphodiesterase activity (7, 10) and to cAMP egress from cells (1). The associated refractile states (in terms of cAMP accumulation) involve all of the cells, typically last hours or days, and are readily reversible for the entire cell population. In contrast, the variants described here occurred at a relatively low frequency (about 10^{-5} after mutagenesis). This is significantly less frequent than the occurrence of cyclic nucleotide variants of the S49 lymphoma system (23) and may reflect important differences in the genetics of the two cell types. This frequency is, however, high enough to be consistent with a dominant hemizygous lesion. The stability of certain clones such as D-1, TD-2, and TD-3 was monitored by testing cAMP accumulation after forskolin challenge after more than 6 months of growth in media without selection. Each of the variants retained its initial pattern of response, indicating that transient regulatory phenomena are not responsible for the phenotypic resistance to 2-chloroadenosine or cholera toxin. It is important to note that certain distinguishing traits of PC12 cells are retained. Variant D-2, for example, continued to express plasma membrane receptors for nerve growth factor (Bothwell and Lanahan, personal communication) and for a specific neural crest antigen (2). Tyrosine hydroxylase is also expressed, at levels somewhat lower than in wild type cells, in many of these variants (unpublished data).

PC12 cells appear to share at least some cyclic nucleotide-regulating properties with other previously characterized cell systems. By analogy to the UNC and cyc^- variants of S49 (21), it can be inferred that PC12 cells also transduce

receptor-cyclase interaction through a GTP-binding coupling factor that may be aberrant in TD-1 and possibly absent in TD-2. GppNHp was an effective stimulator of adenylate cyclase in TD-1 cells. UNC variants of S49 cells contain an N_s protein that is GppNHp activated, but is functionally uncoupled from plasma membrane receptors. Our TD-1 variant presented a pattern of responsiveness consistent with a UNC lesion, although defects in the cyclase catalytic unit or other regulatory sites cannot be excluded. TD-2 expressed little or no activation by GppNHp, and this strongly suggests a dysfunctional or absent N_s protein analogous to the cyc^- defect. Downs and Aurbach (11) have observed impaired forskolin activation of cyc^- adenylate cyclase, and our data from TD-2 are consistent with a similar defect. D-1 expressed membrane-associated cyclase with less activity than did wild-type PC12 cells. The reason for this is unclear, but it is not due to soluble inhibitors or phosphodiesterase isozymes, since washed membrane preparations exhibited the same reduced activity phenotype as did crude cell homogenates. Less functional cyclase polypeptide or the absence of one of a class of cyclase isozymes would confer this phenotype. However, the maximum capacity of the system to respond to forskolin is reduced in D-1 (data not shown), and in some respects this variant resembles the forskolin-resistant mutant mouse adrenocortical tumor Y1 cells recently reported by Schimmer and Tsao (29). They suspected a noncatalytic subunit lesion in the Y1 variants, since the mutant cyclase did respond fully to NaF.

Variant TD-3 possesses an elevated phosphodiesterase. The nature of the increase has not yet been determined, but it is of interest to note that a stable mutant of S49 lymphoma cells expresses an altered species of phosphodiesterase (15).

Clearly, much more detailed biochemical characterization of the variants is needed to assess fully their utility as experimental tools. Several problems of potential general interest to which they might be applied can be readily identified, however. Comprehensive understanding of receptor-cyclase coupling has not yet been attained, and in particular the means by which Ca^{2+} and calmodulin regulate adenylate cyclase activity of nerve tissue remains obscure. It is not known precisely how Ca^{2+} interacts with either the catalytic subunit of adenylate cyclase or with known coupling factors (31), but the magnitude of stimulation by Ca^{2+} is considerable. A second major topic is the participation of cAMP in synaptogenesis. Important advances have already been made using variants of neuroblastoma cells (26), and it is conceivable that related studies of these variants of PC12 cells might be worthwhile. Finally, understanding of the actions of nerve growth factor and of cAMP on the growth and differentiation of cells of the neural crest remains incomplete (14, 16, 24, 34), and it is hoped that the use of these variants will be of value in such research.

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