MOLECULAR AND CELLULAR BIOLOGY, Aug. 1985, p. 1984–1992
0270-7306/85/081984-09$02.00/0

Vol. 5, No. 8

Clonal Variants of PC12 Pheochromocytoma Cells with Defects in cAMP-Dependent Protein Kinases Induce Ornithine Decarboxylase in Response to Nerve Growth Factor but not to Adenosine Agonists

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We have isolated and partially characterized three mutants of the pheochromocytoma line PC12 by using dibutyryl cyclic AMP (cAMP) as a selective agent. Each of these variants, A126-1B2, A208-4, and A208-7, was resistant to both dibutyryl cAMP and cholera toxin when cell growth was measured. In comparison to wild-type PC12 cells, each of these mutants was deficient in the ability to induce ornithine decarboxylase (ODC) in response to agents that act via a cAMP-dependent pathway. In contrast, each of these mutants induced ODC in response to nerve growth factor. To understand the nature of the mutations, the cAMP-dependent protein kinases of the wild type and of each of these mutants were studied by measuring both histone kinase activity and 8-N_3-[^{32}P]cAMP labeling. Wild-type PC12 cells contained both cAMP-dependent protein kinase type I (cAMP-PKI) and cAMP-dependent protein kinase type II (cAMP-PKII). Regulatory subunits were detected in both soluble and particulate fractions. The mutant A126-1B2 contained near wild-type PC12 levels of cAMP-PKI but greatly reduced levels of cAMP-PKII. Furthermore, when compared with wild-type PC12 cells, this cell line had an altered distribution in ion-exchange chromatography of regulatory subunits of cAMP-PKI and cAMP-PKII. The mutant A208-4 demonstrated wild-type-level binding of 8-N_3-[^{32}P]cAMP to both type I and type II regulatory subunits, but only half the wild-type level of type II catalytic activity. The mutant A208-7 had type I and type II catalytic activities equivalent to those in wild-type cells. However, the regulatory subunit of cAMP-PKI occurring in A208-7 demonstrated decreased levels of binding 8-N_3-[^{32}P]cAMP in comparison with the wild type. Furthermore, all mutants were defective in their abilities to bind 8-N_3-[^{32}P]cAMP to the type II regulatory protein in the particulate fraction. Thus, cAMP-PK was altered in each of these mutants. We conclude that both cAMP-PKI and cAMP-PKII are apparently required to induce ODC in response to increases in cAMP. Finally, since all three mutants induced ODC in response to nerve growth factor, the nerve growth factor-dependent induction of ODC was not mediated by an increase in cAMP that led to an activation of cAMP-PK. These mutants will be useful in the elucidation of the many functions controlled by cAMP and nerve growth factor.

The PC12 cell line is a clone isolated from cells that originated from a rat pheochromocytoma (7, 11, 22, 51). This cell line exhibits many properties of adrenal medullary chromaffin cells, including the synthesis and secretion of catecholamines (7, 22). When PC12 cells are treated with nerve growth factor (NGF), they undergo changes that resemble the conversion of chromaffin cells to sympathetic neurons. Some of the NGF-induced alterations in PC12 cells include a change in cellular adhesion (41, 42), membrane conductance (3, 11), membrane structure (8), synthesis of neurotransmitters (13, 21), phosphorylations of proteins (14, 20, 24, 53), and an increase in ornithine decarboxylase (ODC) activity (21, 25, 27).

There have been reports that NGF (41, 42), as well as adenosine and adenosine analogs (17, 23), increase the activity of adenylate cyclase. Cyclic AMP (cAMP) and its derivatives have been shown to increase ODC levels (23), alter nuclear protein phosphorylations (14, 24), elicit extension of neurites (4, 27, 28), and increase tyrosine hydroxylase activity (16). However, the issue of whether NGF alone increases cAMP levels is still not resolved. Hatanaka et al. (25) were unable to detect a substantial increase in cAMP levels in PC12 cells in response to NGF. In addition, our laboratory has recently reported that NGF alone is unable to stimulate adenylate cyclase in PC12 cells. However, when adenylate cyclase is partially activated by adenosine analogs or by cholera toxin (CTX), NGF is able to potentiate the ability of these agents to increase cAMP accumulation (38).

The relationship between the biological actions of NGF and cAMP is also not clear. For example, some proteins are phosphorylated in response to either NGF or cAMP (24), whereas some are phosphorylated in response to NGF alone (20). In another case NGF and cAMP seem to have opposing actions (14).

The effects of cAMP in eucaryotes are thought to be mediated through cAMP-dependent protein kinases (cAMP-PKs) (30, 48). The cAMP-PKs consist of two dissimilar types of subunits, a regulatory subunit and a catalytic subunit (5, 15, 18, 29, 45). The holoenzyme is inactive. Binding of cAMP to the regulatory unit releases the catalytic unit, which is then fully active. This catalytic unit contains an ATP:phosphotransferase activity that has a broad specificity for various protein substrates. The different forms of cAMP-PKs can be distinguished upon ion-exchange chromatography (9, 10). They are referred to as type I (cAMP-PKI) and type II (cAMP-PKII) based on their order of elution from an anion-exchange column. Each tissue has a characteristic chromatographic distribution of protein kinases. Whereas

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some tissues such as beef heart (40) contain primarily only one type, most tissues and cell lines contain both cAMP-PKI and cAMP-PKII (32, 34, 35, 46). The major differences between the two types of protein kinases reside in the regulatory subunits (37). Whereas the catalytic subunits are found to be only slightly different (37, 43), the regulatory subunits differ in a number of aspects, including molecular weight (2, 36, 39, 54), proteolytic fragments (37), and degree of phosphorylation (39).

Since both NGF and adenosine affect many of the same enzymes in PC12 cells, it is of interest to us to determine (i) whether PC12 cells have cAMP-PKI or cAMP-PKII, or both, and (ii) whether these kinases are involved in NGF and adenosine-elicited changes in tyrosine hydroxylase, choline acetyltransferase, acetylcholinesterase, and ODC activities. By isolating and studying mutants with alterations in cAMP-PKs, we show here that both cAMP-PKI and cAMP-PKII must be involved in the induction of ODC by adenosine, but that they do not appear to be directly required for the induction of ODC by NGF.

MATERIALS AND METHODS

Materials. Dibutyryl cAMP (dbcAMP), calf thymus histone type II-a, dithiothreitol, 3-isobutyl-1-methylxanthine, phenylmethylsulfonyl fluoride, and theophylline were from Sigma Chemical Co. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was from Aldrich Chemical Co. The DEAE Bio-Gel A and protein assay were purchased from Bio-Rad Laboratories. 8-N_3-[^{32}P]cAMP (specific activity, 60 to 90 Ci/mmol) was from ICN Pharmaceuticals, Inc., whereas the [γ-^{32}P]ATP was from New England Nuclear Corp. Tissue culture supplies were as described (28).

Cell growth. PC12 cells, maintained at 37°C in a humidified atmosphere of 10% CO_2–90% air, were grown in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal calf serum and 5% horse serum. For both protein kinase assays and 8-N_3-[^{32}P]cAMP incorporations the cells (at 30 to 50% confluency) were washed once with phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate [pH 7.4]), removed from the plate by trituration, and homogenized in a Tris buffer (10 mM Tris-hydrochloride [pH 7.4] containing 1 mM EDTA and 50 μg of phenylmethysulfonyl fluoride per ml) by sonication for 2 s. When homogenization was used (3-cc syringe and a 22-gauge needle) instead of sonication, similar results were noted.

Mutant selection. PC12 cells were mutagenized from 8 to 16 h with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 8 μg/ml. This treatment killed ca. 50% of the cells. The cells were then grown for 1 week in normal medium. The cells were then subcultured and subjected to selection by incubation with 10 mM dbcAMP and 1 mM theophylline (three medium changes) for 1 to 2 weeks. These concentrations were found to be five times those required to inhibit cell growth by 50%. Surviving cells were allowed to grow over several weeks to macroscopically observable colonies that were then transferred to separate plates. They were subsequently recloned before being tested for resistance to dbcAMP, resistance to CTX, induction of ODC, or protein kinase activity. To test for resistance to dbcAMP or CTX, cells were plated at 5×10^4 cells per ml in 24-well plates in medium supplemented with dbcAMP (0 to 5 mM) or CTX (3×10^{-11} M) or CTX plus theophylline (1 mM). The medium was then replaced at ca. 36-h intervals with medium containing the same additives. After 6 to 8 days the cells were counted as described (20).

Protein kinase assay and column chromatography. Cells were washed, collected, and sonicated in Tris buffer. The homogenate was then centrifuged for 1 h at 150,000 × *g* to produce a particulate and a supernatant fraction. Protein determinations were made by using the Bio-Rad protein assay, and 6.25 mg of protein of the supernatant was loaded on each DEAE Bio-Gel A column. The columns (1.5 by 6 cm) were preequilibrated by washing with 20 ml of Tris buffer for 1 h before each separation. A linear gradient of 0 to 0.3 M NaCl was used to separate cAMP-PKI from cAMP-PKII. A total of 45 to 50 fractions of ca. 0.8 ml each were collected over the course of 2.5 h and assayed for cAMP-dependent histone kinase activity. The first eight fractions were collected before the salt gradient was begun. The histone kinase activity of both cAMP-PKs was stimulated with 5 μM cAMP, but less variability between experiments was noted with 100 μM dbcAMP. Thus, this reagent was used for all experiments reported here. Histone kinase activity was assayed by using the method of Witt and Roskoski (52). One unit of histone kinase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of ^{32}P into type II-a histone in 1 min at 30°C. Salt concentrations were determined by comparing the conductivity of the fractions to known buffer standards.

Covalent incorporation of 8-N_3-[^{32}P]cAMP into regulatory subunits. The reaction mixture used contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.2), 10 mM $MgCl_2$, (pH 6.2), 10 mM $MgCl_2$, 0.5 mM IBMX, 1 μM 8-N_3-[^{32}P]cAMP (specific activity, 6 to 10 Ci/mmol), and various amounts of protein originating from either the high-speed particulate or supernatant fractions or the column fractions obtained as described previously. Supernatant and particulate fractions not subjected to column chromatography were dialyzed overnight at 4°C in Tris buffer containing 1 mM dithiothreitol unless otherwise indicated. Samples were incubated at 4°C for 1 h in the dark and then photolyzed by a Mineralite UVS-11 hand lamp at a distance of 4 cm. The preparations were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (31) to determine the extent of incorporation of 8-N_3-[^{32}P]cAMP into various protein bands. Gels were dried and exposed to Kodak X-ray film. cAMP was used at concentrations ranging between 0.01 and 100 μM to illustrate the specificity of 8-N_3-[^{32}P]cAMP binding to the type I and type II regulatory subunits (data not shown).

ODC assay. Wild-type PC12 cells or one of the dbcAMP-resistant mutants were grown to 40 to 50% of confluence in DME containing serum. The medium was never changed the day before an experiment. Cells were washed two times with DME containing 1% horse serum and allowed to incubate in DME containing 1% horse serum plus additives for 5 h (see Fig. 1). Cells were washed at 4°C with a buffer containing 0.32 M sucrose and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.4]), harvested into the same buffer by trituration, and collected by centrifugation (800 × *g*, 15 min). The pelleted cells were resuspended in a buffer containing 50 mM NaH_2PO_4, 0.1 mM EDTA, 5 mM dithiothreitol, and 40 μM pyridoxal phosphate and lysed by sonication. Debris was removed by centrifugation at 8800 × *g* for 15 min at 4°C, and the supernatant fraction was assayed for ODC activity by the method of Djurhuus (12) at the protein concentration of 1 mg/ml. The assay was terminated at 0, 15, 30, 45, and 60 min by spotting an aliquot of the assay mixture onto P81 ion-exchange paper (Whatman, Inc.) and washing in 0.10 M NH_4OH (pH 11.3). Specific activities were determined from the slopes calcu-

lated by linear regression of plots of the ODC activity under the various conditions.

RESULTS

Isolation and characterization of mutants that are deficient in their response to dbcAMP. To determine whether both adenosine and NGF increase ODC activity through the adenylate cyclase pathway, we decided to isolate mutants that were defective in cAMP-PK. PC12 cells were mutagenized and selected for their resistance to the toxic effects of dbcAMP as described above. In this paper we describe the characteristics of three dbcAMP-resistant lines (A126-1B2, A208-4, and A208-7).

As a first step in the characterization of these mutants, we measured the ability of dbcAMP or CTX to inhibit their growth as described above. Increasing concentrations of dbcAMP initially inhibited the growth of PC12 cells and ultimately caused cell death. The concentration required for half maximal effect (IC_{50}) for wild-type cells and each of the three mutant lines is shown in Table 1. There was a clear increase in the ability of each mutant line to grow in the presence of dbcAMP when compared with wild-type cells. The selective agent, dbcAMP, may have both growth-inhibitory effects (at lower concentrations) and toxic effects (at higher concentrations) in an experiment of this type, and the IC_{50} is probably a function of the exact conditions used (i.e., cell density, feeding schedule). We also noted that each of the mutant lines was resistant to the toxic effect of dbcAMP by morphological criteria. Compared with wild-type cultures, the cultures of mutant cells had significantly less debris and individual cells had fewer vacuoles, but these phenomena have not been quantitated.

Since the toxic effects of dbcAMP may be mediated by mechanisms other than its action as a cAMP agonist, we also measured the effect of CTX on the growth of wild-type and dbcAMP-resistant lines. CTX in the presence of theophylline increased the doubling time of wild-type cells by a factor of 1.8, whereas it had a small effect, if any (<20%), on the division rate of each of the mutants (Table 1). In the absence of theophylline, CTX increased the doubling time of wild-type cells by a factor of 1.4, whereas it had a small effect, if any (<5%), on the division rate in each of the mutants.

ODC activity in wild-type and mutant cells. To determine whether the mutant cells were functionally defective in any of the responses thought to be regulated by cAMP, we measured the ability of wild-type PC12 cells and each of the mutants to induce ODC in response to increasing concentrations of phenylisopropyladenosine (PIA) or chloroadenosine. These agents are potent agonists for the adenosine-dependent adenylate cyclase (23). Data shown in Fig. 1 demonstrate that the mutants were defective in the ability to induce ODC in response to PIA (A126-1B2) or chloroadenosine (A208-4 and A208-7). The responses of each mutant to dbcAMP or CTX were also considerably diminished. Whereas the level of ODC in wild-type cells could be induced 10-fold in response to all these agents, mutants A208-4 and A208-7 showed only a small induction of ODC in response to any of these agents. Mutant A126-1B2 did not induce ODC in response to CTX, dbcAMP, or any concentration of PIA tested. Note that for all four of these lines the induction by CTX and dbcAMP was equivalent to that obtained with PIA (CTX and dbcAMP were used at concentrations that were saturating for the response; data not shown). We also found that in mutants A208-7 and A208-4, ODC activity was induced to wild-type levels in response to NGF. Mutant A126-1B2 can also induce ODC in response to NGF, but not to the same levels seen in wild-type cells.

TABLE 1. Growth inhibition of wild-type PC12 cells and variants of PC12 by dbcAMP, CTX, and CTX plus theophylline[a]

Cell line	IC_{50} for dbcAMP[b] (mM)	Doubling time (CTX + theophylline)/ doubling time (control)[c]	Doubling time (CTX)/doubling time (control)[c]
PC12	0.65 ± 0.25 (8)	1.82, 1.82	1.41, 1.47
A126-1B2	2.27 ± 0.62 (4)	0.86, 0.91	0.93, 0.99
A208-7	3.57 ± 1.20 (3)	1.14, 1.09	1.04, 0.99
A208-4	2.53 ± 1.28 (3)	1.02, 0.95	0.94, 1.01

[a] The growth of wild-type PC12 cells and the three mutant lines was determined in the presence of increasing concentrations of dbcAMP, as well as in the presence of CTX (3×10^{-11} M) of CTX plus theophylline (1 mM) as described in the text. Increasing the concentrations of CTX beyond 3×10^{-11} M did not further inhibit cell division.

[b] The IC_{50} for dbcAMP is defined as the concentration of dbcAMP that results in half the number of cells produced in the absence of additives. Shown is the mean ± standard deviation, with the number of determinations given in parentheses.

[c] The data on growth inhibition by CTX and theophylline are from two different experiments, each of which represents the average of two independent cultures of PC12 cells.

Characterization of cAMP-PKs in wild-type and mutant PC12 cells. The cAMP-PKs present in wild-type PC12 cells were assayed both by measuring the cAMP-dependent phosphorylation of histone by extracts prepared from PC12 cells and by affinity labeling of the regulatory subunits of the cAMP-PK. To measure histone kinase activity, PC12 cells were washed with phosphate-buffered saline, harvested, lysed, and fractionated on a DEAE column. The resulting fractions were assayed for their histone kinase activity. There were two peaks of protein kinase activity, one eluting at ca. 0.02 M NaCl and the second being removed from the column at ca. 0.11 M NaCl (Fig. 2A, bottom panel). For the columns that were run for the wild-type cells ($n = 4$) the ratio of the total activity of type II to the total activity of type I was 2.5. Although the protein kinase activity of both peaks was stimulated by dbcAMP, there was clearly a significant level of cAMP-independent protein kinase activity present in both regions. The protein kinase assay was shown to be linear with protein concentrations in the range used (data not shown).

The first and second peaks eluted from the DEAE column corresponded to cAMP-PKI and cAMP-PKII, respectively. The cAMP analog 8-N_3-[^{32}P]cAMP was used to covalently label the regulatory subunits of the cAMP-PK found in PC12 cells. PC12 cells were harvested, a high-speed supernatant was prepared, and the regulatory subunits were covalently labeled with 8-N_3-[^{32}P]cAMP. The proteins were then separated on SDS-polyacrylamide gels and autoradiographed as described above. One aliquot of the supernatant was not dialyzed (Fig. 3, lane A), whereas some of the supernatant (lane B) and the particulate fraction (lane C) was dialyzed overnight as described above. In addition, aliquots were taken from the first and second peaks of the DEAE column elution profiles shown in Fig. 2 and directly labeled with 8-N_3-[^{32}P]cAMP (Fig. 3, lanes D and E, respectively).

In the case of the extracts not subjected to DEAE column chromatography, two major bands (M_r = 47,000 and 54,000) and one minor band (M_r = 52,000) were apparent (Fig. 3, lanes A, B, and C). Although the minor band of 52,000 daltons was usually observed in fractions not subjected to

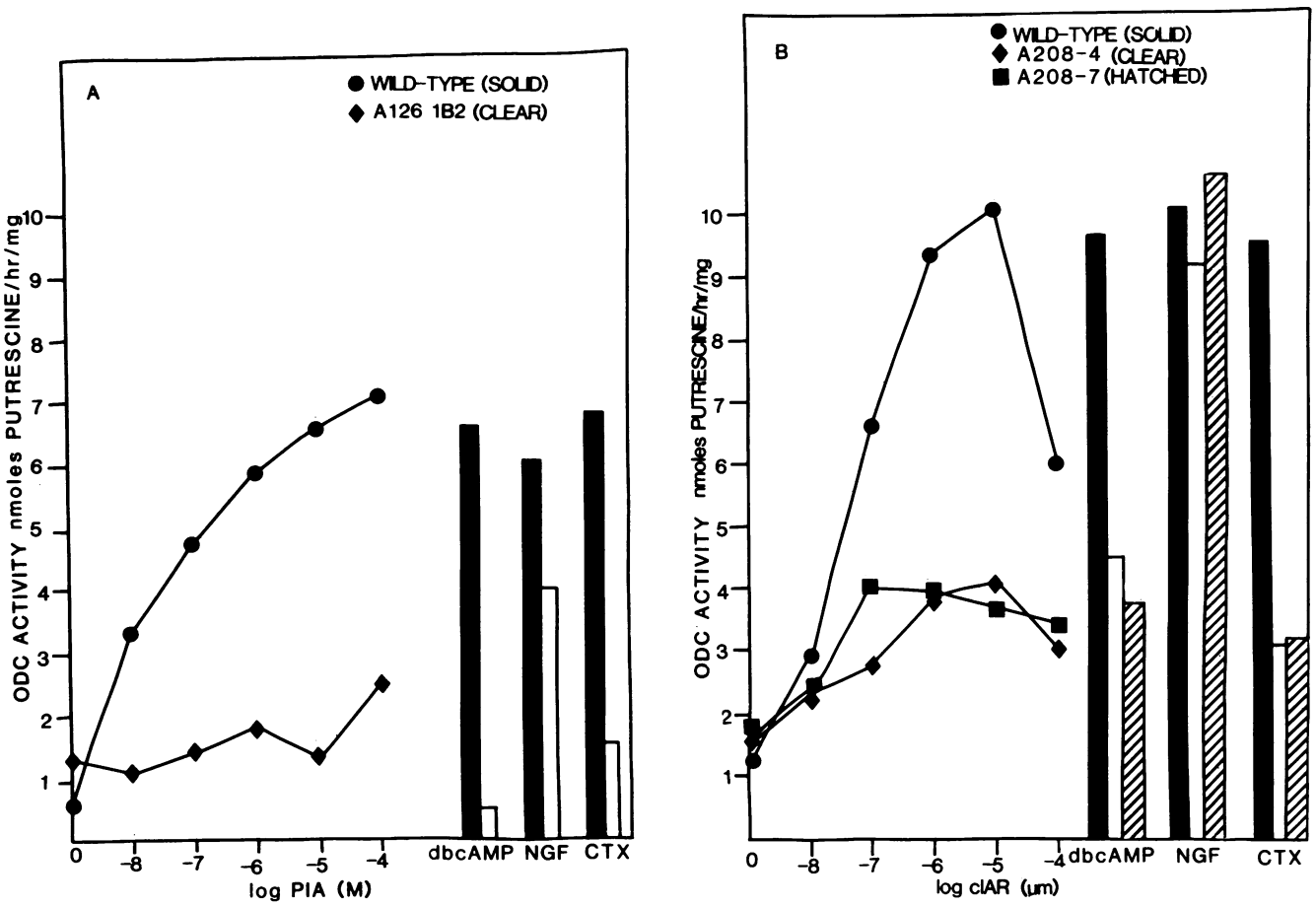


FIG. 1 Induction of ODC by PC12 cells and dbcAMP-resistant variants in response to adenosine agonists, dbcAMP, and NGF. Cultures of wild-type PC12 cells and PC12 mutants were exposed to either PIA (A) or chloroadenosine (clAR) (B) or to NGF (10 ng/ml), dbcAMP (100 μM), and CTX (3×10^{-11} M) for 5 h, and ODC activity was determined as described in the text. Wild-type PC12 cells and the mutant A126-1B2 are shown in (A), and wild-type PC12 cells and mutants A208-4 and A208-7 are shown in (B).

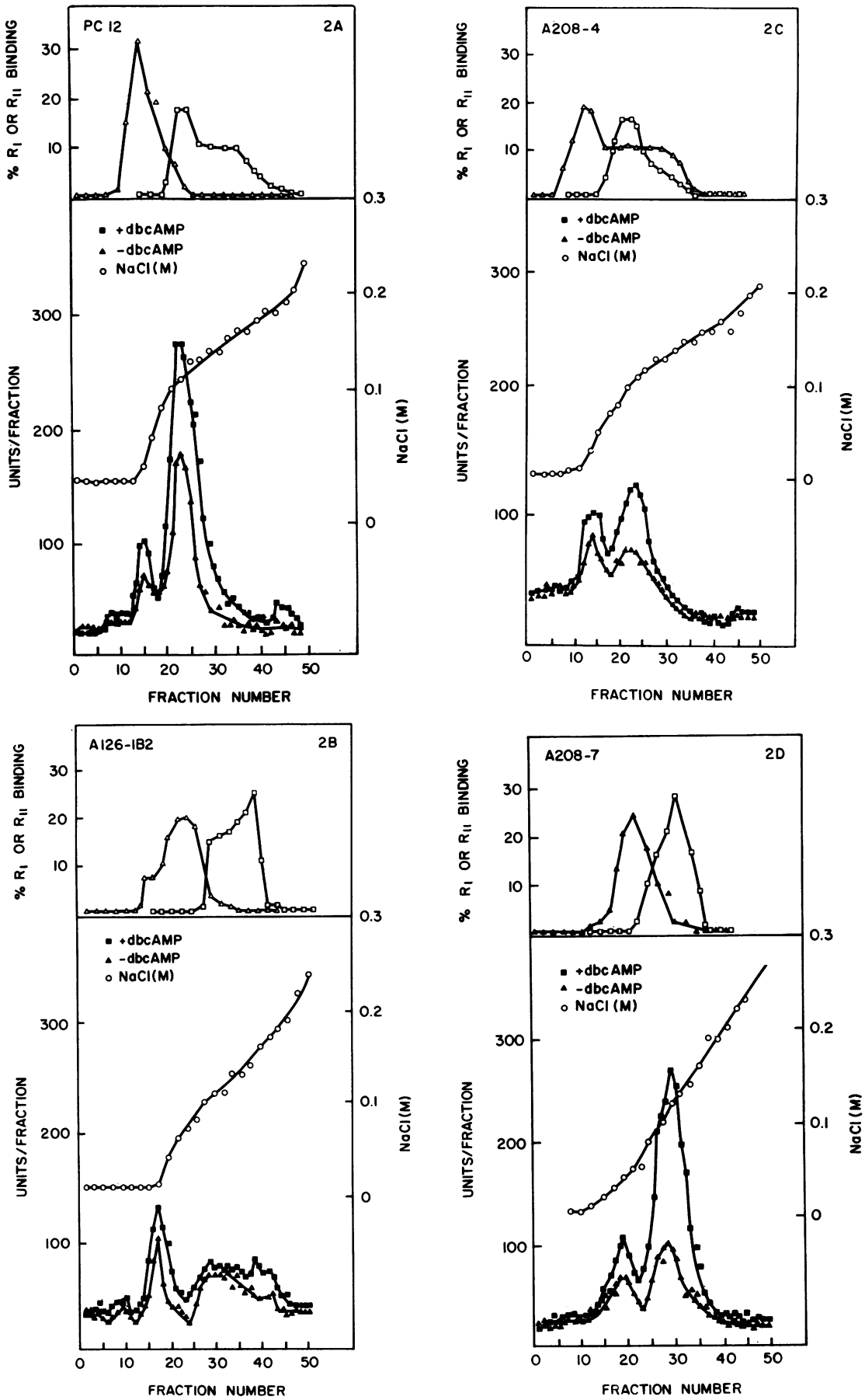
DEAE chromatography, it was usually not apparent in peak fractions chosen for 8-N_3-[^{32}P]cAMP covalent labeling from chromatography experiments (Fig. 3, lanes D and E). The 47,000-dalton labeled protein (designated the regulatory subunit of cAMP-PKI [RI]) corresponded to the first peak of histone kinase activity, and the 54,000-dalton protein (designated the regulatory subunit of cAMP-PKII [RII]) corresponded to the second peak of kinase activity (Fig. 2A). Thus, the designations cAMP-PKI and cAMP-PKII activities will be used.

To determine whether RI and RII copurify with the catalytic activity during DEAE chromatography, the odd-numbered fractions from the DEAE column were labeled with 8-N_3-[^{32}P]cAMP, and the intensity of labeling of both RI and RII was quantitated for each fraction by densitometry of the resulting autoradiographs and plotted in Fig. 2. Under the labeling conditions used, the intensity of labeling was linear with the amount protein (data not shown). In the case of wild-type cells (Fig. 2A), the peaks of the binding of 8-N_3-[^{32}P]cAMP to both RI and RII corresponded to the peaks of the type I and type II histone kinase activity, although the presence of some regulatory subunit at slightly higher salt concentrations than those of the corresponding catalytic activity may suggest the existence of some free RI or RII.

The cytosol fraction from the mutant line A126-1B2 was subjected to DEAE column chromatography and assayed for cAMP-PK and the presence of RI and RII (Fig. 2B). This mutant had a cAMP-PKI histone kinase activity level comparable to that of the wild type, but had a substantially depressed level of cAMP-PKII histone kinase activity. When the odd-numbered fractions were assayed with 8-N_3-[^{32}P]cAMP for the regulatory subunits of cAMP-PK, both RI and RII were found to be present in A126-1B2 extracts. The relative 8-N_3-[^{32}P]cAMP-binding characteristics of each of these subunits is shown in Fig. 2B. Unlike the results with wild-type cell extracts, the fraction showing maximal binding of 8_3-[^{32}P]-cAMP to RI did not correspond to the fraction showing maximal type I histone kinase activity, although there was clearly some RI in the fractions containing the cAMP-PKI enzymatic activity.

A second mutant, A208-4, was also deficient in histone kinase activity. Figure 2C illustrates that this mutant also possessed both type I and type II histone kinase activity; however, type II activity was diminished when compared with wild-type cells, so that type I and type II protein kinase activities were about equal in this mutant. The profile of 8-N_3-[^{32}P]cAMP binding to fractionated extracts of A208-4 cells was very similar to the wild-type profile. That is, the fraction with maximal 8-N_3-[^{32}P]-cAMP binding to RI contained the maximal type I histone kinase activity, and the fraction with maximal 8-N_3-[^{32}P]-cAMP binding to RII showed maximal type II kinase activity (Fig. 2C).

The clone A208-7 was little different from wild-type cells


PC 12
2A
A208-4
2C
A126-182
2B
A208-7
2D
% R_I OR R_{II} BINDING
+dbcAMP
-dbcAMP
NaCl(M)
UNITS/FRACTION
NaCl (M)
FRACTION NUMBER

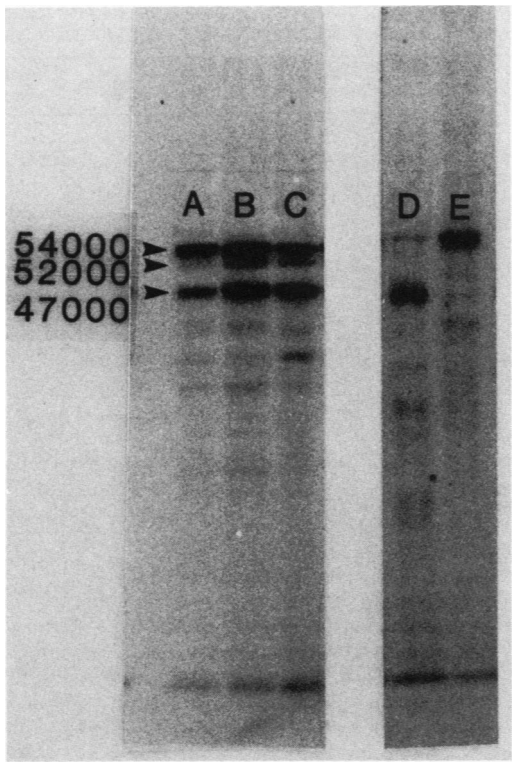


FIG. 3. 8-N_3-[^{32}P]cAMP labeling of RI and RII of wild-type PC12 cells. Lane A, A sample that was prepared under standard conditions but not dialyzed. Lane B, The same sample represented in lane A, but which was dialyzed overnight in Tris-phenylmethylsulfonyl fluoride buffer with 1 mM dithiothreitol. Lane C, The particulate fraction resulting from the centrifugation required to produce the crude supernatant used for the previous two samples. Samples in lanes A through C contained equivalent amounts of total protein. Lane D, A sample taken from the column fraction representing the peak of the type I histone kinase activity (fraction 15). Lane E, A sample taken from the column fraction representing the peak of the type II histone kinase activity (fraction 23). Samples represented in lanes D and E were not dialyzed. Samples were run on an SDS-polyacrylamide gel (12% acrylamide). The proteins designated 47,000 and 52,000-54,000 represent RI and RII, respectively.

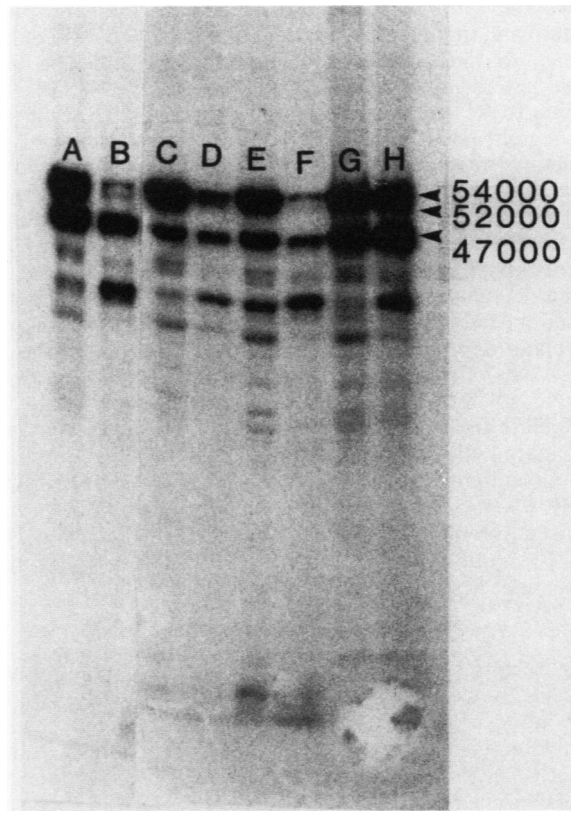


FIG. 4. 8-N_3-[^{32}P]cAMP labeling of RI and RII of wild-type PC12 cells and mutants A126-1B2, A208-4, and A208-7. All cell types were separated into cytosolic and particulate fractions and adjusted for protein content. Fractions were then dialyzed, incubated with 8-N_3-[^{32}P]cAMP as described in the text, and then separated on SDS-polyacrylamide gels (12% acrylamide). All cell lines except A208-4 were run on the same gel. Densitometry data from all cell lines, after normalization to the data from the wild type obtained from the same gel, appear in Table 2. Lanes: A, A208-4 supernatant; B, A208-4 particulate; C, A208-7 supernatant; D, A208-7 particulate; E, A126-1B2 supernatant; F, A126-1B2 particulate; G, wild-type supernatant; H, wild-type particulate.

when assayed for cAMP-dependent histone kinase activity (Fig. 2D). However, when the regulatory subunits of this mutant were covalently labeled with 8-N_3-[^{32}P]cAMP, the mutant was clearly distinguished from wild-type cells. The peak of RI was eluted at a salt concentration distinctly greater (0.08 M) than the peak of the type I catalytic activity, which eluted at 0.02 M NaCl.

To compare the binding characteristics of the regulatory subunits found in the supernatant and pellet fractions of these mutants, high-speed supernatants and particulate fractions prepared from wild-type, A126-1B2, A208-4, and A208-7 cells were affinity labeled with 8-N_3-[^{32}P]cAMP, separated on SDS-polyacrylamide gels, and autoradiographed (Fig. 4). At the protein concentration used, it was determined that there was a linear relationship between the amount of regulatory unit and 8-N_3-[^{32}P]cAMP bound when 10 μM of the photoaffinity ligand was used. The RI and RII analyzed by densitometry are shown in Table 2, which

FIG. 2. DEAE elution profiles of protein kinase activity and distribution of the RI and RII of wild-type and mutant PC12 cytosols. A total of 6.25 mg of protein in Tris-phenylmethylsulfonyl fluoride buffer was prepared from the various cells as described in the text. After the column was equilibrated, the protein was loaded in a volume of ca. 1 ml. A linear gradient of 0 to 0.3 M NaCl (total volume, 30 ml) was used to elute the proteins over the course of 2.5 h. After collection, 50-μl samples were assayed for cAMP-dependent histone kinase activity. Aliquots (70 μl) were used to determine the presence of RI and RII by using 8-N_3-[^{32}P]cAMP in every other fraction. For the latter determinations, samples were not dialyzed but otherwise prepared as described in the text and loaded on SDS-polyacrylamide gels (12% acrylamide). The resulting autoradiograms were scanned with a densitometer, and the areas under the peaks corresponding to RI and RII were determined by integration. The distribution of RI and RII in the column is plotted as a percentage of either RI or RII found in a particular fraction relative to the total RI or RII found in all fractions of the column. Symbols: for densitometry scans: △, RI; □, RII. DEAE elution profiles (bottom panel) of cAMP-PKI and cAMP-PKII and densitometry scans (top panel) of their respective regulatory subunits bound with 8-N_3-[^{32}P]cAMP are as shown. (A) Wild-type PC12 cells; (B) mutant A126-1B2; (C) mutant A208-4; (D) mutant A208-7.

shows the ratio of RI to RII binding in the supernatant fraction and in the pellet fraction, as well as the ratio of each species to the corresponding subunit in wild-type cells. There are significant differences in the 8-N_3-[^{32}P]cAMP binding to RI and RII among the cell types. The wild-type had significant RI and RII both in the supernatant and particulate fractions, and the binding levels of RI and RII were about equivalent. The mutant A126-1B2 had dramatically reduced levels of binding of RI and RII in the particulate fraction, but the ratio of RI to RII was not substantially different from that in wild-type cells in either fraction. The mutant A208-4 had wild-type levels of binding of both regulatory subunits in the supernatant, but RII binding was especially depressed in the particulate fraction. In the mutant A208-7 the binding to RI was significantly depressed relative to wild-type cells in both the supernatant and particulate fractions, but the binding to RII was decreased only marginally. We tentatively concluded from these data that all mutant lines have defects in either cAMP-PKI or cAMP-PKII, or both. Thus, the induction of ODC by cAMP requires functional cAMP-PKI and cAMP-PKII, but NGF does not induce ODC by causing an increase in cAMP and a subsequent activation of the cAMP-PKs.

TABLE 2. Integrated densitometry data from RI and RII of wild-type PC12 cells and clones A126-1B2, A208-4, and A208-7[a]

Cell line	Fraction	RI/RII	Mutant RI/ wild-type RI	Mutant RII/ wild-type RII
PC12	SUP	0.82		
	PART	1.26		
A126-1B2	SUP	0.48	0.45	0.76
	PART	2.0	0.13	0.08
A208-4	SUP	1.03	1.12	0.89
	PART	3.50	0.48	0.18
A208-7	SUP	0.37	0.35	0.78
	PART	0.40	0.20	0.61

[a] After normalization, integrated totals of RI and RII were recorded after densitometry scanning. SUP, Supernatant; PART, particulate.

DISCUSSION

By selecting mutants that are resistant to micromolar concentrations of dbcAMP, we were able to show here that NGF induces ODC activity through a pathway other than the adenylate-cyclase system. All mutants were shown to be (i) defective in their growth responses to CTX and dbcAMP, agents known to alter such characteristics in wild-type cells, (ii) defective in either cAMP-PKI or cAMP-PKII, or both, and (iii) unable to induce ODC activity to wild-type levels in response to the adenosine agonists chloradenosine or PIA. However, these mutant clones were clearly able to induce ODC in the presence of nerve growth factor.

Wild-type PC12 cells contain both cAMP-PKI and cAMP-PKII. The identification of RI and RII by using 8-N_3-[^{32}P]cAMP has been used by several workers (32, 34, 49). Our results with extracts of PC12 cells confirmed that the two peaks eluted from the DEAE columns had regulatory subunits that have molecular weights similar to those found in other systems. However, it is apparent that both types I and II eluted from our column at significantly lower salt concentrations than the cAMP-PKs reported by others (32, 49). It is unclear to us why there is such a large discrepancy. Possibly other proteins are binding to the cAMP-PKs in PC12 cells, thus altering the elution patterns of these kinases.

Whereas RI migrated as a 47,000-dalton band by SDS-polyacrylamide gel electrophoresis, RII appeared as 52,000- and 54,000-dalton proteins. These two bands have also been seen in 3T3-L1 cells (32) and N-18 mouse neuroblastoma cells (34), as well as bovine cardiac muscle (1). Although it has been shown that both the 52,000- and 54,000-dalton proteins can be phosphorylated (1), the function of the 52,000-dalton protein is unknown. It is of interest to note that the 52,000-dalton protein appeared consistently in both the supernatant and particulate fractions. Furthermore, it did not coelute with the 54,000-dalton band in our DEAE columns; but a protein of similar molecular mass, which bound significant amounts of 8-N_3-[^{32}P]cAMP, eluted from fractions 35 through 41 (unpublished data).

In all mutants we investigated there was a significant amount of RI eluting from the column at a higher salt concentration than that for the peak of the type I catalytic activity. This may represent RI not bound in the form of the holoenzyme. The absence of a corresponding peak of cAMP-dependent histone kinase activity in this region suggests that this may be "free" RI. A similar conclusion was reached by Aldao et al. (1) concerning the cAMP-binding protein of bovine cardiac muscle. The idea that free RI exists has been suggested in other systems. In wild-type and cAMP-resistant Chinese hamster ovary cells, free RI eluted between the type I and type II holoenzymes (44), and the concentration of free RI was found to be twice that of RI bound to the holoenzyme. In neuroblastoma cells Walter et al. (50) also reported a similar amount of RI not associated with the holoenzyme. The possibility that the unbound RI represents a sink for a pool of cAMP has been suggested (33). Free RII may also exist in wild-type PC12 cells. Figure 2A illustrates an experiment in which the peak of type II catalytic activity eluted near fractions 23 through 25. This also coincided with the peak of the 8-N_3-cAMP binding to RII (Fig. 2A). However, there was a significant amount of RII in wild-type cells which eluted at a higher salt concentration (Fig. 2A, fractions 28 through 45) than did the peak of 8-N_3-cAMP binding. This may represent free RII, since there was no corresponding plateau of catalytic activity in this area. It is of interest to note that all mutants studied lacked this later-eluting RII.

There have been a number of studies attempting to define precisely the different roles of the cAMP-PKI and cAMP-PKII by using mutants defective in cAMP-PK (19). However, a clear role for the different forms of cAMP-PK has not yet been established. The mutants we isolated and characterized are clearly defective in cAMP-PK, and these defects imparted a functional defect in the ability of the cells to induce ODC in response to agents that modulate cAMP levels. Determination of the exact nature of the defects will require a more detailed analysis of each mutant. A126-1B2 is certainly defective in its type II catalytic ability but has a wild-type level of binding of 8-N_3-[^{32}P]cAMP to RII. However, this mutant may also be defective in RI, since when cytosol supernatants from A126-1B2 were compared with those of wild-type cells, there seemed to be less 8-N_3-[^{32}P]cAMP binding to RI in the mutants. When fractionated on a DEAE column, the peak of binding for RI was displaced to the right and was not coincident with the type I cAMP histone kinase peak, as it was in wild-type cells. Although the biochemical defect that results in these alterations has not yet been established, one possible explanation would be that this mutant carried a defect in the catalytic subunit of cAMP-PK that altered its affinity for both RI and RII. This mutant was unable to induce ODC in response to any concentration of PIA or dbcAMP. This indicates strongly

that cAMP-PKI is not sufficient for the induction of ODC in response to increased cAMP, and it strongly suggests a central role for cAMP-PKII in the induction of ODC. In contrast, this mutant could induce ODC in response to NGF, suggesting that induction of ODC by NGF does not require cAMP-PKII.

The dbcAMP-resistant mutant A208-4 was resistant to the induction of ODC by the cAMP-dependent pathway, but induced ODC normally in response to NGF. This again suggests that NGF does not induce ODC by activating adenylate cyclase. Mutant A208-4 had apparently normal amounts of cAMP-PKI, but had depressed level of cAMP-PKII; cytosolic RI and RII were present at wild-type levels. It is possible that decreased population of catalytic subunit would be sufficient to explain the properties of this mutant, but it is not clear how a modest reduction in cAMP-PKII could prevent induction of ODC. When compared with wild-type PC12, the mutant A208-7 had nearly equal levels of cAMP-PKI and cAMP-PKII catalytic activities, but it appeared to be defective in the association of RI with the catalytic subunit. Furthermore, the cAMP-PKII from mutant A208-7 cells eluted at a higher salt concentration than did wild-type PC12 and mutant A208-4 type II protein kinases. This difference could be due to altered regulatory or catalytic subunits, or both. However, since it has been recently suggested that in the case of lung cytosol (6) proteins may adhere to the cAMP-PKII and consequently alter its affinity for cAMP, the observation that type II protein kinase of mutant A208-7 eluted later than that of the wild type might also be due to an alteration in its association with other cytosolic proteins. Such associations are known to exist with calcineurin (26), as well as microtubule-associated protein 2 (47). These defects in the cAMP-PKs of mutant A208-7 may be sufficient to inhibit the induction of ODC by PIA and cAMP agonists, but not by NGF.

Finally, it is unclear why 100 μM dbcAMP gave more consistent results than 5 μM cAMP when asaying for cAMP-PK activity by using DEAE fractions. Whereas we got comparable levels of cAMP-PK activity with 5 μM cAMP, there was much more experiment-to-experiment variability that was significantly diminished when 100 μM dbcAMP was used. Possibly there was a potent phosphodiesterase present in PC12 cells which had a variable elution pattern, thus resulting in inconsistent data when cAMP was used as the ligand.

cAMP is an important regulator of many eucaryotic functions. In the case of PC12 cells, one function of this nucleotide is to regulate ODC activity through cAMP-PKs. Development and characterization of exclusive type I and type II mutants should lead to elucidation of the different roles of the two classes of protein kinases in the many functions regulated by NGF and cAMP.

ACKNOWLEDGMENTS

We thank Adah Levens for preparing the manuscript and Roger Felix for providing technical assistance.

This work was supported by Public Health Service grant NS17283 from the National Institutes of Health to J.A.W., and R.V.B. is the recipient of Public Health Service fellowship (AM07223) from the National Institute of Health.

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