Nerve Growth Factor-induced Neuronal Differentiation after Dominant Repression of Both Type I and Type II cAMP-dependent Protein Kinase Activities*

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Clonal PC12 lines deficient in cAMP-dependent protein kinase (PKA) were made by stably expressing mutant regulatory subunits (RI) of PKA that are deficient in cAMP binding (Correll, L. A., Woodford, T. A., Corbin, J. D., Mellon, P. L., and McKnight, G. S. (1989) J. Biol. Chem. 264, 16672–16678). Expression of the mutant RIs repressed cAMP-dependent activation of both PKAI and PKAII while having no effects on the cAMP binding to either free RI or RII or the level of catalytic subunit protein. These data suggest that RI and RII compete for the same pool of catalytic subunit and that the level of PKAI and PKAII are interdependent.

We have used these cell lines to examine the requirement for PKA in mediating the effects of nerve growth factor (NGF) and agents that are thought to act exclusively via cAMP-dependent pathways. While several responses to cAMP were strongly compromised in these lines, NGF-dependent responses were comparable in parental and PKA-deficient cells, including: 1) protein phosphorylation, 2) transcriptional induction of the immediate early gene egr1, 3) expression of the gene for GAP-43, 4) induction of ornithine decarboxylase activity, and 5) formation of neurites. Furthermore, transient expression of the cAMP-dependent protein kinase inhibitor (RSVPKI; Day, R. N., Walder, J. A., and Maurer, R. A. (1989) J. Biol. Chem. 264, 431–436) blocked cAMP, but not NGF, induction of regulatory elements derived from the gene for egr1. These experiments support the idea that NGF can regulate neuronal differentiation by pathways that are independent of cAMP-activatable PKA.

Nerve growth factor (NGF) is a polypeptide growth factor

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The abbreviations used are: NGF, nerve growth factor; PKAI, type I cAMP-dependent protein kinase; PKAII, type II cAMP-dependent protein kinase; RI, regulatory subunit of PKAI; RII, regulatory subunit of PKAII; C, catalytic subunit of PKA; PKC, protein kinase C; PKI, heat-stable inhibitor of PKA; Bt,cAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; MES, 2-(N-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ODC, ornithine decarboxylase; kb, kilobase(s).

which plays a role in development of both the peripheral and central nervous system (1). Many studies have focused on the molecular mechanism of action of NGF, and a number of the signal transduction pathways and protein kinases that are activated by NGF have been established. In many cases it has been difficult to determine which of the signal transduction pathways are essential for the expression of specific aspects of the neuronal phenotype, and a consensus about the nature of the key signal transduction events regulated by NGF has not yet been established (2).

PC12 cells, cloned from a rat pheochromocytoma (3), are an excellent model system for studying molecular mechanisms involved in the actions of NGF. PC12 cells respond to NGF by acquiring many characteristics of mature sympathetic neurons (for a review, see Ref. 4). In this system, several putative signal transduction pathways have been implicated in mediating the intracellular components of the NGF signal including protein kinase C (5), phospholipid metabolism (6, 7), and the cAMP-dependent protein kinases (PKAs). NGF also activates a number of other kinases in PC12 cells including N-kinase, MAP2 kinase, a proline directed kinase, S6 kinase (8–11), and tyrosine kinase activity of the trk proto-oncogene product (12, 13) which appears to be a receptor for NGF (13).

The question of whether PKA plays a crucial role in NGF-induced neuronal differentiation has remained controversial. PKA is thought to play a role in NGF-mediated induction of tyrosine hydroxylase phosphorylation (14), fast Na+ channel activity (15), and down-regulation of calmodulin-dependent kinase III activity (16). Likewise, the same set of proteins are phosphorylated in response to NGF and cAMP analogues (17). NGF and Bt,cAMP both elicit neurite formation by PC12 cells, but these neurites appear to be different both in their length and in the mechanism by which they are formed (18). In support of this conclusion, NGF-dependent neurite outgrowth appears to be independent of PKAII (19), and NGF-dependent protein phosphorylation and neurite outgrowth appear to be insensitive to a membrane permeable inhibitor of PKA (20). Increased cAMP levels have been reported in response to NGF by some, but not all, laboratories (21–24), and NGF does not directly activate adenylate cyclase (25). Finally, direct measurement of the activation of PKA by NGF has led to contradictory conclusions (16, 26). Thus, the putative role of PKA in NGF signal transduction has not been fully resolved.

PKA is a tetramer consisting of two regulatory and two catalytic subunits. Upon binding of cAMP to the regulatory subunit, the holoenzyme dissociates to yield a regulatory subunit dimer and two free catalytic subunits which are available to phosphorylate cellular substrates (for review see Ref. 27). Two types of PKA, type I and type II, have been...
described based on their order of elution from anion exchange columns. These enzymes differ in their regulatory subunits, termed RI and RII. RII, but not RI, is autophosphorylated (28) and RI has a high affinity binding site for MgATP (29).

To help establish the potential roles for PKA in PC12 cells, PKA-deficient PC12 cells have been isolated and studied (19, 30, 31). This approach is similar to that taken in other cell systems to study the role of PKA in normal physiological processes (32-36). The most extensively studied of the PKA-deficient PC12 mutants, the A126-1B2 cell line, was isolated after nitrosoguanidine mutagenesis (30). Although A126-1B2 cells have decreased PKAII, PKAI activity levels appear normal, so it remains possible that PKAI may suffice for the requirement for PKA in some of the responses that have been studied. Experiments using these mutants suggest that PKAII is not essential for NGF-dependent neurite outgrowth or ODC induction (19, 30), but PKA does seem to play a significant role in the induction of an early response gene, egr1, in PC12 cells that are deficient in PKC (31). In those experiments, inositol phosphate, remains difficult because there are pronounced differences between A126-1B2 cells and PC12 cells that might not be fully explained by the deficiency of PKAII in this line. Unlike PC12 cells, A126-1B2 cells have a somewhat flattened morphology when grown on tissue culture plastic, and they respond synergistically to the combination of phorbol esters and NGF with the formation of rapid process outgrowth (19).

In addition, unlike parental PC12 cells, A126-1B2 cells also form processes in response to retinoic acid. Because the genetic defects in A126-1B2 cells is not known, the study of cell lines with defined lesions in PKA would provide more clearly interpretable data regarding the role PKA in signal transduction by NGF.

In this report, PC12 cells were rendered PKA-deficient by either stably expressing mutant regulatory subunits of PKAI or by transiently expressing the cAMP-dependent protein kinase inhibitor, PKI. Both approaches render recipient cells insensitive to stimulation by cAMP, thus these cell lines are good model systems in which the requirement for the PKAs in NGF responses can be critically assessed. Using these PKA-deficient cells, we examined the role of PKA in the regulation of several NGF- and cAMP-inducible parameters characteristic of the sympathetic neuronal phenotype.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Transfections, and Isolation of PKA-deficient Cell Lines—Cells were grown in DMEM containing 0.45% glucose supplemented with 10% fetal bovine and 5% horse serum in a humidified 10% CO2 environment. Media was changed every 2-3 days, and cells were harvested and subcultured once a week. After transfection, media was exchanged every 2 days, and cells were washed once with 2 ml of PBS, the blot was incubated for 1 h with 10 ml of PBS containing 2% nonfat dry milk and 5 µl of antibody against C subunit for 2 h at 4°C. Protein concentration in the cell lysates was determined with BCA protein assay (Pierce) and standardized to 50 ug/ml histone H1-S in either the presence or absence of 0.5 µM CaCl2, 10 µM diocanoyl glycerol, and 80 µg/ml phosphatidylcholine in a final volume of 0.5 µl. 0.5 µM EGTA was also used to test if containing CaCl2. The reaction was initiated by the addition of cAMP. Following incubation for 2 min at 30°C, 20 µl of the reaction was spotted onto P81 phosphocellulose strips and phosphopeptides quantitated after washing three times in 10 ml of 75 mM phosphoric acid as previously described (42). In some experiments PkA activity was determined in unfractionated 100,000 x g supernatant from these experiments using 40 µg ATP was used.

Protein kinase C (PKC) activity was determined in the 100,000 x g supernatant fraction of cell lysates. Reactions were carried out in 20 nm Tris-HCl, pH 7.5, 10 mM MgAc, 60 µM ATP, 5.6 nM [γ-32P]ATP (300-600 Ci/mmol), 200 µg/ml histone H1-S in either the presence or absence of 0.5 µM CaCl2, 10 µM diocanoyl glycerol, and 80 µg/ml phosphatidylcholine in a final volume of 0.5 µl. 0.5 µM EGTA was added to samples not containing CaCl2. The reaction was initiated by the addition of enzymes, allowed to proceed 2 min at 30°C, and 25 µl was spotted onto P81 paper strips and radioactivity determined (42). PKC activity was reported as the difference in the amount of [γ-32P]ATP phosphorylated in the presence and absence of CaCl2, diocanoyl glycerol, and phosphatidylserine.

**cAMP Binding**—[3H]cAMP binding was performed after fractionation of extracts on a Mono Q column as described (43).

*Western Blotting—Cell lysates were prepared as above, 150 µg of proteins was separated on 12% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose by electrotransfer (44). After transfer, blots were blocked with 5% nonfat dry milk in PBS for 2 h, and then incubated in 10 ml of PBS containing 2% nonfat dry milk and 5 µl of antibody against C subunit for 2 h at room temperature. After rinsing the filter four times for 5 min with PBS, the blot was incubated for 1 h with 10 ml of PBS containing 2% nonfat dry milk and 1 x 106 cpm [32P]protein A. The filters were then washed four times for 5 min with PBS and subjected to autoradiography.

*Reporter Gene Construct—An egr1 genomic clone was isolated from a rat genomic library in EMBL3. The egr1 clone was digested with BamHI and SacI, and cloned into PBLCAT3 (45) after the addition of BamHI linkers. This construct (egr1CAT) contains nucleotides -380 to +100 of the rat egr1 gene (relative to the transcriptional start) and this sequence has been inserted upstream of the coding region for the CAT gene in the puc18 vector (Pharmacia LKB Biotechnology Inc.) preequilibrated with TE. Proteins were eluted with a 0-0.5 M linear gradient of NaCl in TE at a flow rate of 1.0 ml/min. 0.5-ml fractions were collected and analyzed in each fraction in both the presence and absence of 5 µM cAMP. The reaction mixture contained 24 mM MES (pH 7.0), 4.8 mM MgAc, 2.3 mM NaN3, 0.1 mM Kemptide (Leu-Arg-Ala-Ser-Leu-Gly), 60 µM ATP, 5.6 nM [γ-32P]ATP (300-600 Ci/mmol), and cytosol to a final volume of 52.5 µl. The reaction was initiated by the addition of cytosols. Following incubation for 2 min at 30°C, 20 µl of the reaction was spotted onto P81 phosphocellulose strips and phosphopeptides quantitated after washing three times in 10 ml of 75 mM phosphoric acid as previously described (42). In some experiments PkA activity was determined in unfractionated 100,000 x g supernatant from these experiments using 40 µg ATP was used.

2 Scheibe, R. J., Ginty, D., and Wagner, J. A. (1991) J. Cell Biol. 113, 1173-1182.
Neuronal Differentiation in PKA-deficient Cells

**RESULTS**

Isolation and Characterization of PKA-deficient Cells—To obtain cell lines specifically deficient in PKA activity, PC12 cells were transfected with either the vector HLREV1+2neo or the vector HLREVABneo, each of which is designed to express a mutant regulatory subunit of PKA (36). HLREV1+2neo encodes a protein containing two point mutations in the site B cAMP binding site while HLREVABneo expresses a mutant regulatory subunit of PKAI (36). PKAI (open symbols) and PKAII (closed symbols) for PC12 (circles) and 123.7 cells (squares). Hill plots of the same data are shown as an inset. These data are typical of three to five fractionations and the average levels of cAMP binding and PKA activity are shown in Table 1.
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**TABLE I**

Cyclic AMP binding, PKA activity, EC<sub>50</sub> values for PKAI and PKAII, catalytic subunit protein levels, and protein kinase C activity in PC12, 123.7, and AB.11 cells

|                      | RI(PKAI) | Free RII | RII | PKAI | PKAII | PKAI | PKAII | EC<sub>50</sub> | C Subunit protein | Protein kinase C activity |
|----------------------|----------|----------|-----|------|-------|------|-------|----------------|--------------------|------------------------|
| **PC12**             |          |          |     |      |       |      |       |                |                    |                        |
| Mean                 | 0.16     | 0.37     | 0.61| 1.19 | 6.60  | 0.18 | 0.17  | 100            | 0.37               |                        |
| ± S.E.               | 0.04     | 0.09     | 0.08| 0.22 | 0.45  | 0.01 | 0.01  |                |                    |                        |
| **123.7**            |          |          |     |      |       |      |       |                |                    |                        |
| Mean                 | 0.02     | 0.47     | 0.63| 0.15 | 1.03  | 20.40| 0.18  | 98             | 0.43               |                        |
| ± S.E.               | 0.01     | 0.07     | 0.06| 0.12 | 0.16  | 1.47 | 0.01  |                |                    |                        |
| **AB.11**            |          |          |     |      |       |      |       |                |                    |                        |
| Mean                 | 4.4      | 4.4      | 3.3 | 3.3  | 3.3   | 3.3  | 3.3   | 2.3            | 2.3                |                        |
| **123.7/PC12**       | 0.14     | 1.26     | 1.03| 0.12 | 0.16  | >110 | 1.05  | 0.98           | 1.16               |                        |
| **AB.11/PC12**       | <0.10    | 1.43     | 1.25| <0.10| 0.24  | 1.05 | 0.90  |                |                    |                        |

Parental PC12, 123.7, and AB.11 cell homogenates were separated by fast protein liquid chromatography on a Mono Q column. Cyclic AMP binding and PKA activity were determined in fractions 20-50 as seen in Fig. 2 (see also "Experimental Procedures"). EC<sub>50</sub> is the concentration (μM) of cAMP required to stimulate half-maximal activation of PKA. [3H]cAMP binding and PKA activity were determined from integrated peaks and are expressed as % [3H]cAMP bound and [32P]ATP incorporated into Kemptide per mg protein loaded onto the column, respectively. Catalytic subunit protein levels were determined by Western analysis as described under "Experimental Procedures" and are expressed as percent of PC12 cell controls. Protein kinase C activity was determined in 100,000 x g supernatants of cell homogenates. The level of C subunit protein was not shown) or total RI and RII binding activity.

The [3H]cAMP binding activity of PC12 cells elutes in three peaks (Fig. 1C). The first peak (0.13 M NaCl, fractions 24-25) consists of RI which is associated with catalytic subunit. The second peak (0.22 M NaCl, fractions 28-31) includes both free RI and proteolytic fragments of RI. The third peak (0.34 M NaCl, fractions 35-40) includes both RII which is associated with C as well as free RII. The 123.7 and AB.11 cells appear to contain normal levels of RII (peak 3) and free RI (peak 2) as determined by [3H]cAMP binding, but the level of [3H]cAMP binding associated with PKAI (peak 1) is reduced reflecting the reduced ability of the holoenzyme that contains RII<sub>123.7</sub> to bind cAMP. As expected, [3H]cAMP binding of free RII elutes at approximately the same salt concentration as the PKAI holoenzyme.

To more fully understand the state of PKA in the stably transfected 123.7 cells, the ability of increasing concentrations of cAMP to activate PKAI and PKAII that had been partially purified by fractionation on a Mono Q column was determined. The EC<sub>50</sub> values, i.e. concentrations of cAMP necessary for half-maximal activation of PKA, for PKAII and PKAI are 0.18 and 0.17 μM, respectively, in parental PC12 cells (Fig. 1D and Table I). The EC<sub>50</sub> for PKAII (0.18 μM) in 123.7 cells is comparable to the EC<sub>50</sub> in parental cells, although the absolute level of kinase activity that can be liberated by saturating levels of cAMP is reduced more than 80% (Table I). In contrast, the EC<sub>50</sub> for PKAI in 123.7 cells (＞20 μM) is more than 2 orders of magnitude larger than that found in parental cells. In addition, Hill coefficients for PC12 PKAI, PC12 PKAII, and 123.7 PKAII were 1.33, 1.55, and 1.31, respectively (Fig. 1D, inset), suggesting positive cooperativity as has been previously described for this enzyme (see Ref. 27). In contrast, 123.7 PKAI holoenzyme had a Hill coefficient of 1.36 (Fig. 1D, inset) indicating lack of cooperativity as previously shown for RI<sub>123.7</sub>-containing PKA by Correll et al. (98). Thus, the mutant RI<sub>123.7</sub> protein is translated in 123.7 cells, associates with the catalytic subunit, and elutes at approximately the same salt concentration as the mutant PKA holoenzyme from parental PC12 cells. The dramatic shift in the EC<sub>50</sub> of this holoenzyme is consistent with the alterations in activity and cAMP binding described previously for mutant RI<sub>123.7</sub> (36). The PKAII remaining in both 123.7 cells and AB.11 cells responds to normal concentrations of cAMP (Table I), although the V<sub>max</sub> is greatly reduced suggesting that the mutant PKI probably sequesters the catalytic subunit in a dominant manner from both wild type RI and RII. Finally, although there is a great reduction in the level of PKAI and PKAII activity in 123.7 and AB.11 cells, there is no change in the expression of endogenous RI<sub>a</sub> mRNA, RII<sub>b</sub> mRNA or catalytic (Ca) subunit mRNA (data not shown) or total RI and RII binding activity.

Western blots were performed to detect the relative amounts of catalytic subunit protein in PC12 and PKA-deficient 123.7 and AB.11 cells. There was no difference in the level of C subunit protein in any of these clones (Table 1). Thus, no appreciable change in the expression of the endogenous regulatory or catalytic subunits has occurred in response to the kinase deficiency.

Role of PKA in NGF-mediated and cAMP-dependent Protein Phosphorylation—cAMP analogues and NGF can stim-

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3 These fractions contain both RI and a 29-kDa fragment of RI which is capable of binding cAMP but is uncapable of binding the catalytic subunit (R. Van Buskirk and J. A. Wagner, unpublished data).
ulate the phosphorylation of an identical set of cellular substrates, as detected at the level of a one-dimensional protein gel (17). To help determine the role of PKA in NGF and cAMP-mediated phosphorylation events, phosphorylation of endogenous proteins was examined in parental PC12 cells and PKA-deficient cells. Fig. 2A depicts the results of a typical experiment in which PC12 or PKA-deficient 123.7 cells were treated with no addition (control), 1.0 mM Bt2cAMP, or NGF (100 ng/ml) for 1 h in the presence of [32P]orthophosphate. The average induction of 32P incorporation into four major phosphoproteins in four experiments is summarized in Fig. 1B. The data are presented as the average degree of 32P content in the protein band of interest normalized for 32P content of a 38-kDa protein band whose phosphorylation does not change in the presence of the agents used. In parental cells, Bt2cAMP stimulated the phosphorylation of tyrosine hydroxylase, ribosomal S6, and histone H2b 2–3-fold. This effect was nondetectable in the PKA-deficient 123.7 clone. Thus, 123.7 cells are deficient in PKA activity when the intact cell is challenged with membrane permeable cAMP analogues (Fig. 2) or when the cells are assayed for kinase activity in cell homogenates (Fig. 1 and Table I). Equivalent results have been seen with cells treated with 10 μM forskolin or when another PKA-deficient cell line, AB.11, was used (data not shown). In contrast, NGF induced the phosphorylation of TH, S6, a 25-kDa protein (which is not phosphorylated in response to Bt2cAMP) equivalently in PC12 cells and in the PKA-deficient 123.7 cells. Thus, NGF-mediated phosphorylation of at least three endogenous substrates occurs independent of PKA presumably reflecting the ability of NGF to activate other kinases (8–11). In contrast to previous work (17), our data shows two differences between the NGF- and cAMP-dependent phosphorylations: histone H2B is phosphorylated in response to Bt2cAMP, but not NGF; while p25 is phosphorylated in response to NGF, but not Bt2cAMP.

Role of PKA in Expression of egr1 and GAP-43—To determine the role of PKA in NGF-stimulated gene expression either Bt2cAMP or NGF was added to parental and kinase deficient cells and Northern analysis performed. egr1 is a gene whose expression is rapidly induced at the transcriptional level in PC12 cells (48, 49). Likewise, egr1 was induced by NGF in 123.7 cells (Fig. 3A). The PKA deficiency of the 123.7 cells does not affect either the time course or magnitude of this induction. In contrast, 123.7 cells had greatly attenuated cAMP responsiveness as compared to PC12 cells (Fig. 3B). In parental cells, Bt2cAMP induction of egr1 was half maximal at a concentration of less than 0.1 mM, while Bt2cAMP stimulated only small increases in expression of this gene in 123.7 cells even at a concentration of 1 mM (Fig. 3B). Because PKA-deficient cells do not respond to Bt2cAMP, yet do respond to NGF, it follows that the NGF induction of egr1 is independent of activatable PKA.

To determine the effect of PKA deficiency on the expression of a gene that is induced after a delay, we chose to examine induction of GAP-43 in PKA-deficient PC12 cells. GAP-43 is strongly induced by NGF and this induction seems to occur at a posttranscriptional level (50). Fig. 4 is a graphic representation of a Northern analysis in which parental or 123.7 cells were exposed for varying periods of time to Bt2cAMP or NGF. The relative inductions of GAP-43 seen here are comparable to those seen by others in response to NGF (50). Exposure to Bt2cAMP increased GAP-43 expression in parental cells, but not 123.7 cells. In contrast, NGF induced expression of the gene in both cell lines indicating that this NGF event also occurs independent of PKA. Thus, NGF does not require PKA to activate representative early (egr1) or late (GAP-43) gene expression.

Effects of PKA Deficiency on the NGF- and cAMP-mediated Increases in ODC Activity—ODC activity is rapidly induced in response to both NGF and Bt2cAMP in PC12 cells. To determine if PKA influenced the induction of an enzyme
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15330

A.

PC12

123.7

Time after NGF, hr

0 1 5 10 24 96

0 1 5 10 24 96

3.3 Kb

1.8 Kb

egr1

alpha tubulin

B.

PC12 Cells

123.7 Cells

dbCAMP Concentration, mM

0 0.4 0.8 1.2 1.6 2.0 2.4 2.8 3.2 3.6 4.0

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0

GAP3 alpha tubulin (Relative OD Units)

Fig. 3. NGF, but not dbCAMP, induces egr1 expression in PKA-deficient cells. A, RNA was isolated from cells treated with NGF for indicated times and Northern analysis performed. The filter was probed for egr1 and reprobed for a-tubulin as a control for the amount of RNA loaded per lane. Size determinations were made by comparison of the filter with ethidium bromide-stained RNA ladder (BRL). B, dihydrocAMP dose-dependently increased the expression of egr1 in PC12 cells but not 123.7 cells. Cells were treated with indicated concentrations of dibutyryl cAMP for 1 h and Northern analysis performed as above. The autoradiograph was scanned by densitometry and the area under the curve for the egr1 signal was divided by that of a-tubulin.

activity by NGF both PC12 and the PKA-deficient AB.11 cells were exposed to either dbCAMP, cholera toxin (a potent activator of adenylate cyclase), or NGF for 5 h. Parental PC12 cells responded with a 10–15-fold induction of specific activity of ODC after treatment with dbCAMP, cholera toxin, or NGF (Fig. 5). In contrast, AB.11 cells responded to NGF but to none of the other agents, demonstrating that there is insufficient PKA activity in these cells to induce ODC by this pathway. Thus, NGF induction of ODC activity also occurs in the absence of PKA, supporting the idea that NGF can induce this enzyme by a PKA-independent pathway.

Role of PKA in Morphological Differentiation—Both NGF and cAMP agonists induce neurite outgrowth in PC12 cells, although the cAMP-induced processes never become as long as those induced by NGF (18, 51). Therefore, it was of interest to examine the role of PKA in NGF-mediated neurite outgrowth. Fig. 6 shows the effects of NGF and dbCAMP on neurite outgrowth in PC12, 123.7, AB.11, and 123.3 cells. The 123.3 cells were selected for G418 resistance after transfection with HLREV,neo. Although they were subjected to the same transfection protocol as 123.7 and AB.11 cells, they did not express mutant RI and had normal levels of PKA activity (data not shown). Untreated PC12, 123.3, 123.7, and AB.11 cells have a similar morphology, indicating that PKA deficiency does not itself have a dramatic effect on cell morphology. When treated with dbCAMP, both PC12 and 123.3, but neither 123.7 or AB.11, formed neurites within 2 days. Similar results were observed with cholera toxin (not shown). In contrast, all four clones produced neurites when exposed to NGF for 5 days. The fraction of each cell type that formed a process in response to NGF or dbCAMP is presented in the legend to Fig. 6.

To determine the effects of NGF on the activity of PKA in cells expressing mutant regulatory subunits, PC12 and 123.7

Fig. 4. NGF induces GAP-43 in both PC12 and PKA-deficient 123.7 cells. RNA was isolated from PC12 cells (circles) or 123.7 cells (squares) that had been exposed to 100 ng/ml NGF (open symbols) or 1.0 mM dbCAMP (closed symbols) for various times as indicated in the figure. Northern analysis was used to quantitate the expression of GAP-43 and a-tubulin as described under "Experimental Procedures." Densitometric analysis of the expression of GAP-43 mRNA which has been normalized to a-tubulin mRNA is shown.

Fig. 5. NGF induces ODC activity in both PC12 and PKA-deficient 123.7 cells. PC12 cells (open bars) or AB.11 cells (closed bars) were exposed to either dbCAMP (1.0 mM), cholera toxin (3 X 10^-11 M), or NGF (100 ng/ml), and the activity of ODC was measured as described under "Experimental Procedures." These data are the means ± S.E. of three separate experiments.
cells were treated with NGF for 24 h and PKA activity measured (Fig. 7). PKA activity in parental cells was not affected by NGF treatment, however, NGF treatment of 123.7 cells led to a greater decrease in PKA activity. Similar results were seen in cells treated with NGF for 48 h as well as another PKA-deficient clone (data not shown). This effect may be explained by the observation that NGF markedly increased the expression of the mutant RI, but not endogenous RI, mRNA level (Fig. 7A). Regardless, these experiments demonstrate that cells expressing mutant regulatory subunits remain PKA deficient after NGF treatment. Therefore NGF-dependent neurite outgrowth is not dependent on PKA, while PKA is required for the formation of neurites in response to Bt2cAMP or cholera toxin.

**Effects of Inhibition of PKA with RSVPKI in a Transient Expression System**—As an alternative approach to test our conclusion that NGF is not dependent on PKA for the induction of certain genes, PC12 cells were transiently transfected with a vector that expresses PKI (the heat-stable inhibitor or the Walsh inhibitor), which binds with high affinity to the free catalytic subunit and competitively inhibits kinase activity (52,53). RSVPKI is an expression vector that encodes the full length PKI, and RSVPKI is an equivalent expression vector. The most likely explanation for the effect of the expression of the mutant RI on PKAI and PKAII activity but had no effect on the expression of other subunits of PKA examined. The most likely explanation for the effect of the expression of the mutant RI on PKAI activity is that the mutant RI sequestered catalytic subunit from both endogenous RI and endogenous RII. If this explanation is correct, this means that RI and RII compete for the same pool of catalytic subunit, and that pool is in limiting supply. The most obvious alternative explanations for the decrease in the level of PKAII are inconsistent with our data. For example, the reduced level of PKAI does not appear to effect the level of PKAII within the cell.

**DISCUSSION**

We have described the effects of expression of mutant regulatory subunits of PKA on PKAI and PKAII holoenzymes and the subsequent effects of PKA deficiency on NGF and cAMP responsiveness in PC12 cells. Expression of the mutant RI subunits in PC12 cells repressed both PKAI and PKAII activity but had no effect on the expression of other subunits of PKA examined. The most likely explanation for the effect of the expression of the mutant RI on PKAI activity is that the mutant RI sequestered catalytic subunit from both endogenous RI and endogenous RII. If this explanation is correct, this means that RI and RII compete for the same pool of catalytic subunit, and that pool is in limiting supply. The most obvious alternative explanations for the decrease in the level of PKAII are inconsistent with our data. For example, the reduced level of PKAI does not appear to effect the level of PKAII as determined by cAMP binding activity (Fig. 1C, Table 1). Thus, our data demonstrate that the absolute levels of PKAI and PKAII are interdependent. Furthermore, expression of mutant RI would also be expected to reduce the amount of CAMP-responsive PKAII associated with structural elements (e.g. p150 or MAP2) within the cell.

These cells deficient in both PKAI and PKAII are a useful model for determining potential roles of PKA in mediating the effects of NGF. While some previous studies have suggested a requirement for PKA in at least some NGF-stimulated events, the exact role of this kinase has remained unexplained.
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Controversial. Previous results have indicated that NGF does not activate adenylate cyclase (25), but different laboratories have reported conflicting observations about whether NGF activates PKA activity (16, 26) or increases the level of cAMP (21-24). It is plausible that changes in cAMP metabolism or PKA activity may be subtle and therefore difficult to detect. The use of well defined lines with a defect in the cAMP-dependent protein kinases that results in a resistance to Bt2cAMP or agents that clearly act via PKA provides a powerful approach to determining if the PKAs play an essential role in eliciting any particular response. In the present study, we demonstrate that NGF is not absolutely dependent on PKA for a wide range of responses including: activation of transcription of egr1, increasing the expression of GAP-43, enhancement of phosphorylation of at least three proteins, induction of ODC activity, or the initiation of morphological differentiation and neurite formation. These responses were chosen to represent a broad spectrum of the potential actions of NGF. They include responses that are very rapid (egr1 expression) as well as responses that occur only after a long delay (induction of GAP-43 or the formation of neurites). The responses also range from protein modifications to changes in transcriptional activity to changes in cell shape. None of these responses seems to show an unambiguous requirement for PKA.

Recent work has implicated PKA in mediating at least part of the NGF signal transduction pathway in PC12 cells. A126-1B2 cells, a PC12-derived clonal line that is deficient in Type II PKA (30), has a diminished capacity to phosphorylate one of the peptides of tyrosine hydroxylase that is normally phosphorylated in response to NGF (14). Because NGF stimulates phosphate incorporation into a number of sites on tyrosine hydroxylase by several different kinases (55), the analysis on one-dimensional gels that was reported in this paper would not be expected to be sensitive enough to clearly demonstrate reduced phosphorylation in PKA-deficient cells; however, tyrosine hydroxylase phosphorylation was somewhat less intense in PKA-deficient cells than in parental cells.

A potential role of PKA in NGF-dependent gene expression was recently revealed by a study of PKA and PKC in the regulation of early response genes in PC12 cells. NGF induces early gene expression in A126-1B2 cells as well as PC12 cells deficient in PKC (31). In contrast, when A126-1B2 cells were rendered deficient in PKA, leading to decreased levels of both PKC and PKA, induction of egr1 in response to NGF was substantially diminished (31). These observations support a role for PKAI in NGF-dependent gene expression under specific circumstances, but they also suggest that PKA and PKC may play functionally redundant roles in NGF-dependent gene expression. The experiments employing A126-1B2 cells are useful, but they are ambiguous because A126-1B2 cells were selected for resistance to Bt2cAMP from a mutagenized population of cells and, thus, A126-1B2 cells may contain additional mutations of unknown effect (30). In addition, while A126-1B2 cells have decreased PKAI, they have normal levels of PKAl (30). In contrast, the nature of the mutation that leads to the deficiency of PKA in both 123.7 cells and AB.11 cells is well understood. Both 123.7 cells and AB.11 cells are deficient in both PKAI and PKAII activity, yet these PKA-deficient cells have normal levels of PKC as well as endogenous catalytic and regulatory subunits of PKA (Table I). The similarity of the responses of these two lines to one another and to A126-1B2 cells supports the conclusions that neither PKAI nor PKAII plays a central role in the responses of PC12 cells to NGF.

Even if the PKAs are not activated directly as part of the NGF-dependent signal transduction cascade involving cAMP, it remains possible that they may play an indirect role in the responses of PC12 cells to NGF. For example, phosphorylation of a particular protein by a kinase that is directly activated as part of the NGF-dependent cascade may make that protein a better substrate for PKA. Several precedents for this scenario exist, e.g. phosphorylation of a sequence in glycogen synthetase by casein kinase II apparently creates a recognition site for glycogen synthetase kinase 3 (37). It is conceivable that an analogous situation might occur in which basal PKA (i.e. free catalytic subunit) might phosphorylate a protein that has recently been modified by another NGF dependent kinase (e.g. PKC or MAP kinase). Thus, basal PKA may be necessary for mediating part of the NGF signal in this hypothetical situation. Alternatively, the catalytic subunit of PKA may be activated by a mechanism independent of cAMP metabolism. Although possibilities such as these exist, our data clearly suggest that cells lacking cAMP activatable PKA respond normally to NGF and argue against a central role of this kinase in the regulation of neuronal

**Fig. 8. Transient expression of PKI inhibits cAMP-**

**A**. egr1CAT (2.5 µg/plate) was cotransfected with increasing concentrations of RSVPKI or RSVPKImut, an expression vector encoding an inactive PKI into PC12 cells. Numbers preceding treatments are amounts (µg) of indicated DNA per plate used in the transfections. RSVneo was added to maintain the total transfected DNA constant at 12.5 µg/plate. Cells were treated 2 days after transfection by changing media to fresh media containing either no addition or 1.0 mM Bt2cAMP, and CAT activity was determined in cell extracts after 12 h. **B**, PC12 cells were cotransfected with 2.5 µg/plate egr1CAT and 10 µg of either RSVneo or RSVPKI. After 3 days, cells were treated with either no addition, Bt2cAMP (1.0 mM), cholaera toxin (3 × 10⁻⁷ M), PMA (100 nM), or NGF (100 ng/ml) for a subsequent 12 h. CAT assays were performed and normalized for protein concentration. These data are representative of three (A) or two (B) experiments with similar results.
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differentiation that is initiated by NGF.

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