Nerve Growth Factor Action Is Mediated by Cyclic AMP- and Ca^{+2}/Phospholipid-dependent Protein Kinases

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Abstract. Nerve growth factor (NGF) mediates the phosphorylation of tyrosine hydroxylase in PC12 cells on two distinct peptide fragments, separable by two-dimensional tryptic phosphopeptide mapping (phosphopeptides T1 and T3). Phorbol diester derivatives capable of activating Ca^{+2}/phospholipid-dependent protein kinase (C-kinase) cause a specific phosphorylation of peptide T3 in a dose-dependent, saturable manner. Derivatives of the endogenous C-kinase activator diacylglycerol, also cause the phosphorylation of tyrosine hydroxylase on peptide T3. The C-kinase inhibitors chlorpromazine and trifluoperazine inhibit the phorbol diester stimulated phosphorylation of site T3 in a dose-dependent manner. These agents inhibit the phosphorylation of T3 in response to NGF, but have no effect on NGF's ability to cause T1 phosphorylation.

In a PC12 mutant deficient in cAMP-dependent protein kinase activity, NGF mediates the phosphorylation of tyrosine hydroxylase on peptide T3 but not on T1. We conclude that NGF mediates the activation of both the cAMP-dependent protein kinase and the C-kinase to phosphorylate substrate proteins. These kinases can act independently to phosphorylate tyrosine hydroxylase, each at a different site, and each of which results in the enzyme activation. A molecular framework is thus provided for events underlying NGF action.

NERVE growth factor (NGF),[1] a protein growth hormone, is required for the survival and differentiation of sympathetic and sensory neurons, both in vivo and in vitro (23). More recently, requirements of NGF for the survival of adrenal chromaffin cell precursors (2) and for the differentiation of central nervous system neurons (see references 22 and 24) have been demonstrated. PC12, a clonal cell line derived from a rat pheochromocytoma, does not require NGF for survival, yet responds to NGF by differentiating into a sympathetic neuron-like cell (10). A wide range of morphological and biochemical changes accompany NGF-induced neuronal differentiation (see references 9 and 46). Some of these include the extension of neuronal processes, elevation of neurotransmitter-synthesizing enzyme activities, establishment of a Na^+-based action potential, and a decline of cell division.

While the molecular mechanism of NGF action has been largely unknown, a number of schemes have been proposed to mediate NGF actions. These include phospholipid or protein methylation (31, 37), phosphatidylinositol turnover (20, 39), elevation of cAMP, and a mobilization of intracellular Ca^{+2} (35). We have reported a specific enhancement of a number of protein phosphorylations within minutes of NGF treatment of PC12 cells (12). Analysis of the total phosphoprotein patterns and in particular of ribosomal protein S6 phosphorylation induced by a variety of agents, suggested that NGF may activate cAMP-dependent protein kinase. Another protein phosphorylation stimulated by NGF as well as by other agents was that of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine neurotransmitter biosynthesis. NGF-induced phosphorylation of tyrosine hydroxylase results in an activation of the enzyme (8, 25).

Tyrosine hydroxylase contains at least four potential sites of phosphorylation (25). The use of two-dimensional tryptic phosphopeptide mapping, which separates the sites, has recently allowed us to distinguish between different patterns of tyrosine hydroxylase phosphorylation generated in PC12 cells in response to various agents (25). We demonstrated that NGF and cAMP elevation each result in qualitatively similar but quantitatively distinct patterns of tyrosine hydroxylase phosphorylation. The identity of the kinases through which NGF is acting to phosphorylate two peptide fragments within tyrosine hydroxylase has been further investigated. By differentially activating and inhibiting Ca^{+2}/phospholipid-dependent protein kinase (C-kinase) and cAMP-dependent protein kinase, we demonstrate that NGF-induced phosphorylation of tyrosine hydroxylase is independently mediated by both of these kinases.

1. *Abbreviations used in this paper*: C-kinase, Ca^{+2}/phospholipid-dependent protein kinase; NGF, nerve growth factor; PMA, phorbol-12-myristate-13-acetate.

Materials and Methods

Materials

The 2.5-S form of NGF (β-NGF) was purified as described previously (26). Dibutyryl cAMP, all phorbol derivatives, chlorpromazine, trifluoperazine, and Nonidet P-40 were from Sigma Chemical Co. (St. Louis, MO). 1,2-dioctanoyl-3-glycerol and 1-oleoyl-2-acetyl-3-glycerol were obtained from Avanti Polar Lipids (Birmingham, AL). Carrier-free $H_3\ ^{32}PO_4$ was obtained from New England Nuclear (Boston, MA). *N*-tosyl-*L*-phenylalanine chloromethylketone-trypsin was purchased from Worthington Biochemical Corp., Freehold, NJ. All other reagent grade chemicals were obtained from either Malinckrodt Inc. (Science Products Div., St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA).

Cell Culture

Clone PC12 (10) and the mutant A126-1B2 (40) were grown in Dulbecco's modified Eagle's medium (41) containing 10% horse serum and 5% fetal calf serum (KC Biologicals Inc., Lenexa, KS). Cultures were plated on 60-mm or 100-mm tissue culture dishes (Falcon Labware, Oxnard, CA) and kept at 37°C in 10% CO_2 and 90% air.

Labeling of Cells with $^{32}PO_4$ and Sample Processing

These procedures were carried out according to the basic design described previously (12, 25). Cells were grown to a density of $<10^6$ cells/60-mm dish at which time the growth medium was removed and the cells suspended in Eppendorf microtubes in Dulbecco's modified Eagle's medium lacking phosphate but supplemented with 5% fetal calf serum and 5% horse serum, or Dulbecco's modified Eagle's medium containing only 0.09 mM phosphate (for experiments with diacylglycerols). After 1 h, carrier-free $^{32}PO_4$ was then added to 500 μCi/ml if only gel electrophoresis analysis was to be carried out or to 5 mCi/ml if phosphopeptide mapping was to be carried out. Various agents were added as indicated in the figure legends or in the text and the cells were incubated for 1–2 h. Since chlorpromazine or trifluoperazine were found to cause some cell lysis at the higher concentrations required, experiments involving these inhibitors were carried out at 22°C, which circumvented this lysis problem. The cells were incubated at 22°C, for 10 min before the addition of, and in the continual presence of the inhibitors. After incubations, the cells were pelleted by centrifugation at 11,000 *g* for 30 s at 4°C. The pellet was then resuspended in ice cold lysis buffer (0.01 M NaP [pH 7.1], 0.1 M NaF, 0.24 M sucrose, 0.01 M EDTA, and 1% Nonidet P-40) and allowed to stand for 1 min on ice. The lysate was centrifuged for 3 min at 11,000 *g* to pellet the nuclei. The supernatant was then immediately solubilized by the addition of 1 vol twice-concentrated solubilizing solution (1× buffer contains 0.08 M Tris HCl [pH 6.8], 2% SDS, 10% glycerol, and 5% freshly added 2-mercaptoethanol) and placement in a boiling water bath for 5 min.

Gel Electrophoresis and Autoradiography

All samples were placed in a boiling water bath for 3 min before electrophoresis. One-dimensional electrophoresis was carried out in 10 or 17.5% acrylamide slab gels in 0.1% SDS as previously described (3). Autoradiography was carried out at −70°C with preflashed Kodak X-Omat R film (21). Densitometry was carried out using the Chromoscan III densitometer (Joyce Loebl, Division of Vickers Limited, Tyne and Wear, England).

Table I. Stimulation of Tyrosine Hydroxylase Phosphorylation by Phorbol Derivatives

Phorbol derivative	Maximal stimulation
	%
4β-phorbol-12-myristate-13-acetate (PMA, 200 nm)	100
4β-phorbol-12,13-dibenzoate (270 nm)	100
4β-phorbol-12-monomyristate (250 nm)	1
4β-phorbol (380 nm)	0

Cells were labeled with $^{32}PO_4$ in the presence or absence of the indicated phorbol derivatives. Proteins were subjected to SDS PAGE, autoradiography, and densitometric analysis as described in Materials and Methods. The increase in ^{32}P-labeling of tyrosine hydroxylase was quantitated as described in the legend to Fig. 1.

Phosphopeptide Mapping of Tyrosine Hyroxylase

Purification of tyrosine hydroxylase, performic acid oxidation, tryptic digestion, and two-dimensional separation of resulting peptides were all carried out by a modification of the methods of Hunter and Sefton (14) according to McTigue, Cremins, and Halegoua (25). The four phosphopeptides (T1–T4) were shown to be tryptic-derived fragments of tyrosine hydroxylase by immunoprecipitation and two-dimensional gel analyses (25). Autoradiograms were quantitated using a Joyce Loebl densitometer as before (25).

Results

C-Kinase–mediated Phosphorylation of Tyrosine Hydroxylase

Agents that have been shown to phosphorylate tyrosine hydroxylase at tryptic phosphopeptide site T3 such as NGF, epidermal growth factor, cAMP, and K^+ depolarization (25) have also been shown to result in an increased turnover of phosphatidylinositol in responsive cells (34, 38). The resulting liberation of diacyglycerol would be expected to result in C-kinase-directed protein phosphorylation. Phorbol-12-myristate-13-acetate (PMA), a direct activator of C-kinase also causes the phosphorylation of tyrosine hydroxylase (Fig. 1; see also reference 25). Cellular proteins labeled with $^{32}PO_4$ in the presence or absence of increasing amounts of PMA were subjected to SDS PAGE and autoradiography. Quantitation of specific tyrosine hydroxylase labeling as described in Materials and Methods revealed that the PMA-enhanced phosphorylation of tyrosine hydroxylase was dose-dependent and saturable. The maximal phosphorylation of tyrosine hydroxylase by PMA in various experiments ranged from between 1.5- and 3.5-fold. As little as 2 nM PMA caused a significant phosphorylation of the enzyme with maximal phosphorylation occurring at ~70 nM PMA. These dose-response characteristics are compatible with those reported for other C-kinase–directed protein phosphorylations induced by PMA in vivo (see reference 43). The PMA-mediated increase in tyrosine hydroxylase phosphorylation was seen within 1 min of treatment and maximal phosphorylation was maintained for at least 2 h in the presence of PMA.

To further determine the specificity of the PMA-induced phosphorylation of tyrosine hydroxylase, other phorbol esters that have been shown to vary in their ability to activate C-kinase were examined. As documented in Table I, 4β-phorbol-12-monomyristate and 4β-phorbol (each at over 200 nM), which are ineffective in activating C-kinase (6), did not result in an enhancement of tyrosine hydroxylase phosphorylation. In contrast, 4β-phorbol-12,13-dibenzoate, which has been found to activate C-kinase to the same degree as PMA (6), resulted in the same degree of stimulation of tyrosine hydroxylase phosphorylation as PMA. To demonstrate the site of phosphorylation within tyrosine hydroxylase, the cellular proteins from PC12 cells that had been ^{32}P-labeled and PMA-treated or not, were subjected to SDS PAGE. The band migrating at 60,000 D (Fig. 1 *A*), shown by immunoprecipitation and two-dimensional gel analysis to consist of tyrosine hydroxylase (12, 25), was cut out of the gel, extracted, and subjected to complete tryptic digestion and two-dimensional phosphopeptide mapping as described in Materials and Methods. As can be seen from the phosphopeptide maps (Fig. 1 *B*), the increase in tyrosine hydroxylase phosphoryla-

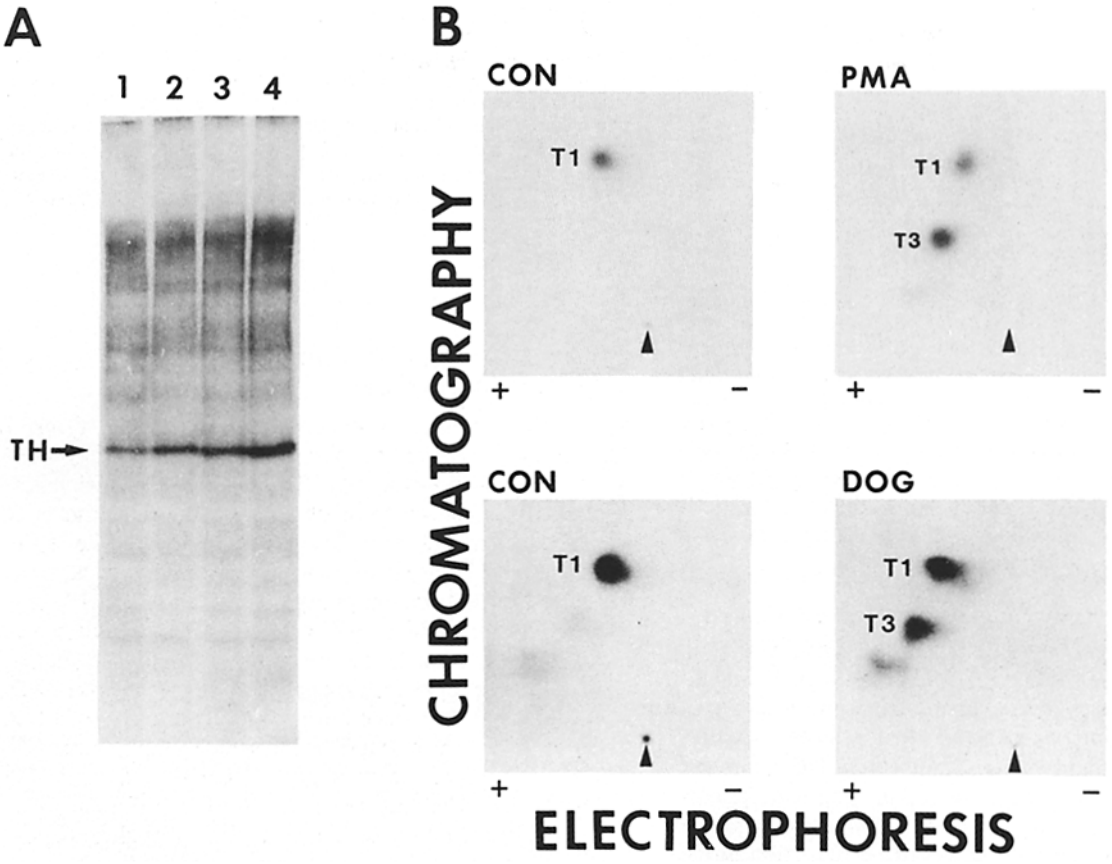

Figure 1. SDS PAGE and tryptic phosphopeptide mapping analysis of tyrosine hydroxylase phosphorylation stimulated by PMA and diacylglycerols. Cells were ^{32}P-labeled in the presence or absence of the indicated agents. Cellular proteins were subjected to SDS PAGE and autoradiography (*A*). Tyrosine hydroxylase was prepared and subjected to tryptic phosphopeptide mapping (*B*) all as described in Materials and Methods. (*A*) Lane *1*, control; lane *2*, + PMA (200 nM); lane *3*, + 1-oleyl-2-acetyl-3-glycerol (0.3 mM); lane *4*, + 1,2-dioctanoyl-3-glycerol (0.3 mM). (*B*) Tryptic phosphopeptide map autoradiograms; DOG, 1,2-dioctanoyl-3-glycerol (0.3 mM).

tion caused by PMA is due to an increase in phosphorylation on peptide T3 (see also reference 25).

Since diacylglycerol is the physiological activator of C-kinase, its ability to effect tyrosine hydroxylase phosphorylation was also examined. Cells were ^{32}P-labeled in the presence or absence of 1-oleoyl-2-acetyl-3-glycerol or 1,2-dioctanoyl-3-glycerol and the labeling of tyrosine hydroxylase assayed as described in Materials and Methods. These derivatives traverse the cell membrane and activate C-kinase (7, 16). As can be seen from the SDS polyacrylamide gel autoradiogram shown in Fig. 1, these agents resulted in an increased phosphorylation of tyrosine hydroxylase. In this experiment, PMA, 1-oleoyl-2-acetyl-3-glycerol and 1,2-dioctanoyl-3-glycerol each resulted in a 2.3-, 2-, and 4.5-fold stimulation of ^{32}P incorporation into tyrosine hydroxylase, respectively. The greater potency of the 1,2,-dioctanoyl-3-glycerol compared with the 1-oleoyl-2-acetyl-3-glycerol agrees with previously reported results (7). To determine which tryptic fragment of tyrosine hydroxylase is phosphorylated in response to diacylglycerol, [^{32}P]tyrosine hydroxylase was prepared from cells labeled in the presence or absence of 1,2-dioctanoyl-3-glycerol and subjected to two-dimensional phosphopeptide mapping as above. As Fig. 1 *B* shows, this diacylglycerol resulted in an increase in T3 phosphorylation. This pattern is similar to that induced by PMA (Fig. 1 *B*).

To further assess whether the phosphorylation of tyrosine hydroxylase at a site within T3 was directed by C-kinase, the drugs chlorpromazine and trifluoperazine, each of which were reported to inhibit C-kinase in vitro and in vivo (17, 26, 27, 44), were used. Cells were ^{32}P-labeled in the presence of PMA and varying concentrations of either chlorpromazine or trifluoperazine. The ^{32}P-labeling of tyrosine hydroxylase was quantitated as described in Materials and Methods. The inhibitors were found to block PMA-induced phosphorylation of tyrosine hydroxylase in a dose-dependent manner (Fig. 2).

Inhibition of NGF-mediated T3 Phosphorylation

The above results demonstrated that phosphorylation of phosphopeptide T3 was directed by C-kinase in PC12 cells. It thus seems likely that the NGF-mediated phosphorylation of T3 occurs through activation of this kinase. As Fig. 2 shows, both chlorpromazine and trifluoperazine were able to inhibit PMA-directed phosphorylation of T3. To more directly determine if T3 phosphorylation by NGF was a result of C-kinase activation, we tested the ability of these inhibitors to block the T3 phosphorylation seen in response to NGF. Cells were labeled with $^{32}PO_4$ in the presence of NGF, with or without 0.4 mM chlorpromazine. [^{32}P]tyrosine hydroxylase was prepared and subjected to tryptic phos-

Figure 2. Concentration dependence of trifluoperazine and of chlorpromazine-mediated inhibition of PMA-stimulated phosphorylation of tyrosine hydroxylase. Cells were ^{32}P-labeled in the presence or absence of the indicated agents. Proteins were subjected to SDS PAGE (10% acrylamide), autoradiography, and densitometric analysis all as described in Materials and Methods. Stimulation of tyrosine hydroxylase phosphorylation by PMA (200 nM) was determined as described in Fig. 1. The maximal phosphorylation of tyrosine hydroxylase by PMA was 1.8-fold over the control. For each concentration of inhibitor, the stimulation seen by PMA was determined relative to the inhibitor alone. ●, chlorpromazine; △, trifluoperazine.

phopeptide mapping analysis. As can be seen in Fig. 3 *B*, NGF-mediated a threefold and 15-fold increase in T1 and T3 phosphorylation, respectively. In the experiment shown in Fig. 3, *C* and *D* chlorpromazine resulted in an 80% inhibition of the T3 phosphorylation stimulated by NGF. Greater than 90% inhibition has been observed in other experiments using 0.5 mM chlorpromazine or 0.1 mM trifluoperazine (data not shown). There was, however, no reduction in the degree of NGF-stimulated T1 phosphorylation by these inhibitors (Fig. 3, *C* and *D*). These results indicate that the NGF-induced phosphorylation of T3 but not T1 results from the activation of C-kinase.

Phosphorylation of Tyrosine Hydroxylase in cAMP-dependent Protein Kinase-deficient PC12

We had previously suggested that the phosphorylation of ribosomal protein S6 (12) and of tyrosine hydroxylase peptide T1 (25) in response to NGF resulted from an activation of cAMP-dependent protein kinase. To more conclusively establish that NGF mediated these phosphorylations through cAMP-dependent protein kinase, we examined the ability of NGF to elicit protein phosphorylations in mutant PC12 cells that are deficient in cAMP-dependent protein kinase activity (40). Cells were labeled with $^{32}PO_4$ in the presence or absence of NGF, and the cellular proteins subjected to SDS PAGE as described in Materials and Methods. From densitometric quantitation of the gel autoradiogram (shown in Fig. 4 *A*, lanes *1* and *2*), a 2.5-fold increase in tyrosine hydroxylase phosphorylation as well as an increase in ribosomal protein S6 phosphorylation were caused by NGF in wild-type PC12 cells (12). In the mutant line, NGF mediated a 1.5-fold increase in tyrosine hydroxylase phosphorylation, whereas no increase in the phosphorylation of ribosomal protein S6 was observed (Fig. 4 *A*, lanes *3* and *4*). Furthermore, addition of dibutyryl cAMP or cholera toxin to the mutant cells did not result in an observable increase in the phosphorylation of any cellular proteins (not shown). These results confirm the reported deficiency of cAMP-dependent kinase in the mutant cells.

To determine if the NGF-mediated phosphorylation of tyrosine hydroxylase at site T1 was due to cAMP-dependent protein kinase, mutant cells were ^{32}P-labeled in the presence or absence of NGF. The [^{32}P]tyrosine hydroxylase was prepared and subjected to phosphopeptide mapping as described in Materials and Methods. As seen in the phosphopeptide maps, NGF caused a multifold increase in the phosphorylation of T3 in both the mutant (Fig. 4 *B*) and the wild-type cells (Fig. 3, *A* and *B*). However the increase in T1 phosphorylation was specifically lacking in the mutant cells. These results demonstrate that T1 phosphorylation in response to NGF is mediated by a cAMP-dependent protein kinase, whereas T3 phosphorylation induced by NGF can be attributed to a cAMP-independent mechanism, namely C-kinase activation.

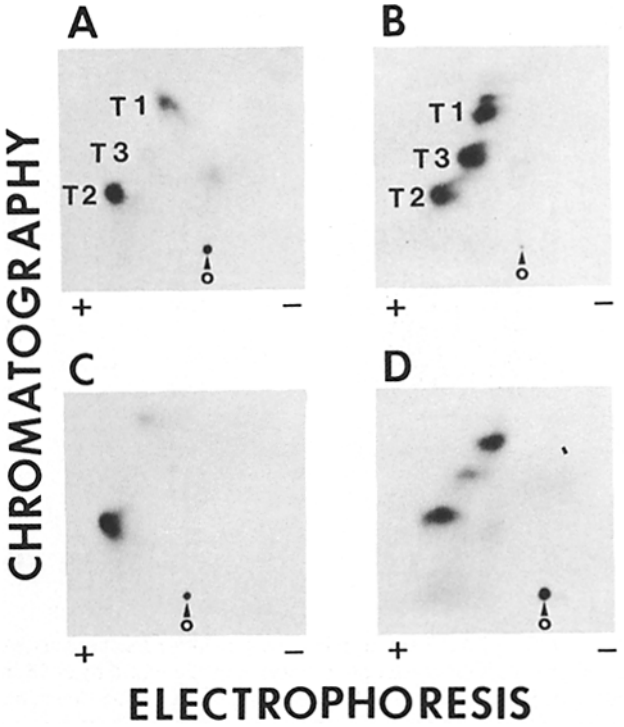

Figure 3. Tryptic phosphopeptide mapping of tyrosine hydroxylase stimulated by NGF in the presence or absence of chlorpromazine. Cells were labeled with $^{32}PO_4$ in the presence or absence of the indicated agents. Tyrosine hydroxylase was prepared and subjected to tryptic phosphopeptide mapping as described in Materials and Methods. *A*, control; *B*, + NGF (50 ng/ml); *C*, + chlorpromazine (0.4 mM); *D*, + NGF (50 ng/ml) + chlorpromazine (0.4 mM).

Discussion

The following conclusions may be drawn from the data presented above. (*a*) The phosphorylation of tyrosine hydroxylase within tryptic-derived phosphopeptide T3 can be directed by C-kinase in PC12 cells. (*b*) The NGF-induced phosphorylation of T3 is directed by C-kinase. (*c*) The NGF-induced phosphorylation of T1 is directed by cAMP-dependent protein kinase. (*d*) The action of either kinase and resulting site-specific phosphorylation of tyrosine hydroxylase can occur independently in response to NGF.

NGF mediates the phosphorylation of a number of proteins in PC12 cells in a rapid and reversible manner (12). One of

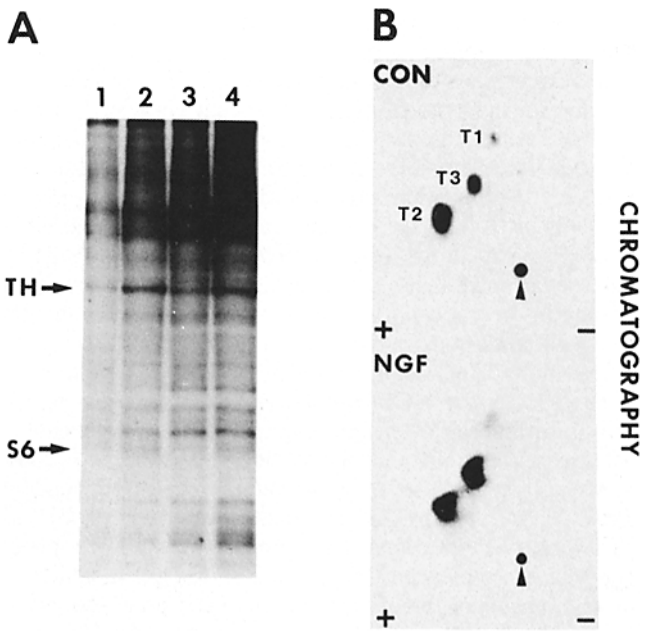

Figure 4. SDS PAGE and tryptic phosphopeptide mapping analyses of tyrosine hydroxylase phosphorylation stimulated by NGF in wild-type PC12 and in cAMP-dependent protein kinase–deficient, mutant PC12. Cells were ^{32}P-labeled in the presence or absence of NGF. Proteins were subjected SDS PAGE and autoradiography (*A*). Tyrosine hydroxylase was prepared and subjected to tryptic phosphopeptide mapping (*B*). (*A*) Lane *1*, wild-type control; lane *2*, wild-type + NGF (50 ng/ml); lane *3*, mutant control; lane *4*, mutant + NGF (50 ng/ml). (*B*) Mutant; CON, control; NGF, + NGF (50 ng/ml).

these proteins, tyrosine hydroxylase, is a multiply phosphorylated protein in which site-specific phosphorylation can occur on each of four distinct peptide fragments in response to different agents (25). NGF mediates the phosphorylation of tyrosine hydroxylase on two fragments (T1 and T3). From an analysis of tyrosine hydroxylase phosphorylation resulting from NGF treatment of PC12, we have determined that an activation of two kinases by NGF results in the phosphorylation of tyrosine hydroxylase. This is clearly indicated by the ability of NGF to elicit the phosphorylation of each fragment independently (in the presence of site-specific inhibitors, see Figs. 3 and 4).

The phosphorylation of tyrosine hydroxylase within peptide T3 is directed by C-kinase. This conclusion is supported by a variety of data. Specific activation of C-kinase by phorbol diester derivatives or by diacylglycerol derivatives result in T3 phosphorylation. Consistent with these results is the report of C-kinase-mediated phosphorylation and activation of tyrosine hydroxylase in vitro (1, 33). In addition, each of two C-kinase inhibitors, chlorpromazine or trifluoperazine, are able to block the phosphorylation of T3 by the phorbol ester, PMA. NGF treatment of PC12 cells and sympathetic neurons has been shown to cause phosphatidylinositol turnover (20, 39). Thus the resulting accumulation of diacylglycerol would activate C-kinase. We have shown that trifluoperazine and chlorpromazine are each able to specifically block the phosphorylation of T3 by NGF. This result additionally leads to the conclusion that NGF mediates the phosphorylation of T3 through C-kinase. However, since these compounds are also potent inhibitors of Ca^{+2}/calmodulin-dependent protein kinase, another kinase that phosphorylates tyrosine hydroxylase in vitro (42, 45), the possibility that NGF uses this kinase to mediate T3 phosphorylation would have to be ruled out. The following evidence indicates that Ca^{+2}/calmodulin-dependent protein kinase phosphorylates tyrosine hydroxylase at phosphopeptide site T2, a site distinct from those phosphorylated in response to NGF. The phosphorylation of T2 results from a membrane depolarization–induced influx of Ca^{+2} (25). The enhanced phosphorylation of T2 under this condition is more sensitive to inhibition by trifluoperazine (median infective dose 10 μM; Cremins and Halegoua, unpublished data) than is the PMA-stimulated phosphorylation of T3 (median infective dose 50 μM, from Fig. 2). Last, in vitro studies using purified components have indicated that the phosphorylation of tyrosine hydroxylase by Ca^{+2}/calmodulin-dependent protein kinase occurs at a site distinct from that phosphorylated by C-kinase (1, 42).

NGF treatment of PC12 cells results in the stimulation of cAMP-dependent protein kinase activity. This conclusion is supported by a variety of data. NGF causes a rise in cAMP levels in PC12 cells (35). NGF and agents that raise cAMP concentrations result in qualitatively similar patterns of protein phosphorylation in PC12 cells as analyzed by one- and two-dimensional gel electrophoresis (12). In the same study, additivity experiments that were performed using NGF and a number of other agents that result in ribosomal protein S6 phosphorylation, indicated that only the NGF plus cAMP combination is not additive. NGF and elevators of cAMP result in the phosphorylation of tyrosine hydroxylase within phosphopeptide T1 (13, 25). T1 phosphorylation in response to NGF occurs even in the presence of C-kinase inhibitors (Fig. 3). cAMP-dependent protein kinase mediates the phosphorylation of tyrosine hydroxylase in vitro (15). In a PC12 mutant, deficient in cAMP-dependent protein kinase activity, NGF is unable to mediate the phosphorylation of either T1 or of ribosomal protein S6.

An apparent inconsistency exists between some of our results and those of others using an in vitro approach to study tyrosine hydroxylase phosphorylation. Although our data indicate that cAMP-dependent protein kinase and C-kinase mediate the phosphorylation of distinct sites on tyrosine hydroxylase in PC12 cells, the sites phosphorylated by these kinases in vitro were reported to be identical (1, 42). One possible explanation may lie in the different phosphopeptide mapping systems used. We have previously shown that a separation of all phosphopeptides derived from tyrosine hydroxylase is dependent upon the use of the appropriate system for two-dimensional separation (25). Alternatively, the sites within tyrosine hydroxylase that are accessible to phosphorylation by the various kinases in vitro may be different from those accessible in living cells. Within this latter context, C-kinase activation in vivo may result in the activation of another kinase which phosphorylates T3, and hence C-kinase may be indirectly responsible for T3 phosphorylation.

An outcome of the present studies is the demonstration that NGF can independently stimulate two different kinase pathways. The phosphorylation of T1 by cAMP-dependent protein kinase appears to be independent of phosphatidylinositol turnover and C-kinase activation because it occurs even in the presence of C-kinase inhibitors. This is further

apparent because several agents that cause phosphatidylinositol turnover and/or C-kinase activation and a resulting phosphorylation of T3, do not mediate an increased phosphorylation of T1 (25). In contrast, T3 phosphorylation in response to NGF appears to be only partially dependent upon cAMP-dependent protein kinase activation. Whereas cAMP elevation results in T3 phosphorylation in wild-type cells (25) but does not do so in a PC12 mutant deficient in cAMP-dependent protein kinase, NGF is still able to mediate T3 phosphorylation in the mutant cells. It is possible that cAMP-dependent protein kinase may phosphorylate sites in both T1 and T3. However, cAMP-dependent protein kinase activation results in only T1 phosphorylation in adrenal chromaffin cells (site E in reference 13; see reference 25). Thus a more likely and interesting alternative is that elevation of cAMP-dependent protein kinase activity in PC12 cells leads indirectly to the activation of C-kinase. Consistent with this interpretation is the finding that cAMP elevation in PC12 cells results in phosphatidylinositol turnover (38).

A framework for the molecular events underlying NGF action emerges from existing data. NGF may activate C-kinase through two distinct but converging pathways. Both pathways involve increased turnover of phosphatidylinasitol, one through a more direct means (i.e., NGF receptor–G protein interaction) and the other indirectly through cAMP-dependent protein kinase activation. The resulting accumulation of diacylglycerol would activate C-kinase and thus result in the phosphorylation of substrate proteins such as tyrosine hydroxylase (within T3) and others. One candidate for the putative G-protein involvement is the proto-oncogene product, ras p21. A monoclonal antibody directed against and inhibitory for ras p21 proteins inhibit NGF-directed neurite extension (11), whereas activated ras protein mimics NGF action on PC12 cells (4, 29). The pathway for the activation of cAMP-dependent protein kinase by NGF has also not yet been elucidated. NGF caused a small rise in the cAMP level in PC12 cells (35) and sympathetic neurons (28, 36). However, stimulation of adenylate cyclase by NGF has not been detectable, although NGF potentiates cAMP elevation by other agents (32). It also remains a possibility that cAMP phosphodiesterase activity is affected by NGF. The stimulation of cAMP-dependent protein kinase would result in the phosphorylation of tyrosine hydroxylase (within T1), ribosomal protein S6, and others. The analysis of tyrosine hydroxylase phosphorylation should continue to provide a useful means of assessing the differential activation of multiple cellular kinases in PC12 cells.

What are the cellular consequences of the activation of two protein kinases by NGF? In one case, two major kinase activities converge to phosphorylate a common substrate, tyrosine hydroxylase, at two sites. Phosphorylation at each unique site has been shown to result in the enzyme activation (13, 25). The significance of tyrosine hydroxylase activation by NGF and the resulting increased catecholamine neurotransmitter synthesis has been discussed (8, 25). Activation of these kinases may mediate other NGF actions. For example, NGF induces the synthesis of a number of enzymes and other proteins. The induction of the proto-oncogene *fos* product by NGF has been suggested to occur via activation of C-kinase (18). The induction of ornithine decarboxylase by NGF occurs even in mutant PC12 cells deficient in cAMP-dependent protein kinase (40). Furthermore, ornithine decarboxylase has been shown to be induced by C-kinase activators (30). A major morphological change induced by NGF is the extension of neuronal processes. NGF-mediated neurite outgrowth has been suggested to occur via a combination of rapid, short term events and long term transcription-dependent events (see reference 5). The elevation of cAMP and Ca^{++} mobilization have also been suggested to play a role in NGF-induced neurite extension (35), possibly through phosphatidylinositol turnover (38, 39). We have further determined that neurite extension is preceded by a change in the phosphorylation and distribution of vinculin and focal adhesions by NGF (S. Halegoua, manuscript in preparation). The role of this and other protein phosphorylations, directed by NGF-stimulated protein kinases, in the process of neurite extension is being actively investigated.

Increased protein phosphorylation in response to NGF may result not only from the activation of cAMP-dependent and C-kinases. A third distinct kinase stimulated by NGF has been observed in cytoskeletal preparations, which phosphorylates a cytoskeletal protein of 230 kD (19). In addition, protein phosphorylation caused by NGF at a somewhat longer time course has been previously reported (47). It is further likely that some actions of NGF are elicited by other cellular effectors either independently or in concert with protein kinases.

We thank Drs. J. Levine, J.McKelvy, and J. White for valuable discussions and critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health No. NS-18218 (to Simon Halegoua) and No. CA-40929 (to John Wagner).

Received for publication 19 March 1986, and in revised form 19 May 1986.

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