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Regulation of Tyrosine Hydroxylase Phosphorylation in PC12 Pheochromocytoma Cells by Elevated K^+ and Nerve Growth Factor

Evidence for Different Mechanisms of Action

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SUMMARY

A specific antiserum was used to compare phosphorylation of tyrosine hydroxylase (TH) (EC 1.14.16.2, tyrosine 3-monooxygenase) as regulated by elevated K^+ and nerve growth factor (NGF) in cultured PC12 pheochromocytoma cells. Exposure of cultures to either elevated K^+ or to NGF significantly enhanced the incorporation of [^{32}P]orthophosphate into TH. The effect of elevated K^+ was evident at 10 mM and was maximal by 40–80 mM. Increased phosphorylation of TH was detected at 0.1 nM (3 ng/ml) NGF and reached a maximal level by 0.3–1 nM (10–30 ng/ml) NGF. Elevated K^+ showed a biphasic time course of action with one maximum of phosphorylation at about 30 sec of exposure and a second after about 10 min of exposure. In contrast, the NGF effect showed an initial lag of several minutes followed by a monophasic increase in phosphorylation to reach a plateau. Both treatments enhanced TH activity, but in each case the time courses of this did not strictly correlate with that of phosphorylation. The effect of elevated K^+ on TH phosphorylation required the presence of extracellular Ca^{2+} and was suppressed by trifluoperazine (100 μM). *N*-(6-Aminohexyl)-5-(chloronaphthalene)-1-sulfonamide (W-7) (100 μM), a potent inhibitor of calmodulin activity, also blocked the enhancement of phosphorylation by elevated K^+, whereas *N*-(6-aminohexyl)-1-(naphthalene)sulfonamide (W-5) (100 μM), a less potent analogue of W-7, did not. In contrast to these findings, the increase in TH phosphorylation brought about by NGF did not require extracellular Ca^{2+}, and was only slightly affected by trifluoperazine or W-7. When TH phosphorylated under various conditions (control medium, elevated K^+, NGF) was subjected to peptide mapping after exposure to *Staphylococcus aureus* protease V8, multiple phosphorylated peptides were observed. Elevated K^+ and NGF each produced increases in labeling of each of the peptides. However, the relative degree of labeling of different peptides was distinct for each condition. These data suggest that elevated K^+ and NGF bring about rapid enhancement of the phosphorylation of TH by means of different mechanisms.

INTRODUCTION

There is much evidence that TH[4] is a substrate for phosphorylation and that phosphorylation, in turn, results in changes of the kinetic properties of this enzyme (1–12). For instance, TH activity *in vitro* is stimulated by cAMP-dependent phosphorylation (1–4, 10, 11), and this stimulation is associated with an increased affinity of the enzymes for pterine cofactor and reduction of feedback inhibition by catecholamines (1, 2, 5, 8, 9). It

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[4] The abbreviations used are: TH, tyrosine hydroxylase; NGF, nerve growth factor; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-(chloronaphthalene)-1-sulfonamide; W-5, *N*-(6-aminohexyl)-1-(naphthalene)sulfonamide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; KRH, HEPES-buffered (pH 7.4) phosphate-free modified Krebs-Ringer saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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has also been shown that cAMP-dependent phosphorylation and activation of TH can occur in intact cells (8, 10, 13). Electrical stimulation or treatment of cells with depolarizing levels of K^+ also leads to activation of TH (1, 11–14) and does so via a mechanism that appears to be due to phosphorylation of the enzyme. Finally, NGF, a protein that exerts major influences on the survival, development, and function of sympathetic and certain sensory neurons (for review, see Ref. 15), has in addition been shown to cause a rapid enhancement of TH phosphorylation in cultured PC12 pheochromocytoma cells (16). Exposure of such cultures to NGF also brings about an acute activation of TH (17).

Various types of experiments suggest that cAMP exerts its effects on TH by mechanisms that are distinct from those involved in the actions of depolarization and of NGF. For example, (a) the activation of TH brought about in intact cells by elevated K^+ is additive with that produced by exogenously supplied cAMP analogues (10–12); (b) cholinomimetic agents and cAMP analogues appear to bring about phosphorylation at different sites on TH (6); and (c) cAMP-dependent phosphorylation of TH is associated with changes in K_m for cofactor and catecholamines (2, 5, 9, 11, 12), whereas depolarizing conditions and NGF each appear to alter only the V_{max} of the enzyme (12, 17).

To further investigate the mechanisms of phosphorylation and regulation of TH in intact cells, we have carried out experiments to compare the effects of depolarizing levels of K^+ with those of NGF. To do so, we have employed the PC12 clonal line of rat pheochromocytoma cells (18). These cells represent an easily grown source of homogeneous material, have high levels of TH (2, 19), and possess voltage-sensitive Ca^{2+} channels (19) and receptors for (and response to) NGF (15, 18). Use has been made also of specific antiserum prepared against TH purified from PC12 cells (2). This reagent enabled the selective identification of TH and its isolation from other cell phosphoproteins. By these means, evidence was obtained that elevated K^+ and NGF regulate the phosphorylation of TH through distinguishable mechanisms. Preliminary results were previously reported (20–22).

METHODS

Materials. The following materials were purchased from commercial sources: *Staphylococcus aureus* protease V8 (Miles Laboratories); W-5 and W-7 (Calbiochem); TFP (Smith Kline and French); [^{35}S]methionine, carrier-free [^{32}P]orthophosphate, and L-[3,5-^{3}H]tyrosine (New England Nuclear); 8-bromo-cAMP and N^6,$O^{2'}$-dibutyryl-cAMP (Boehringer); DL-6-methyl-5,6,7,8-tetrahydropterine (Calbiochem); catalase (Sigma); ^{14}C-labeled molecular weight markers (Bethesda Research Laboratories); protein A-Sepharose (Pharmacia); Trasylol (Mobay Chemicals); L-tyrosine and Triton X-100 (Eastman Kodak); and cation and anion exchange resin (Bio-Rad AG 50W-X8 and Bio-Rad 9). Nerve growth factor was isolated by the method of Mobley *et al.* (23). Antiserum to purified PC12 cell TH was prepared as previously described (2).

Cell maintenance. Clonal pheochromocytoma cells (PC12 cells) were cultured as previously described (18). For experiments, cells were grown on 35-mm collagen-coated plastic tissue culture dishes.

Phosphorylation. In most experiments, cultures were washed free of growth medium and preincubated for 1–2 hr in a humidified atmosphere at 37° with 50–100 μCi of [^{32}P]orthophosphate in 1 ml of KRH (24).

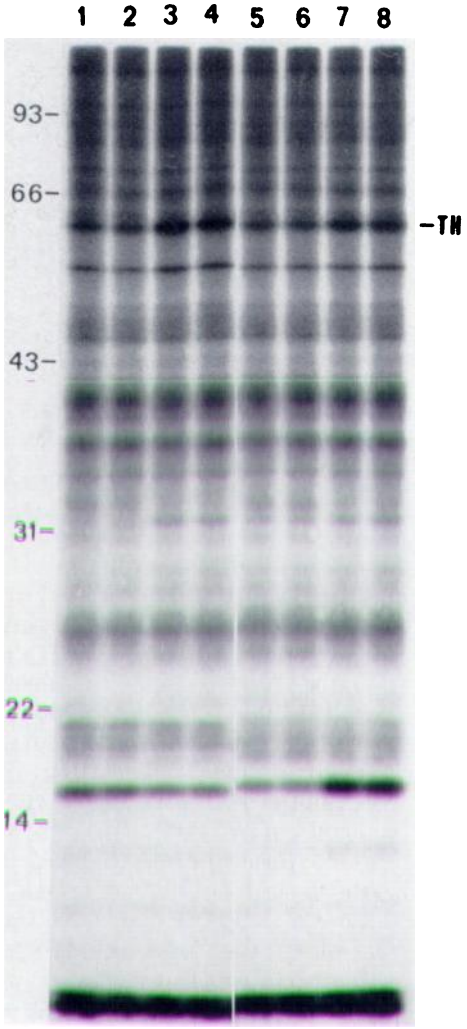

FIG. 1. *SDS-PAGE patterns of phosphorylated proteins in PC12 cell homogenates after various treatments*

An autoradiograph is shown. In each case, equal numbers of trichloroacetic acid-precipitable counts (200,000 cpm) were loaded onto a 15% acrylamide gel. Lanes *1* and *2*, control (no additives); lanes *3* and *4*: NGF-treated (50 ng/ml); lanes *5* and *6*: dibutyryl cAMP-treated (1 mM); lanes *7* and *8*: treated with 40 mM K^+. Details of labeling and treatment are given in the text. *Numbers* indicate positions of marker proteins (as $M_r \times 10^{-3}$); the position of TH (M_r = 60,000) is indicated. Comparable results were obtained in six independent experiments.

This solution was then removed and replaced either with KRH containing additives or with KRH modified to contain various concentrations of K^+ (by iso-osmotic replacement of NaCl with KCl). For experiments in which EGTA was present, KRH was prepared without Ca^{2+}. The cultures were then incubated for an additional 30 min to 1 hr unless otherwise specified. In some experiments, preincubation was not carried out and the cultures were incubated for the entire 2 hr in the presence of label and additives or elevated K^+. This protocol gave similar results to those obtained for preincubation with label.

Immunoprecipitation. At the end of the incubation period, the cultures were washed three times with phosphate-buffered saline (pH 7.2). The cells were then harvested by scraping them with a rubber policeman into a solution (pH 6.8) containing 30 mM sodium phosphate, 50 mM NaF, 1 mM EDTA, 3% SDS, 10% glycerol, and 0.0001% bromphenol blue. The cell extracts were then immediately placed in boiling water for 5 min and were either used at once or quick-frozen on dry ice and stored at −20° until use. For immunoprecipitation, the samples

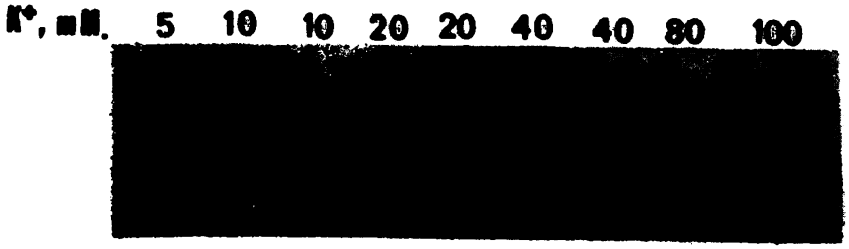

FIG. 2. *The effects of various concentrations of K^+ on phosphorylation of TH in cultured PC12 cells*

Cultures were exposed to [^{32}P]orthophosphate and to various concentrations of K^+. Equal numbers of trichloroacetic acid-precipitable counts (of total cell proteins) were subjected to immunoprecipitation with anti-TH serum, and the immunoprecipitates were dissolved and analyzed by SDS-PAGE and autoradiography, all as described in the text. Only the region of the autoradiograph containing the TH band is shown. The *numbers* above each lane indicate the concentration of K^+ in the medium. Comparable results were obtained in three independent experiments. The relative densities of the bands as determined by densitometric scanning are (from *left* to *right*): 1, 2, 2.1, 2.3, 3.2, 4.3, 4.5, 6.25, and 4.5.

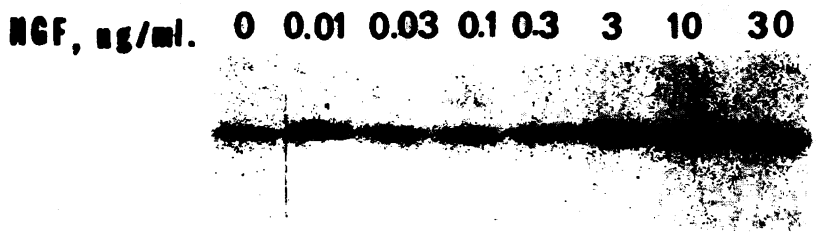

FIG. 3. *Effect of various concentrations of NGF on phosphorylation of TH in cultured PC12 cells*

Details of the experiment are given in the text. This and the following figures show the regions of autoradiographs of SDS-polyacrylamide gels containing TH bands which were immunoprecipitated from aliquots containing equal numbers of trichloroacetic acid-precipitable counts of whole cell extracts. *Numbers* above each lane indicate the concentration of NGF in nanograms/milliliter. The relative densities of the bands are (from *left* to *right*): 1, 1.3, 1.5, 1.6, 1.5, 2.7, 3.0, and 3.3. Comparable results were obtained in two independent experiments.

(freshly prepared or thawed) were sonicated and diluted with 4 volumes of a solution containing 190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 2.5% Triton X-100, 50 mM NaF, 0.02% NaN_3, and 100 units/ml Trasylol (solution A). An excess of anti-TH serum (5 μl), was added and the mixture was incubated for 1 hr at room temperature followed by incubation overnight at 4° (25). Subsequently, preswollen protein A-Sepharose beads were added in excess of the antigen-antibody complex and the solution was mixed slowly for 2 hr. The beads were washed four times with solution A containing 0.1% SDS, and the bound proteins were dissolved by boiling for 5 min in 50 μl of 50 mM Tris-phosphate buffer (pH 6.7) containing 1 M dithiothreitol, 10% sucrose, 10% SDS, and 20 mM EDTA. The solution was centrifuged at 12,000 $\times g$ for 3 min, and the supernatant was loaded on SDS-polyacrylamide gels.

SDS-PAGE. The immunoprecipitated samples (prepared as above) were loaded onto SDS-polyacrylamide slab gels (26, 27) containing 7.5% polyacrylamide and electrophoresed to separate TH from nonspecifically precipitated material. The proteins were fixed by soaking the gels for 1 hr in a solution containing 30% methanol and 7% acetic acid, and the gels were dried under vacuum. The radioactive bands were visualized by exposing the dried gels to X-ray film (Kodak AR5). Densities of radioactive bands on the X-ray film were quantified using a GS 300 Densitometer (Hoeffer Scientific). In one experiment, the bands (localized by means of autoradiography) were excised from the dried gel with a razor blade and the gel was digested overnight with a solution containing 99 parts of 30% H_2O_2 and 1 part of NH_4OH at 60° in a closed scintillation vial (28). The radioactivity was determined in a scintillation spectrometer.

Assay of TH activity. Cells for Th assays were treated the same as those for phosphorylation except that radioactive phosphate was omitted. The cultures were harvested in 50 mM phosphate buffer (pH 6.1) containing 50 mM NaF and were immediately frozen on dry ice. The assays were performed by published procedures (2, 17, 29).

Double labeling with ^{35}S and ^{32}P. For double labeling with ^{35}S and ^{32}P, the PC12 cultures were exposed to 40 μCi of [^{35}S]methionine/ml in complete medium for 1–2 days and then, after washing, to [^{32}P] orthophosphate as indicated above. Radioactivity incorporated into macromolecules was determined by trichloroacetic acid precipitation in combination with paper chromatography (30).

Proteolysis of ^{32}P-labeled tyrosine hydroxylase. After immunoprecipitation and SDS-PAGE, the ^{32}P-labeled bands corresponding to TH were localized by autoradiography and cut from the dried gel. These were then digested with *S. aureus* protease V8 (50–200 μg/gel slice), and the fragments were resolved by SDS-PAGE (10–20% gradients of polyacrylamide) all as previously described (31). The ^{32}P-containing peptides were localized by autoradiography.

RESULTS

Acute regulation of TH phosphorylation in PC12 cells. PC12 cell cultures were exposed to [^{32}P]orthophosphate

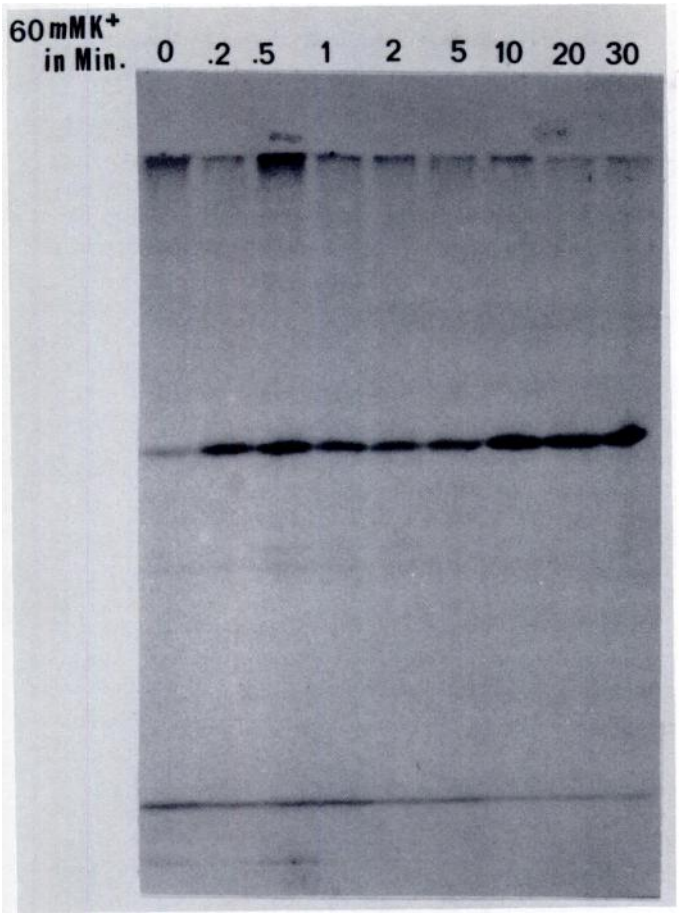

FIG. 4. *Time course of effect of elevated K^+ (60 mM) on the phosphorylation of TH in cultured PC12 cells*

The times of incubation (in minutes) are indicated above each lane. Comparable results were obtained in two independent experiments.

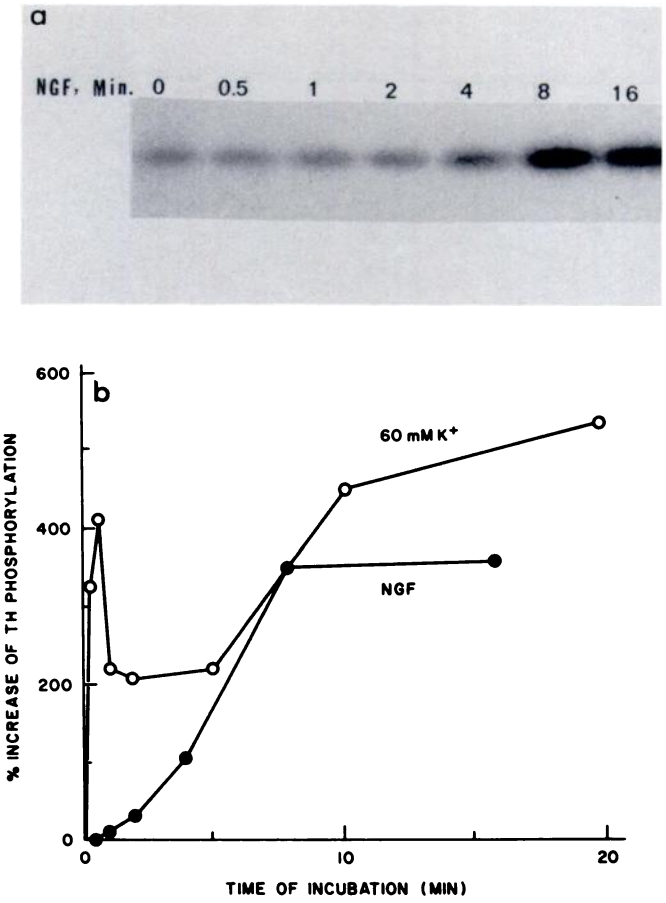

FIG. 5. *Time course of the effect of NGF (50 ng/ml) on phosphorylation of TH in PC12 cultures (a) and comparative time courses of the effects of NGF and elevated K^+ on phosphorylation of TH (b)*

a. Other details are the same as in the legend to Fig. 4. Comparable results were obtained in two independent experiments. b. The relative densities of the bands in Figs. 4 and 5a are shown. For each condition, the values are normalized to the control (zero time) value.

for 1–2 hr and then for an additional 30 min to no additives (control), 50 ng/ml (approximately 2 nM) NGF, elevated (40–60 mM) K^+, or permeant cAMP derivatives (1 mM $N^6,O^{2'}$-dibutyryl-cAMP or 8-bromo-cAMP). After rapid washing to remove free label, the cells were immediately dissolved in SDS-containing sample buffer. The samples were then boiled, and their phosphoproteins were resolved by SDS-PAGE and autoradiography. Fig. 1 shows that both NGF and elevated K^+ caused increases in the relative phosphorylation of a band of apparent M_r = 60,000. This change also occurred in the presence of the cAMP derivatives. A past study has noted these effects in PC12 cells (16) and has indicated that at least a portion of the responsive protein comprising the 60,000-dalton band is immunoprecipitable TH. To study and to compare the effects of elevated K^+ and NGF in more detail, further experiments were carried out by us using a specific antiserum raised against PC12 cell TH.

Effect of various concentrations of K^+ and of NGF on TH phosphorylation. PC12 cell cultures were exposed for 30 min to various concentrations of either NGF or K^+ after exposure to $^{32}PO_4$. After harvesting and boiling, aliquots containing equal numbers of trichloroacetic acid-precipitable counts were subjected to immunoprecipitation with anti-TH serum, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Fig. 2 shows that exposure of the cells to concentrations of K^+ greater than 10 mM results in an enhanced incorporation of $^{32}PO_4$ into the immunoprecipitated TH band. The degree of phosphorylation was dependent on the concentration of K^+ and reached a maximal level by 40–80 mM K^+. Scanning densitometry of the autoradiograms revealed that the maximal stimulation of incorporation of $^{32}PO_4$ was 4–6-fold that in controls.

The results presented in Fig. 3 show that NGF also enhances the incorporation of $^{32}PO_4$ into TH in a concentration-dependent manner. Phosphorylation of TH is considerably increased at an NGF concentration of 3 ng/ml (0.1 nM) and reaches its maximum (3–5-fold stimulation) between 10 and 30 ng/ml (0.3–1 nM). This dose-dependent relationship falls in a range quite similar to that of other NGF-elicited responses in PC12 cells (15) and closely resembles the dose-response relationship for activation of TH by NGF (17).

Time courses of the K^+- and NGF-induced phosphorylation of TH. PC12 cell cultures were pre-exposed to [^{32}P] orthophosphate for 1 hr and then incubated for various times with either elevated K^+ or NGF (60 mM and 50

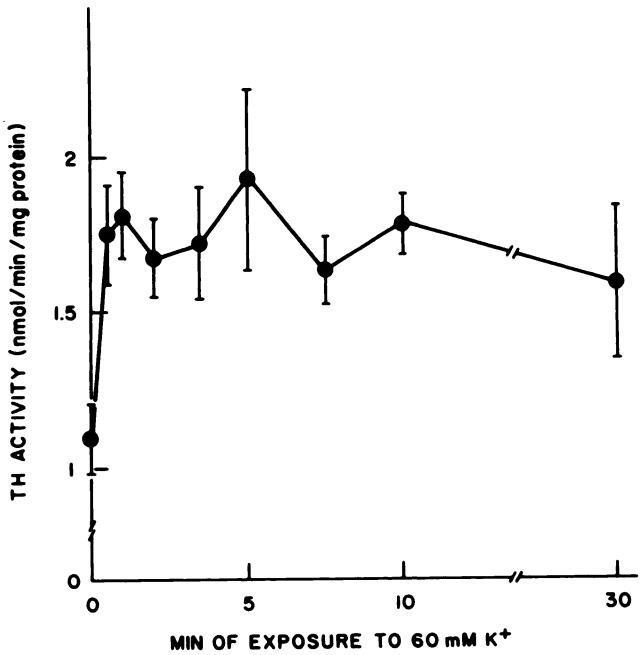

FIG. 6. *Time course of the effect of elevated K^+ (60 mM) on the activity of TH in PC12 cells*

Cells were exposed to elevated K^+ for various times, harvested, and assayed for TH activity as described in "Methods." Data points are means ± SE ($n = 3$).

ng/ml, respectively, in this and all subsequent experiments). As is evident in Figs. 4 and 5b, the K^+-induced enhancement of incorporation of $^{32}PO_4$ follows a biphasic time course. Labeling reaches a maximum (5-fold stimulation) at about 30 sec of exposure and then undergoes a second increase at about 10 min of treatment. The NGF-dependent response shows a rather different time course. The results presented in Fig. 5, a and b, show that the level of incorporation of $^{32}PO_4$ into TH shows little change in the first several minutes after addition of NGF. After 4 min of treatment, phosphorylation of TH increases by about 80% and subsequently reaches its maximum (4–5-fold stimulation) by about 8 min of incubation time. This time course with NGF is in good agreement with that presented by Halegoua and Patrick (16).

Time course of activation of TH. PC12 cultures were exposed to elevated K^+ for various times, and their soluble contents were assayed for TH activity. The data in Fig. 6 show that the enzyme was activated to a plateau level of about 70% over controls within 30 sec. Thus, phosphorylation and activation of the enzyme, although concurrent, do not follow identical time courses (compare Figs. 5b and 6).

A previous time course study (17) has revealed that NGF increases PC12 cell TH activity by about 60% within 10 min of exposure and that activation is half-maximal by about 2–3 min of treatment. This was confirmed in the present experiments, thus indicating that in the case of NGF also, the time courses of TH phosphorylation and activation are not strictly superimposable.

Effect of removal of extracellular Ca^{2+} and of several calmodulin inhibitors on K^+ and NGF-induced phosphorylation of TH. To determine whether the observed enhancements of TH phosphorylation require extracellular Ca^{2+}, cultures were pre-exposed to [^{32}P]orthophosphate and then to either elevated K^+ or NGF in the presence of the Ca^{2+}-chelating agent, EGTA (1 mM) (in Ca^{2+}-free medium). As shown in Fig. 7a, Ca^{2+}-free conditions appeared to completely inhibit the enhancement of TH phosphorylation elicited by elevated K^+, but appeared to have minimal effects on that caused by NGF (Fig. 7b). To quantify these findings more precisely, in one experiment cultures were double-labeled by exposure to [^{35}S] methionine for 3 days and subsequently to [^{32}P]orthophosphate for 1 hr. The cells were then treated (in the continued presence of labeled phosphate) with elevated K^+ or with NGF for 30 min in the presence either of 1 mM EGTA in Ca^{2+}-free medium or of 2.6 mM Ca^{2+} (control medium). TH was then analyzed by immunoprecipitation and SDS-PAGE as above. The TH band was cut from the gels and analyzed for radioactivity. The results are presented in Table 1. The $^{32}P/^{35}S$ ratio in immunoprecipitated TH was only slightly altered for cells incubated in the absence of extracellular Ca^{2+} alone. NGF caused an approximate 2.5-fold increase in the $^{32}P/^{35}S$ ratio of TH (as compared to controls), and this was only marginally affected by the absence of Ca^+. Elevated K^+ also brought about an increase (3.2-fold) in the $^{32}P/^{35}S$ ratio of TH; in contrast, however, this effect was substantially reduced by removal of Ca^{2+}.

Several drugs that are known to inhibit the activity of calmodulin and/or Ca^{2+}- and phospholipid-dependent kinase (kinase C) were tested for their influence on the capacity of elevated K^+ or NGF to enhance the phosphorylation of TH (32, 33). TFP (100 μM) reduced $^{32}PO_4$ incorporation into TH in control cultures by approximately 37% (Fig. 8, a and b). At this concentration, TFP appeared to completely block the K^+-elicited effect on TH (Fig. 8a), while it inhibited the NGF-induced effect on TH (after deduction of the effect on basal phosphorylation) only by approximately 33% (Fig. 8b). Experiments were also carried out with two compounds of closely related chemical structures known as W-5 and W-7. The latter, but not the former, potently interferes with the actions of calmodulin (32). As shown in Fig. 9, W-5 and W-7 (100 μM) have only slight effects on the basal levels of incorporation of $^{32}PO_4$ into TH in control cultures and only marginal effects on the enhanced levels of phosphorylation of this enzyme in NGF-treated cultures. However, while the enhanced $^{32}PO_4$ labeling elicited by high K^+ was reduced approximately 15% by W-5, it was reduced approximately 62% by W-7 (Fig. 9).

Peptide analysis of phosphorylated TH. To determine whether elevated K^+ and NGF enhance the phosphorylation of TH at the same or different sites, $^{32}PO_4$-labeled TH was isolated from each type of culture (control and after various times of exposure to NGF or elevated K^+) by immunoprecipitation and SDS-PAGE and then subjected to electrophoretic peptide analysis after digestion with protease *S. aureus* V8 (31). It is evident from Fig. 10, a and b, that several different ^{32}P-containing peptides could be resolved after digestion with apparent M_r values

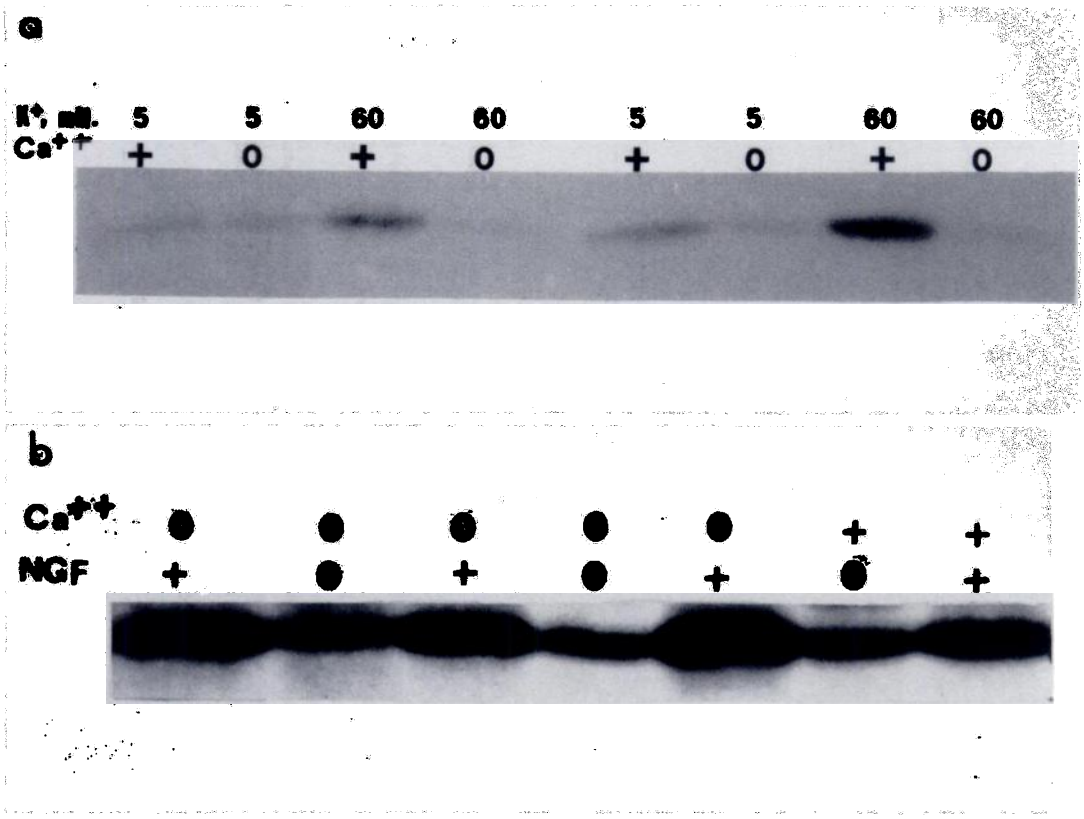

FIG. 7. *Effect of removal of extracellular Ca^{2+} on K^+-elicited enhancement of TH phosphorylation (a) and on NGF-elicited phosphorylation of NGF in cultured PC12 cells*

a. Cultures were incubated for 30 min either in the presence of control Ca^{2+} (2.6 mM in KRH) or in the absence of Ca^{2+} (1 mM EGTA in Ca^{2+}-free KRH) in either control (5 mM) or elevated (60 mM) levels of K^+ after labeling with [^{32}P]orthophosphate. Details of the experiment are given in "Methods." The various parameters of incubation are indicated. The relative densities of the bands are (from *left* to *right*): 1, 1, 3.3, 0.4, 1.6, 0.8, 6.8, and 0.7. Comparable results were obtained in three independent experiments. b. Details are as for a, except that the K^+ concentration was 5 mM in all cultures and NGF (50 ng/ml) was added as indicated. Comparable results were obtained in two independent experiments.

TABLE 1

Effects of removal of extracellular Ca^{2+} on phosphorylation of TH in response to elevated K^+ or NGF

PC12 cells were double-labeled with [^{35}S]methionine and [^{32}P]orthophosphate as described in the text and then treated for 30 min in the presence of the additives as indicated. Equal numbers of trichloroacetic acid-precipitable ^{32}P counts from each culture type were then immunoprecipitated with anti-TH serum, and the immunoprecipitates were analyzed by SDS-PAGE. The TH gel bands were localized by autoradiography and then cut from the gel and analyzed for ^{32}P/^{35}S in a scintillation counter. An area of the gel showing no immunoprecipitable material was also analyzed in order to determine background (which was subtracted from data presented below). No Ca^{2+} was present in the medium unless indicated.

Additives	^{32}P/^{35}S	$+Ca^{2+}/-Ca^{2+}$
Ca^{2+} (2.6 mM)	0.29	
		1.1
EGTA (1 mM)	0.26	
NGF (50 ng/ml) + Ca^{2+} (2.6 mM)	0.73	
		1.4
NGF (50 ng/ml) + EGTA (1 mM)	0.54	
K^+ (60 mM) + Ca^{2+} (2.6 mM)	0.94	
		5.2
K^+ (60 mM) + EGTA (1 mM)	0.18	

ranging from 7,800 to 12,300. The bands will be referred to numerically in order of decreasing mobility. Fig. 10a shows that from 0.5 min and onward of exposure of the cells to elevated K^+, the enhanced phosphorylation of TH is associated with an increased incorporation of ^{32}P into three distinct bands. After 30 min of treatment, the same three bands are affected, although band 2 seems to be phosphorylated to a somewhat greater degree. Two bands at somewhat higher molecular weight (which are also detectable at earlier times) are also enhanced at the 30-min time point; these could represent additional sites of phosphorylation, or incomplete digestion products as suggested by the inconsistency of their presence or relative abundance in different experiments.

The enhanced phosphorylation of TH elicited by exposure of the cells to NGF also resulted in a distinctive pattern of peptide labeling. Fig. 10b shows that following exposure of the cells to NGF for 2, 10, and 20 min, the increase is mainly associated with the higher incorporation of ^{32}P into band 2.

DISCUSSION

In the present study, we have compared the phosphorylation of TH in PC12 cells elicited by exposure to depolarizing levels of K^+ with that mediated by acute

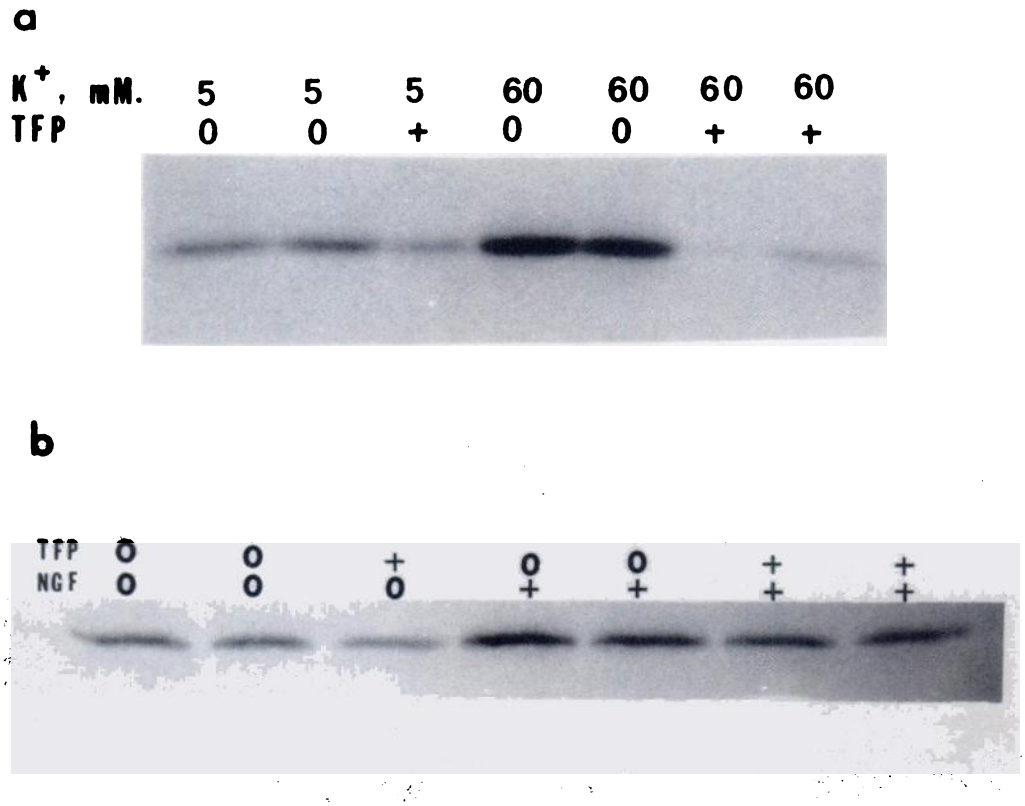

FIG. 8. *Effect of trifluoperazine (TFP) (100 μM) on the phosphorylation of TH in PC12 cultures under various conditions (i.e., the presence and absence of 60 mM K+ (a) or 50 ng/ml NGF (b) for 30 min)*

The various parameters of incubation are as indicated. Relative densitives of the bands are (from *left* to *right*) in a: 1, 1.3, 0.7, 3, 2.8, 0.1, and 0.3; and in b: 1, 0.9, 0.6, 1.6, 1.2, 0.9, and 0.9. Comparable results were obtained in three independent experiments.

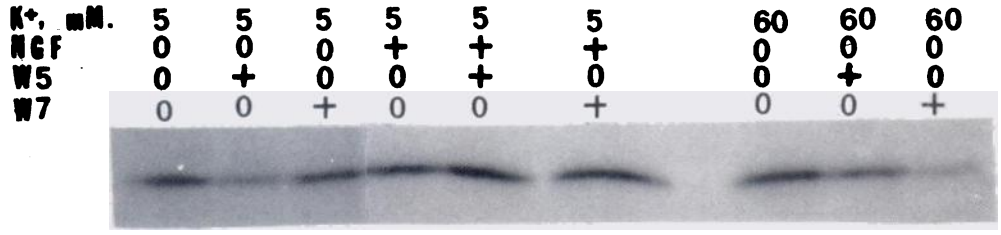

FIG. 9. *Effects of W-5 (100 μM) and W-7 (100 μM) on the phosphorylation of TH in PC12 cultures under various conditions (i.e., the presence and absence of 60 mM K+ or of 50 ng/ml NGF for 30 min)*

The various parameters of incubation are as indicated. The relative densities are (from *left* to *right*): 1, 0.6, 1, 1.3, 1.6, 1.4, 1.3, 1.1, and 0.5. Comparable results were obtained in three independent experiments.

exposure to NGF. Several types of evidence indicated that each affected TH phosphorylation by distinct mechanisms. First, the time courses of each effect were different. Elevated K^+ exhibited a biphasic time course of action, while after addition of NGF, phosphorylation remained unaffected for several minutes and then monotonically increased to a plateau level. Second, the effect of elevated K^+, but not that of NGF, was almost completely abolished when extracellular Ca^{2+} was removed. Third, the actions of elevated K^+ were inhibited in the presence of TFP and W-7, whereas those of NGF were not. Finally, peptide analysis indicated that the two treatments yield different patterns of phosphorylation.

The precise mechanism(s) involved in the phosphorylation of TH in response to elevated K^+ or to NGF are presently unknown. As reviewed in the Introduction, past evidence (1, 11–13, 17) suggests that these treatments work by means that are distinct from those involved in the phosphorylation and activation of TH by cAMP or its analogues. The present as well as past (12, 20) exper-

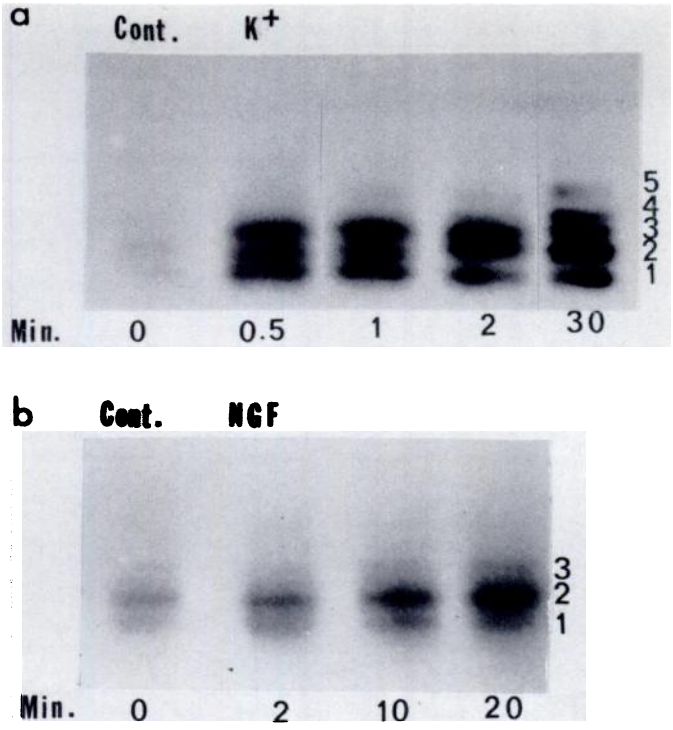

FIG. 10. *Patterns of phosphorylated peptides of PC12 cell TH at various time intervals of exposure of the cells to elevated K^+ or NGF*

The peptides were produced as described in "Methods" by digestion with *S. aureus* protease V8. Cultures were labeled in the presence of [^{32}P]orthophosphate under the indicated conditions for various time intervals. Aliquots containing equal numbers of trichloroacetic acid-precipitable counts were subjected to immunoprecipitation and SDS-PAGE. The ^{32}P-labeled TH obtained in this manner was digested with *S. aureus* protease V8 and then electrophoresed as described in "Methods." a. An autoradiograph of the final gel of cultures exposed to elevated K^+ for 0, 0.5, 1, 2, and 30 min. b. An autoradiograph of the final gel of cultures exposed to NGF for 0, 2, 10, and 20 min.

iments indicate that the effects of depolarizing levels of K^+ on TH phosphorylation require extracellular Ca^{2+} and are probably mediated by its entry. It has been previously demonstrated that PC12 cells possess voltage-sensitive Ca^{2+} channels and that exposure of these cells to elevated K^+ causes a large increase in Ca^{2+} influx (19). Entry of Ca^{2+} may, in turn, enhance TH phosphorylation by means of Ca^{2+}-activated or -dependent kinases (34). It is noteworthy that the phosphorylation of a variety of brain cytosolic proteins is mediated by either phospholipid-sensitive (kinase C) (35) or calmodulin-activated (36) kinases in a Ca^{2+}-dependent manner. Evidence has also been presented that the activation of tryptophan hydroxylase that occurs in depolarized serotonergic neurons is due to Ca^{2+}-dependent phosphorylation of the enzyme (37). It will be of interest to determine whether similar or different kinases are involved in the depolarization-induced phosphorylation of the two enzymes.

As noted above, our present data suggest that NGF enhances TH phosphorylation by means of kinase activity that does not require entry of extracellular Ca^{2+} and that it may be independent of calmodulin. A variety of peptide factors have been found to evoke rapid selective increases in phosphorylation of cellular proteins (cf. Ref. 38), and in several cases the receptors for these factors have themselves been shown to possess kinase activity (cf. Ref. 39). It may then be that NGF enhances TH phosphorylation by activating a distinct kinase or set of kinases. On the other hand, it is conceivable that the changes in phosphorylation elicited by NGF or elevated K^+ are not mediated via kinases alone, but rather also, or exclusively, by the increased phosphorylation of TH due to inhibition of selective phosphatases.

Of relevance to these issues, recent studies[5] indicate that exposure of PC12 cells to NGF activates a protein kinase, the enhanced activity of which can be detected in broken cell preparations. Such experiments indicate, in agreement with the present work, that the NGF-activated kinase is not activated by and does not require either Ca^{2+} or calmodulin and that it is insensitive to chlorpromazine.

This and previous (6) peptide mapping studies have indicated that TH is phosphorylated at multiple sites, even in unstimulated cells. In our study, exposure of PC12 cells to high K^+ or NGF yielded an increased incorporation of $^{32}PO_4$ to several peptides as compared to the controls. However, the patterns of enhanced incorporation of $^{32}PO_4$ into various peptide bands were different for these two treatments. Exposure of the cells to high K^+ resulted in an intense increase of labeling in bands 1–3, while exposure of the cells to NGF resulted in a selective increase of labeling into peptide band 2. We do not presently know the number of different sites phosphorylated within each peptide. Thus, it is unknown whether NGF or elevated K^+ leads to phosphorylation of either the same or different sites within each peptide. Also, it remains to be determined whether or how phosphorylation at each site in an individual peptide chain influences the activity of the tetrameric enzyme molecule.

The present and previous (17, 40) data indicate that for both elevated K^+ and for NGF the time courses of phosphorylation and activation of TH are not strictly superimposable. There are several possible explanations for this. One is that although TH contains multiple sites for phosphorylation, only one is involved in activation of the enzyme. The occurrence of TH as a tetramer raises the possibility that phosphorylation of even less than one site per TH molecule could regulate activity. Thus, activation could take place before full phosphorylation is observed. Alternatively, the differences in time courses could be generated by differences in experimental handling of cell extracts required for the immunoprecipitation and activity measurements.

We have obtained a different time course for phosphorylation and activation of TH after exposure of the cells to high K^+ than that recently reported by Yanagihara *et al.* (41). This discrepancy might also be due to differences in experimental conditions, especially since TH activity was measured by different procedures in these two studies. To resolve these issues, further investigations are in progress.

[5] T. H. Muller, E. M. Rowland, M. Goldstein, and L. A. Greene, manuscript submitted.

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