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Relationship between Activation and Phosphorylation of Tyrosine Hydroxylase by 56 mM K^+ in PC12 Cells in Culture

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SUMMARY

Incubation of rat pheochromocytoma PC12 cells with 56 mM K^+ is associated with increased activity and enhanced phosphorylation of tyrosine hydroxylase in the cells. The increase in the phosphorylation of tyrosine hydroxylase is observed after 30 sec of incubation with 56 mM K^+; maximal phosphorylation is observed after 1 min of incubation. In contrast, although a significant increase in the activity of tyrosine hydroxylase is demonstrable after 30 sec of incubation with 56 mM K^+, maximal activation is not attained until 3 min of incubation. Both the activation and increased phosphorylation of tyrosine hydroxylase exhibit a similar dependence upon potassium concentration in the incubation medium and are dependent on the presence of extracellular calcium. These results suggest that, although there is a relationship between activation and phosphorylation of tyrosine hydroxylase after potassium-evoked depolarization of rat pheochromocytoma PC12 cells in culture, this relationship may be complex.

INTRODUCTION

Tyrosine hydroxylase (tyrosine 3-monooxygenase: L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) is the enzyme that catalyzes the rate-limiting step in the biosynthesis of catecholamines (1, 2). Stimulation of the adrenal medulla and adrenergic neurons results in both enhanced secretion of catecholamines and an increase in catecholamine biosynthesis (2). This increase in catecholamine synthesis is associated with an increase in tyrosine hydroxylase activity in the cells. However, the molecular mechanisms for the activation of tyrosine hydroxylase following stimulation of adrenergic cells have not been unequivocally established.

Recent studies have shown that tyrosine hydroxylase is activated by cyclic AMP-dependent protein kinase *in vitro* (3, 4) and by cyclic AMP analogues *in situ* (5, 6). Moreover, it has been demonstrated that tyrosine hydroxylase activation is associated with increased phosphorylation of the enzyme in various tissues *in vitro* (7–9). Vulliet *et al.* (9) have shown that there is a high correlation between cyclic AMP-dependent phosphorylation and activation of tyrosine hydroxylase purified from rat pheochromocytoma. However, it still remains to be demonstrated that increased phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent protein kinase is involved in the activation of tyrosine hydroxylase in intact cells. Meligeni *et al.* (10) and Haycock *et al.* (11) recently reported that exposure of isolated bovine adrenal chromaffin cells to either cyclic AMP analogues or acetylcholine is associated with increased phosphorylation and activation of tyrosine hydroxylase and that different protein kinases and sites of enzyme phosphorylation are involved in these effects.

In this study, we have examined the effect of 56 mM K^+ on the activity and phosphorylation of tyrosine hydroxylase in rat pheochromocytoma PC12 cells in culture. The PC12 cells are an excellent model system in which to study the regulation of catecholamine synthesis (12, 13). Our studies demonstrate that 56 mM K^+ stimulates the activity of tyrosine hydroxylase and that this is only partially correlated with an increased phosphorylation of tyrosine hydroxylase in intact PC12 cells in culture.

MATERIALS

Tissue culture flasks and dishes were obtained from Falcon Plastics Company (Los Angeles, Calif.). RPMI medium 1640 was obtained from GIBCO Laboratories (Grand Island, N. Y.). Horse serum was from KC Biologicals, Inc. (Lenexa, Kan.). Fetal bovine serum was from Sterile System, Inc. (Logan, Utah.). L-[1-^{14}C]Tyrosine and ^{32}P-labeled inorganic phosphate (carrier-free) were purchased from New England Nuclear Corporation (Boston, Mass.). Poly-L-lysine hydrobromide was from Sigma Chemical Company (St. Louis, Mo.). *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem-Behring Corporation (San Diego, Calif.). The ATP bioluminescence assay kit was obtained from Boehringer-Mannheim GmbH (Mannheim, West Germany). Polyethylene imine-cellulose sheets were from J. T. Baker Chemical Company (Phillipsburg, N. J.). All other chemicals were of the highest purity available from commercial sources.

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METHODS

Cell culture. The rat pheochromocytoma PC12 cells were kindly provided by Dr. Rosanne Goodman (University of Pennsylvania, Philadelphia, Pa.). The PC12 cells were grown as described by Greene and Tischler (14) in plastic tissue culture flasks (Falconware) in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, streptomycin (50 μg/ml), penicillin (50 units/ml). Cultures were maintained at 37° in a water-saturated atmosphere containing 5% CO_2. Usually the cells were resuspended by pipetting and subcultured on 35-mm polylysine-treated dishes at a density of 1 × 10^6 cells/dish. After 3–4 days in culture, cells were washed twice with oxygenated Krebs-Ringer-Hepes[1] buffer (KRH buffer) (pH 7.4), containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, and 5.6 mM glucose, and preincubated for 15 min at 37°. The cells were washed once more with prewarmed KRH buffer and then treated with the indicated compounds. When 56 mM KCl was used, the NaCl content was decreased to keep the tonicity of KRH buffer constant.

Assay of tyrosine hydroxylase activity. After incubation, the medium was removed and the cells were immediately frozen on dry ice and scraped into a buffer containing 30 mM potassium phosphate (pH 6.8), 50 mM NaF, and 1 mM EDTA. The suspension was homogenized, and the homogenate was centrifuged at 20,000 × *g* for 10 min. In control studies, it has been demonstrated that, in homogenates of PC12 cells, at least 80–90% of tyrosine hydroxylase activity is present in the supernatant fraction. The supernatant was collected and subjected to gel filtration on a Sephadex G-25 column previously equilibrated with 30 mM potassium phosphate buffer (pH 6.8), 10 mM NaF, and 0.1 mM EDTA. Tyrosine hydroxylase activity was determined by a modification of the decarboxylase-coupled assay (6). In this assay, L-[1-^{14}C]tyrosine is converted to L-[1-^{14}C]dopa by the action of tyrosine hydroxylase. The generated dopa is then decarboxylated by added aromatic amino acid decarboxylase purified from hog kidney (6). The standard incubation mixture (final volume, 100 μl) contained 10 μmoles of potassium phosphate buffer (pH 6.8), 0.5 μmole of ascorbate, 0.5 μmole of EDTA and 6500 units of catalase (Boehringer Mannheim), 0.01–0.1 μmole of 6-$MePtH_4$ (Calbiochem-Behring), and 0.01 μmole (0.1 μCi) of L-[1-^{14}C] tyrosine. The reaction was allowed to proceed at 30° for 5–10 min. After incubation, the hydroxylation reaction was terminated by the addition of 35 μl of a solution containing 0.2 μmole of 3-iodotyrosine in 10 μmoles of potassium phosphate buffer (pH 6.8). The second reaction was initiated by the addition of 15 μl of L-aromatic amino acid decarboxylase and pyridoxal phosphate to produce quantitative decarboxylation of the L-[1-^{14}C]dopa formed in the first step. The reaction was allowed to proceed at 37° for 30 min and was terminated by injection of 0.1 ml of 0.8 N perchloric acid into the reaction mixture. The $^{14}CO_2$ liberated was collected for 1 hr at 37° in a well containing paper wicks (Whatman 1 MM) and 0.2 ml of NCS tissue solubilizer (Amersham), which was suspended from a rubber septum covering the vessel. The well was then transferred to counting vials, and the radioactivity was determined by liquid scintillation spectrometry. Counting efficiency was approximately 95%. Protein was determined according to the method of Bradford (15), using bovine serum albumin as standard. The specific activity of tyrosine hydroxylase is expressed as nanomoles of $^{14}CO_2$ formed per minute per milligram of protein.

Measurement of phosphorylation of tyrosine hydroxylase. After preincubation for 15 min, the cells in the dish were incubated with [^{32}P] phosphate (carrier-free, 0.125–0.5 mCi/ml) for 30 min at 37°. The cells were then washed twice with 1 ml of prewarmed KRH buffer and incubated for different periods of time with or without 56 mM K^+. After incubation, the medium was removed by aspiration, and the cells were immediately frozen on dry ice. The cells were scraped into a solution containing 30 mM potassium phosphate buffer (pH 6.8), 50 mM NaF, and 1 mM EDTA, and homogenized. The homogenate was centrifuged at 20,000 × *g* for 10 min. Aliquots of the resulting supernatant were mixed with antiserum (60 μl) specific for tyrosine hydroxylase (16) in the presence of 5 mM NaF, 10 mM EDTA, 50 mM sodium pyrophosphate, and 50 mM potassium phosphate buffer (pH 7.6) in a final volume of 300 μl. This mixture was incubated at room temperature for 60 min. After incubation, an appropriate volume (75 μl) of *Staphylococcus aureus* cells (see below) was added, and the mixture was incubated for 15 min at room temperature. The sample was centrifuged at 12,000 × *g* for 3 min. The pellet was resuspended and centrifuged, successively in (a) 0.8 M NaCl/0.8% Triton X-100; (b) twice in 0.12 M NaCl/16 mM Tris-HCl (pH 7.8) 0.8% Triton X-100/0.8% deoxycholate/0.08% SDS/0.8 M urea; and finally, (c) in 0.8 M urea. All washing media contained 50 mM potassium phosphate buffer (pH 7.6), 20 mM EDTA, 5 mM NaF, and 50 mM sodium pyrophosphate. After the washing procedure, tyrosine hydroxylase was eluted from the *S. aureus* cells by suspending the pellet in 45 μl of a solution containing 3% SDS, 5% glycerol, 0.02% bromophenol blue, 10 mM Tris-HCl (pH 7.8), and 15 μl of 500 mM dithiothreitol. After boiling at 95° for 10 min and centrifuging at 12,000 × *g* for 5 min, supernatants were applied to SDS-polyacrylamide slab gels.

Gel electrophoresis was performed according to the method described by Rudolph and Krueger (17). After electrophoresis, the gels were stained and destained. The gels were dried with heat and vacuum (by Slab Gel Dryer: Hoffer Scientific, Inc.) on Whatman 3 MM paper. Autoradiography of the dried gels was performed by a modification of the method of Rudolph and Krueger (17). The [^{32}P]phosphate incorporated into tyrosine hydroxylase was assessed both from the density of the appropriate autoradiographic band on the gel and by cutting out the protein band corresponding to purified tyrosine hydroxylase standard and counting the ^{32}P by liquid scintillation spectrometry (16).

Preparation of S. aureus cells (Pansorbin). The *S. aureus* cells were prepared according to the method of Kessler (18). The cells (75 μl/sample) were centrifuged at 3000 rpm for 30 min, and the resulting pellet was resuspended in a solution containing 0.11 M NaCl, 7 mM Tris-HCl (pH 7.6), 14 mM EDTA, 0.35% Triton X-100, 50 mM NaF, 50 mM potassium phosphate (pH 7.6), and 50 mM sodium pyrophosphate. After incubation at room temperature for 15 min, this suspension was centrifuged at 3000 rpm for 30 min, and the pellet was resuspended in a solution containing 0.12 M NaCl, 8 mM Tris-HCl (pH 7.6), 16 mM EDTA, 0.04% Triton X-100, bovine serum albumin (0.8 mg/ml), 5 mM NaF, 50 mM potassium phosphate (pH 7.6), and 50 mM sodium pyrophosphate. This suspension was stored at 2° until use.

Assay of endogenous ATP and newly synthesized [^{32}P]ATP in the cells. The PC12 cells were incubated with or without [^{32}P]phosphate (carrier-free; 0.5 mCi/ml) and washed twice with KRH buffer (pH 7.4). The cells were scraped off the dishes with 0.25 M sucrose/10 mM NaF/2 mM EDTA/10 mM Hepes buffer (pH 7.4) and homogenized at 2°. The homogenate was centrifuged at 20,000 × *g* for 20 min. The supernatant was incubated at 95° for 10 min and used for the assay of ATP and [^{32}P]ATP. The ATP content in the supernatant was determined by the firefly luciferin-luciferase method with ATP bioluminescence assay kit (Boehringer-Mannheim), using a scintillation counter to measure emitted light. The [^{32}P]ATP in the supernatant was separated by thin-layer chromatography on polyethylene imine-cellulose sheets using 1.6 M LiCl as the resolving solvent (19). After drying, the [^{32}P]ATP was localized by autoradiography, and the spots were cut off from the plates and counted by liquid scintillation spectrometry. For measurement of recovery, either ATP or [^{32}P]ATP was added to the suspension of harvested cells as an internal standard. The recoveries were 48.8 ± 12.4% and 55.6 ± 6.5% for ATP and [^{32}P]ATP, respectively.

RESULTS

Stimulation of tyrosine hydroxylase phosphorylation by 56 mM K^+ in PC 12 cells. [^{32}P]Phosphate (0.125–0.5 mCi/ml) added to the incubation medium was rapidly taken

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dopa, dihydroxyphenylalanine; 6-$MePtH_4$, D,L-2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine HCl; SDS, sodium dodecyl sulfate.

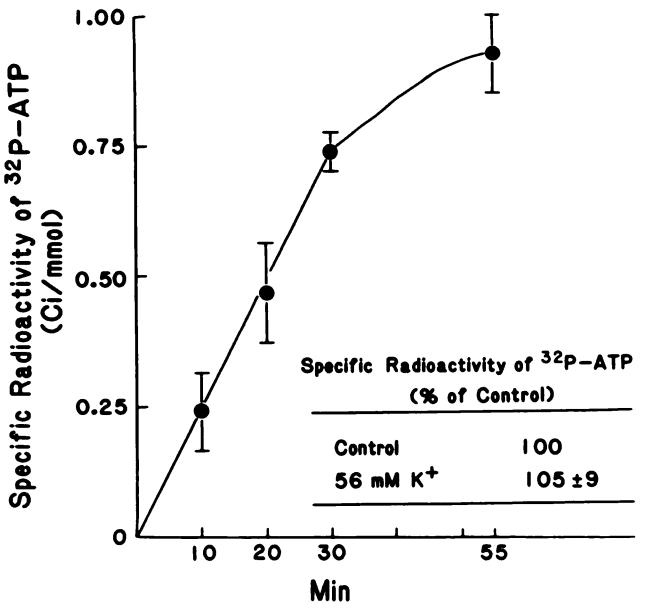

FIG. 1. *Specific radioactivity of ATP in PC12 cells incubated in the presence of inorganic [^{32}P]phosphate*

The PC12 cells were incubated for various times at 37° with [^{32}P] phosphate (carrier-free, 0.5 mCi/ml) and washed twice with KRH buffer (pH 7.4). The cells were scraped into a buffer containg 0.25 M sucrose, 10 mM NaF, 2 mM EDTA, and 10 mM Hepes (pH 7.4). The ATP content and [^{32}P]ATP in supernatant of cells were determined as described under Methods. The values are expressed as the mean ± standard deviation of three experiments.

Inset. After incubation of the cells for 30 min at 37° with [^{32}P] phosphate (carrier-free, 0.5 mCi/ml), the cells were washed twice with KRH buffer (pH 7.4) and incubated for 2 min at 37° with or without 56 mM K^+. The ATP content and [^{32}P]ATP in supernatant of cells were determined as described under Methods. The specific radioactivity of [^{32}P]ATP in cytosol of control cells was 0.757 ± 0.032 Ci/mmole. The values are expressed as the mean ± standard deviation of three experiments.

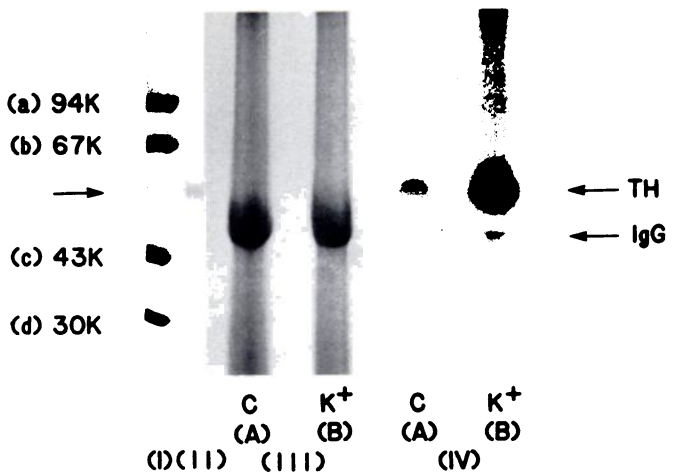

FIG. 2. *Coomassie blue stain and autoradiogram of SDS-polyacrylamide gel electrophoretic patterns of antiserum-treated supernatants from control and 56 mM K^+-stimulated cells*

The cells were preincubated at 37° with [^{32}P]phosphate (0.5–0.125 mCi/ml) for 30 min and then washed with prewarmed KRH buffer twice. The cells were then incubated for 3 min at 37° with or without 56 mM K^+. The supernatants from control cells and 56 mM K^+-stimulated cells were allowed to react with anti-tyrosine hydroxylase antiserum. Immunoprecipitates were dissolved in 3% SDS and 125 mM dithiothreitol and subjected to electrophoresis in 10% SDS-polyacrylamide gels for approximately 5 hr at 180 V (constant voltage) (17). Coomassie blue stain of SDS-PAGE: *(I)* molecular weight standard (subunits in daltons), phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000 (Pharmacia); *(II)* partially purified pheochromocytoma tyrosine hydroxylase (*TH*); *(III)* immunoprecipitate of control *(A)* and 56 mM K^+-stimulated *(B)* cells. Autoradiogram of SDS polyacrylamide gel electrophoresis: *(IV)* immunoprecipate of control *(A)* and 56 mM K^+-stimulated *(B)* cells.

up into the PC12 cells. As shown in Fig. 1, [^{32}P]ATP was synthesized from [^{32}P]phosphate in the PC12 cells. Following incubation with [^{32}P]phosphate for 30 min, there was no significant difference in the specific radioactivity of [^{32}P]ATP in cytosol isolated from cells incubated for 2 additional min in the presence or absence of 56 mM K^+ (Fig. 1). Incubation of the cells with [^{32}P]phosphate for 30 min resulted in a small amount of ^{32}P incorporation into a prominent band which was demonstrable on the SDS gel following immunoprecipitation of the PC12 cell supernatant with antiserum against tyrosine hydroxylase. The band (M_r = approximately 60,000) migrated to a position coincident with that of tyrosine hydroxylase purified from rat pheochromocytoma. Treatment of the cells with 56 mM K^+ resulted in a 3- to 6-fold increase in the incorporation of [^{32}P]phosphate into the same band (Fig. 2).

Time course of activation and phosphorylation of tyrosine hydroxylase by 56 mM K^+. Basal activity of tyrosine hydroxylase did not significantly change during incubation of the cells for at least 10 min at 37°. An increase in tyrosine hydroxylase activity was observed after 30 sec of incubation with 56 mM K^+. The maximal activation of tyrosine hydroxylase was observed after incubation with 56 mM K^+ for 3 min. This activation was still present after incubation for 10 min in the presence of 56 mM K^+ (Fig. 3).

An increase in the incorporation of [^{32}P]phosphate into tyrosine hydroxylase was observed after incubation of the cells with 56 mM K^+ for 30 sec. The maximal phosphorylation of tyrosine hydroxylase (3- to 6-fold greater than that observed in untreated cells) was observed after incubation with 56 mM K^+ for 1 min and persisted for at least 5 min of incubation (Fig. 4). K^+ (56 mM) also stimulated the phosphorylation of several other proteins in the cells when we measured phosphorylation of total cellular proteins and did not utilize the immunoprecipitation step. However, in PC12 cells, the M_r 60,000 tyrosine hydroxylase band was the major soluble protein phosphorylated (data not shown).

Effect of external Ca^{2+} on activation and phosphorylation of tyrosine hydroxylase by 56 mM K^+. The effect of 56 mM K^+ on activation and phosphorylation of tyrosine hydroxylase in the PC12 cells was examined in medium containing different concentrations of calcium. Basal tyrosine hydroxylase activity was not dramatically changed in cells treated with different concentrations of Ca^{2+} (Fig. 5). However, omission of Ca^{2+} from the incubation medium abolished the activation of tyrosine hydroxylase caused by 56 mM K^+. At higher concentrations of Ca^{2+} (5.2 mM), tyrosine hydroxylase activation by 56 mM K^+ was maintained. Similarly, the K^+-stimulated

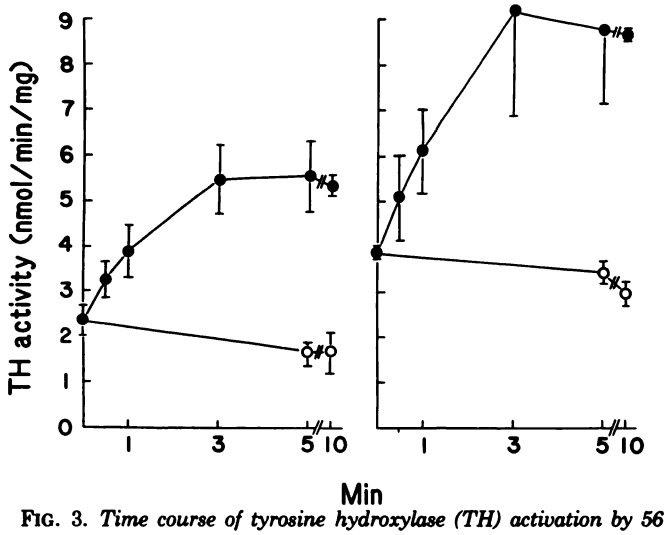

FIG. 3. *Time course of tyrosine hydroxylase (TH) activation by 56 mM K^+*

The PC12 cells were preincubated for 15 min (37°) and washed with prewarmed KRH buffer. The cells were then incubated at 37° for different periods of time in the presence (●) or absence (○) of 56 mM K^+. Tyrosine hydroxylase was assayed as described under Methods.

Left. Tyrosine hydroxylase activity using 0,1 mM 6-$MePtH_4$ as cofactor. Basal tyrosine hydroxylase activity at 0 min = 2.27 ± 0.32 nmole/min/mg. Right. Tyrosine hydroxylase activity using 1 mM 6-$MePtH_4$. Basal tyrosine hydroxylase activity at 0 min = 3.85 ± 0.19 nmole/min/mg. Results are given as the mean ± standard deviation (n = 4–8 experiments).

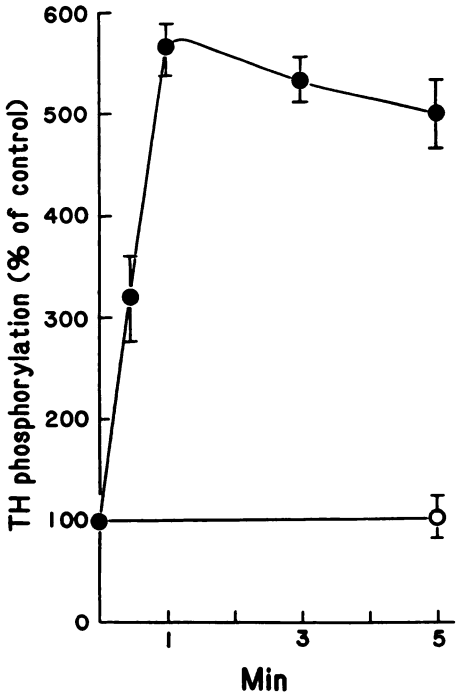

FIG. 4. *Time course of the phosphorylation of tyrosine hydroxylase (TH) by 56 mM K^+*

The PC12 cells were incubated for 30 min at 37° with [^{32}P]phosphate (0.125–0.5 mCi/ml). After washing the cells with prewarmed KRH buffer, the cells were incubated for different periods of time in the presence (●) or (○) absence of 56 mM K^+. The supernatants of control and 56 mM K^+-stimulated cells were treated with antiserum to tyrosine hydroxylase and the immunoprecipitates were analyzed for ^{32}P after SDS-polyacrylamide gel electrophoresis. The autoradiogram of the gel was exposed for 15–20 hr at −90° in the presence of a light-intensifying screen. ^{32}P incorporation into the 60,000-dalton band was determined by liquid scintillation spectrometry after identifying and removing from the gel the band corresponding to tyrosine hydroxylase, as described under Methods. The incorporation of [^{32}P]phosphate into control tyrosine hydroxylase was 213–904 cpm/dish. The increase in the ^{32}P phosphorylation of tyrosine hydroxylase is expressed as the percentage of control at 0 min (means ± standard deviation) (n = 4 or 5 experiments).

phosphorylation of tyrosine hydroxylase (Fig. 6) was abolished when Ca^{2+} was omitted from the incubation medium. Phosphorylation of tyrosine hydroxylase was comparable in cells incubated in the presence of either 2.6 or 5.2 mM Ca^{2+}. Thus, the stimulatory effects of 56 mM K^+ on activation and phosphorylation of tyrosine hydroxylase were dependent upon the presence of Ca^{2+} in the incubation medium.

Effect of different concentrations of K^+ in the medium on the activation and phosphorylation of tyrosine hydroxylase. Figures 7 and 8 depict the effects of different K^+ concentrations on activation and phosphorylation of tyrosine hydroxylase. There was a similar relationship between K^+ concentration and both the activation and phosphorylation of tyrosine hydroxylase.

DISCUSSION

We investigated the relationship between activation and phosphorylation of tyrosine hydroxylase in rat pheochromocytoma PC12 cells in culture. [^{32}P]Phosphate was taken up into these cells and was converted to [^{32}P]ATP. After 30 min of incubation with [^{32}P]phosphate, the specific radioactivity of [^{32}P]ATP in the cytosol of the cells was approximately 0.75 Ci/mmole. After preincubation of the cells with [^{32}P]phosphate, there was no significant difference in the specific radioactivity of [^{32}P]ATP in the cytosol isolated from control cells and cells that had been treated with 56 mM K^+ for an additional 2 min. Depolarization of the PC12 cells with 56 mM K^+, followed by immunoprecipitation with specific tyrosine hydroxylase antiserum and SDS-polyacrylamide gel electrophoresis of the supernatant prepared from homogenates of these cells, revealed that there was an increased incorporation of [^{32}P]phosphate into one prominent protein band which migrated to a position coincident with that of tyrosine hydroxylase purified from rat pheochromocytoma tissue. Using the specific radioactivity of [^{32}P]ATP (0.75 Ci/mmol) in the cells and the specific activity of tyrosine hydroxylase purified from rat pheochromocytoma tumor (500 nmoles/min/mg of protein), we estimate that approximately 0.2–0.3 mole of [^{32}P]phosphate was incorporated into 1 mole of 60,000 M_r subunit of tyrosine hydroxylase after 1–3 min of incubation of PC12 cells in the presence of 56 mM K^+ (9). This value must be regarded as approximate, since we cannot be certain about the precise specific activity of either cytosolic ATP or tyrosine hydroxylase in PC12 cells. It is possible that a more restricted pool of ATP, of which the specific radioactivity is not ascertainable by present methodology, is directly involved in the phosphorylation of tyrosine hydroxylase. Furthermore, it is possible that a fraction of the [^{32}P]ATP is labeled in the α or β, rather than the γ position.

Enhanced phosphorylation of tyrosine hydroxylase

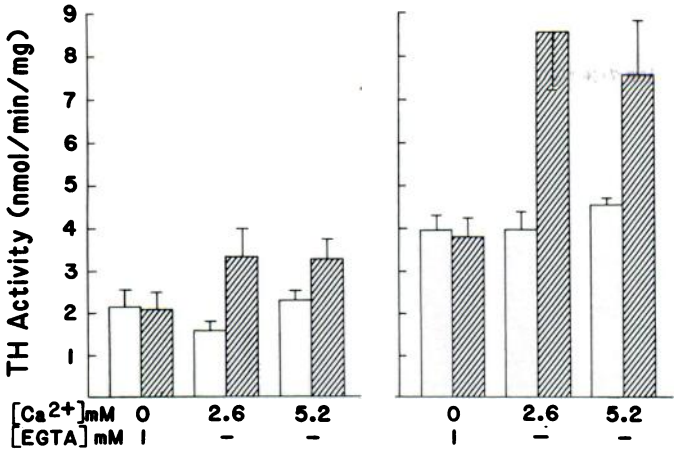

FIG. 5. *Effect of external Ca^{2+} on tyrosine hydroxylase (TH) activation by 56 mM K^+*

After preincubation (as described in the legend to Fig. 3), the cells were washed twice with prewarmed KRH buffer lacking Ca^{2+} or containing 2.6 mM Ca^{2+} and incubated for 3 min at 37° with (▨) or without (□) 56 mM K^+ in the presence of different concentrations of Ca^{2+} (0 mM, 2.6 mM, 5.2 mM). Tyrosine hydroxylase activity was assayed as described under Methods.

Left, Tyrosine hydroxylase activity with 0.1 mM 6-MePtH$_4$; right, tyrosine hydroxylase activity with 1 mM 6-MePtH$_4$. Results are given as the mean ± standard deviation (n = 4 experiments).

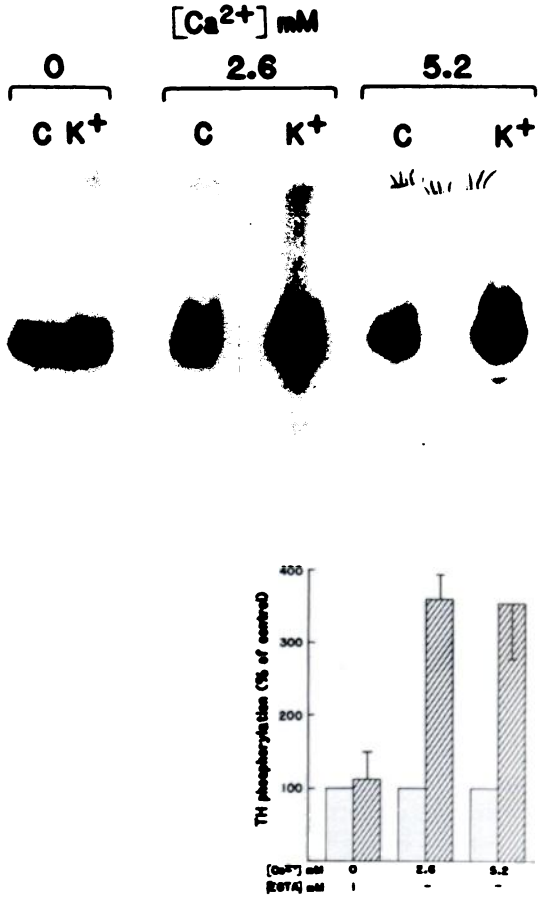

FIG. 6. *Effect of external Ca^{2+} on the phosphorylation of tyrosine hydroxylase (TH) by 56 mM K^+*

The PC12 cells were incubated for 30 min at 37° with [^{32}P]phosphate (0.125–0.5 mCi/ml). The cells were washed twice with prewarmed KRH buffer lacking Ca^{2+} or containing Ca^{2+} (2.6 mM) and incubated for 3 min with 4.8 mM or 56 mM K^+ in the presence of different concentrations of Ca^{2+} (0 mM, 2.6 mM, 5.2 mM). Supernatants from these cells were treated with antiserum and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis as described under Methods. Autoradiography of the gel and determination of counts per minute (*inset*) of ^{32}P incorporated into the band corresponding to tyrosine hydroxylase were performed as described in the legend to Fig. 4. *C*, Control cell; *K*$^+$, 56 mM K^+-stimulated cell. Inset, ▨ = 56 mM K^+; □ = 4.8 mM K^+. Incorporations of [^{32}P]phosphate into control tyrosine hydroxylase were 256–293 cpm/dish (at 0 mM Ca^{2+}), 217–573 cpm/dish (at 2.6 mM Ca^{2+}), 207–354 cpm/dish cells (at 5.2 mM Ca^{2+}). Results are given as the mean ± standard deviation (n = 4 experiments).

was observed after incubation with 56 mM K^+ for 30 sec. The maximal phosphorylation of tyrosine hydroxylase was observed after incubation for 1 min. The time course of the phosphorylation of tyrosine hydroxylase by 56 mM K^+ was more rapid than that of the activation of tyrosine hydroxylase by 56 mM K^+ (compare Figs. 3 and 4). If the phosphorylation of tyrosine hydroxylase is responsible for the activation of the enzyme in the PC12 cells, and this involves a simple bimolecular reaction, then the rate of phosphorylation and activation should coincide. Although the reason(s) for this discrepancy is not clear, there are several possible explanations: (a) following the phosphorylation of tyrosine hydroxylase in intact cells, a second reaction must occur which results in enzyme activation [Yamauchi *et al.* (20)]; (b) the phosphorylation of tyrosine hydroxylase may take place at multiple sites on the tetrameric enzyme, and more than one subunit must be phosphorylated before enzyme activation can be expressed; (c) each subunit is phosphorylated at several sites and more than one site must be covalently modified before enhanced enzyme activity is expressed; (d) dephosphorylation may be occuring at sites which are not critical to activation while phosphorylation proceeds more slowly at the sites which, when phosphorylated, are associated with enzyme activation; or (e) there may be a mechanism other than phosphorylation which is involved in the activation of tyrosine hydroxylase. For explanations b and c, because of a lack of complete precision in our estimates of phosphorylation, we may not be capable of distinguishing between 85% phosphorylation and 95% phosphorylation. If phosphorylation of three or more sites produces no activation, but subsequent phosphorylation of an additional site results in activation, then we might detect "full phosphorylation," as far as the precision of our analysis permits, before we detect "full activation."

Another possible explanation for the discordant time courses for the activation and phosphorylation of tyrosine hydroxylase is that subsequent alterations in the enzyme may take place following homogenization that are associated with enzyme activation. One possible mechanism is limited proteolysis of the enzyme, which is known to be associated with activation of tyrosine hydroxylase (2). However, the time course of the activation of tyrosine hydroxylase in PC12 cells by 56 mM K^+ was not affected by addition of 2 mM EGTA, leupeptin

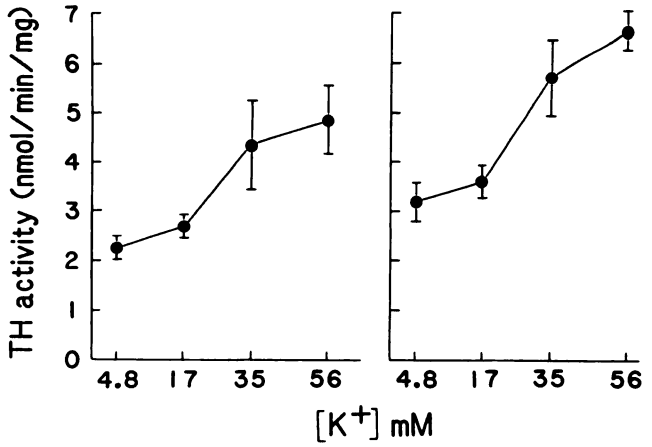

FIG. 7. *Effect of different concentrations of K^+ on tyrosine hydroxylase (TH) activation*

The PC12 cells were preincubated for 30 min (37°) and washed with prewarmed KRH buffer. After washing, the PC12 cells were incubated for 3 min (37°) with different concentrations of K^+ (4.8 mM, 17 mM, 35 mM, 56 mM K^+). TH activity was assayed as described under Methods. Tyrosine hydroxylase activity was measured in the presence of 0.1 mM 6-MePtH$_4$ (*left*) and 1 mM 6-MePtH$_4$ (*right*). Results are given as the mean ± standard deviation (n = 3 experiments).

(50 μg/ml), and 0.1 mM phenylmethylsulfonyl fluoride to the homogenizing buffer (data not shown). Furthermore, the behavior of tyrosine hydroxylase on SDS-polyacrylamide gel electrophoresis was not altered following *in situ* activation by 56 mM K^+, suggesting that a peptide fragment is not cleaved from the enzyme subunits during the experimental manipulations. These results suggest that *in vitro* proteolysis is not a significant problem under the experimental conditions employed in these studies.

The activation of tyrosine hydroxylase mediated by phosphorylation by cyclic AMP-dependent protein kinase is generally associated with a decrease in the K_m of the enzyme for the pterin cofactor. However, our experiments (Figs. 3, 5, and 7) indicate that the degree of activation of tyrosine hydroxylase is identical when the enzyme is assayed at either 0.1 or 1 mM 6MePtH$_4$. These results suggest that 56 mM K^+ stimulates the activity of tyrosine hydroxylase by an increase in the apparent V_{max} of the enzyme. This interpretation is in agreement with the data of Chalfie *et al.* (21).

Both activation and increased phosphorylation of tyrosine hydroxylase by 56 mM K^+ were abolished when Ca^{2+} was omitted from the incubation medium. Recent studies have shown that 56 mM K^+ stimulates $^{45}Ca^{2+}$ uptake into rat pheochromocytoma cells (22); thus, it is possible that 56 mM K^+ increases the phosphorylation and the activity of tyrosine hydroxylase by increasing the activity of a calcium-dependent protein kinase. Yamauchi *et al.* (20) and Mestikawy *et al.* (23) have reported that the activity of tyrosine hydroxylase in rat brain is stimulated by a Ca^{2+}/calmodulin-dependent protein kinase *in vitro*. There is also evidence that a calcium-dependent, phospholipid-sensitive protein kinase activates tyrosine hydroxylase *in vitro* (24).

There have been many reports demonstrating that cyclic AMP plays an important role in the regulation of tyrosine hydroxylase from various tissues (2, 5, 10, 21,

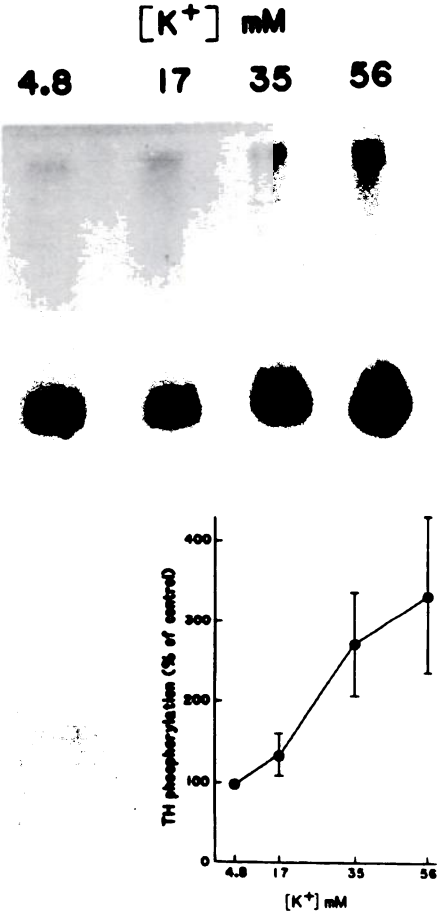

FIG. 8. *Effect of different concentrations of K^+ on tyrosine hydroxylase (TH) phosphorylation*

The PC12 cells were preincubated for 30 min at 37° in the presence of [^{32}P]phosphate (0.5–0.125 mCi/ml) and washed with prewarmed KRH buffer. After washing, the cells were incubated for 3 min with different concentrations of K^+, as described in the legend to Fig. 7. Autoradiography of the gel and determination of counts per minute (*inset*) of ^{32}P incorporated into the band corresponding to tyrosine hydroxylase were performed as described in the legend to Fig. 4. The incorporation of [^{32}P]phosphate into control cells (4.8 mM K^+) was 208–419 cpm/dish. Results are given as the mean ± standard deviation (n = 3 experiments).

25, 26). Cyclic AMP-dependent protein kinase increases the activity and catalyzes the phosphorylation of tyrosine hydroxylase purified from rat pheochromocytoma tumor (9). Furthermore, we have observed that dibutyryl cyclic AMP stimulates both the activity and the phosphorylation of tyrosine hydroxylase in intact PC12 cells in culture (data not shown). K^+ (56 mM) increases the levels of cyclic AMP in PC12 cells (27). Therefore, it is possible that this K^+-stimulated increase in cyclic AMP is responsible for the phosphorylation and activation of tyrosine hydroxylase in the cells via activation of cyclic AMP-dependent protein kinase. However, recent data suggest that depolarizing agents or secretagogues and cyclic AMP stimulate catecholamine synthesis by different mechanisms (4, 11, 21, 28). For example, tyrosine hydroxylase in bovine chromaffin cells in culture is phosphorylated *in situ* at more than one site, and phosphorylation of these sites is affected differently by acetyl-

choline and 8-bromo cyclic AMP (29). Furthermore, Andrews *et al.* (30) have shown that striatal tyrosine hydroxylase can be activated by a protein kinase which is cyclic AMP-independent and may be calcium-independent. From these data, it appears likely that multiple protein kinases may be involved in the regulation of tyrosine hydroxylase in brain and in chromaffin cells, including rat pheochromocytoma PC12 cells.

REFERENCES

1. Nagatsu, T., M. Levitt, and S. Udenfriend. Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239**:2910–2917 (1964).
2. Weiner, N. Regulation of norepinephrine biosynthesis. *Annu. Rev. Pharmacol.* **10**:273–290 (1970).
3. Morgenroth, V. H., III, L. R. Hegstrand, R. H. Roth, and P. Greengard. Evidence for involvement of protein kinase in the activation by adenosine 3′:5′-monophosphate of brain tyrosine 3-monooxygenase. *J. Biol. Chem.* **250**:1946–1948 (1975).
4. Goldstein, M., R. L. Bronaugh, B. Ebstein, and C. Roberge. Stimulation of tyrosine hydroxylase activity by cyclic AMP in synaptosomes and in soluble striatal enzyme preparations. *Brain Res.* **109**:563–574 (1976).
5. Anagnoste, B., C. Shirron, E. Friedman, and M. Goldstein. Effect of dibutyryl cyclic adenosine monophosphate on ^{14}C-dopamine biosynthesis in rat brain striatal slices. *J. Pharmcol. Exp. Ther.* **191**:370–376 (1974).
6. Weiner, N., F.-L. Lee, E. Dreyer, and E. Barnes. The activation of tyrosine hydroxylase in noradrenergic neurons during acute nerve stimulation. *Life Sci.* **22**:1197–1216 (1978).
7. Joh, T. H., D. H. Park, and D. J. Reis. Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: mechanism of enzyme activation. *Proc. Nalt. Acad. Sci. U. S. A.* **75**:4744–4748 (1978).
8. Yamauchi, T., and H. Fujisawa. *In vitro* phosphorylation of bovine adrenal tyrosine hydroxylase by adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **254**:503–507 (1979).
9. Vulliet, P. R., T. A. Langan, and N. Weiner. Tyrosine hydroxylase: a substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* **77**:92–96 (1980).
10. Meligeni, J. A., J. W. Haycock, W. F. Bennett, and J. C. Waymire. Phosphorylation and activation of tyrosine hydroxylase mediate the cAMP-dependent increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.* **257**:12632–12640 (1982).
11. Haycock, J. W., J. A. Meligeni, W. F. Bennett, and J. C. Waymire. Phosphorylation and activation of tyrosine hydroxylase mediate the acetylcholine-induced increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.* **257**:12641–12648 (1982).
12. Chalfie, M., and R. L. Perlman. Regulation of catecholamine biosynthesis in a transplantable rat pheochromocytoma. *J. Pharmacol. Exp. Ther.* **200**:588–597 (1977).
13. Greene, L. A., and G. Rein. Short-term regulation of catecholamine biosynthesis in a nerve growth factor responsive clonal line of rat pheochromocytoma cells. *J. Neurochem.* **30**:549–555 (1978).
14. Greene, L. A., and A. S. Tischler. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2424–2428 (1976).
15. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
16. Meligeni, J., A. W. Tank, J. K. Stephens, E. Dreyer, and N. Weiner. *In vivo* phosphorylation of rat adrenal tyrosine hydroxylase during acute decapitation stress. *Cold Spring Harbor Conf. Cell Prolif.* **8**:1377–1389 (1981).
17. Rudolph, S. A., and B. K. Krueger. Endogenous protein phosphorylation and dephosphorylation. *Adv. Cyclic Nucleotide Res.* **10**:107–133 (1979).
18. Kessler, S. W. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol* **115**:1617–1624 (1975).
19. Randerath, K., and E. Randerath. Thin-layer separation methods for nucleic acid derivatives. *Methods Enzymol.* **12A**:323–347 (1967).
20. Yamauchi, T., and H. Fujisawa. Tyrosine 3-monooxygenase is phosphorylated by Ca^{++}-, calmodulin-dependent protein kinase, followed by activation by activator protein. *Biochem. Biophys. Res. Commun.* **100**:807–813 (1981).
21. Chalfie, M., L. Settipani, and R. L. Perlman. The role of cyclic adenosine 3′:5′-monophosphate in the regulation of tyrosine 3-monooxygenase activity. *Mol. Pharmacol.* **15**:263–270 (1979).
22. Toll, L. Calcium antagonists: high affinity binding and inhibition of calcium transport in a clonal cell line. *J. Biol. Chem.* **257**:13189–13192 (1982).
23. Mestikawy, S. E., J. Glowinski, and M. Hamon. Tyrosine hydroxylase activation in depolarized dopaminergic terminals: involvement of Ca^{++}-dependent phosphorylation. *Nature (Lond.)* **302**:830–832 (1983).
24. Iuvone, P. M., and B. K. J. Butler. Activation of striatal tyrosine hydroxylase by calcium, ATP and magnesium: possible involvement of endogenous calcium-dependent phospholipid-sensitive protein kinase. *Fed. Proc.* **42**:379 (1983).
25. Oka, M., and F. Izumi. Effect of cyclic adenosine 3′:5′-monophosphate and its dibutyryl derivatives on catecholamine synthesis in bovine adrenal medullary slices. *F. E. B. S. Lett.* **51**:253–256 (1975).
26. Masserano, J. M., and N. Weiner. The rapid activation of adrenal tyrosine hydroxylase by decapitation and its relationship to a cyclic AMP-dependent phosphorylating mechanism. *Mol. Pharmacol.* **16**:513–528 (1979).
27. Baizer, L., and N. Weiner. Regulation of catecholamine release from PC12 cells during stimulation with elevated K^+ or carbachol. *Fed. Proc.* **42**:378 (1983).
28. Simon, J. R., and R. H. Roth. Striatal tyrosine hydroxylase: comparison of the activation produced by depolarization and dibutyryl-cAMP. *Mol. Pharmacol.* **16**:224–233 (1979).
29. Haycock, J. W., W. F. Bennett, R. J. George, and J. C. Waymire. Multiple site phosphorylation of tyrosine hydroxylase: Differential regulation *in situ* by 8-bromo-cAMP and acetylcholine. *J. Biol. Chem.* **257**:13699–13703 (1982).
30. Andrews, D. W., T. A. Langan, and N. Weiner. Evidence for the involvement of a cyclic AMP-independent protein kinase in the activation of soluble tyrosoine hydroxylase from rat striatum. *Proc. Natl. Acad. Sci. U. S. A.* **80**:2097–2101 (1983).

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