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Phoshorylation of Tyrosine Hydroxylase on at Least Three Sites in Rat Pheochromocytoma PC12 Cells Treated with 56 mM K^+: Determination of the Sites on Tyrosine Hydroxylase Phosphorylated by Cyclic AMP-Dependent and Calcium/Calmodulin-Dependent Protein Kinases

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SUMMARY

Incubation of rat pheochromocytoma PC12 cells with the calcium ionophore, A23187 (10^{-5} M), 56 mM K^+, or dibutyryl cAMP (2 mM) is associated with increased activity and enhanced phosphorylation of tyrosine hydroxylase in the cells. Both the activation and the increased phosphorylation of tyrosine hydroxylase produced by A23187 and 56 mM K^+ are dependent on the presence of extracellular calcium, whereas similar effects produced by dibutyryl cAMP are independent of calcium. The effects of 56 mM K^+ plus dibutyryl cAMP or A23187 plus dibutyryl cAMP on the activation and phosphorylation of tyrosine hydroxylase are additive. In contrast, the effects of 56 mM K^+ plus A23187 on either the activation or the phosphorylation of the enzyme are not additive. Following stimulation of intact PC12 cells with $^{32}P_i$, in order to label ATP stores, and tryptic digestion of the phosphorylated enzyme, separation of the tryptic phosphopeptides by high pressure liquid chromatography yields four distinct ^{32}P-peptide peaks. Incubation of the cells in the presence of either 56 mM K^+ or A23187 is associated with increased $^{32}P_i$ incorporation into three peptides whereas, in the presence of dibutyryl cAMP, increased $^{32}P_i$ incorporation is observed in only one of these peptides. When tyrosine hydroxylase purified from rat pheochromocytoma tumor is incubated *in vitro* with [γ-^{32}P]ATP and either cAMP-dependent or calcium/calmodulin-dependent protein kinase under appropriate conditions, increased phosphorylation of tyrosine hydroxylase is observed. However, even though *in vitro* phosphorylation by cAMP-dependent protein kinase is associated with activation of tyrosine hydroxylase, *in vitro* phosphorylation by calcium/calmodulin-dependent protein kinase does not lead to activation of the enzyme. Tryptic digestion of tyrosine hydroxylase phosphorylated by calcium/calmodulin-dependent protein kinase yields three distinct ^{32}P-peptide peaks, which are identical to those phosphorylated by treatment of intact PC12 cells with either high K^+ or A23187. In contrast, cAMP-dependent protein kinase phosphorylates only one peptide, which is identical to that phosphorylated by treatment of the intact cells with dibutyryl cAMP. These results indicate that tyrosine hydroxylase is activated and phosphorylated at multiple sites in PC12 cells exposed to 56 mM K^+ or A23187. The results suggests that the *in situ* phosphorylation of these sites is catalyzed by calcium/calmodulin-dependent protein kinase; however, phosphorylation by this protein kinase is not sufficient to activate the enzyme. cAMP-dependent protein kinase also can phosphorylate tyrosine hydroxylase *in situ*, and phosphorylation by this protein kinase is sufficient to activate the enzyme.

Depolarization of catecholaminergic neurons or cells leads to an increased release of catecholamines and a concomitant increase in the activity of tyrosine hydroxylase [EC 1.14.16.2, tyrosine 3-monooxygenase: L-tyrosine, tetrahydropteridine oxidoreductase (3-hydroxylating)], the enzyme that catalyzes the rate-limiting step in the biosynthesis of the catecholamines (1). This activation of tyrosine hydroxylase has been observed in a number of systems, including rat and bovine adrenal medulla (2, 3), rat sympathetic ganglia (4, 5), rat striatal synaptosomes and slices (6–8), guinea pig vas deferens (9), rat pheochromocytoma tumor (10), and PC12 cells (11, 12). In most cases, this

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ABBREVIATIONS: HPLC, high pressure liquid chromatography; KRH buffer, Krebs-Ringer-Henseleit buffer; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 6-MePtH$_4$, D,L-2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

increase in activity is due to an increased affinity of the enzyme for pterin cofactor. In some of these systems, activation of the enzyme has been shown to be associated with phosphorylation of the enzyme (3, 5, 11).

Incubation of intact cells with cAMP analogs also is associated with an increase in the activity of tyrosine hydroxylase, which is characterized as a decrease in the K_m for the pterin cofactor (2–4, 6–9). The addition of cAMP, Mg^{2+}, and ATP to homogenates of catecholaminergic tissues results in the activation of the enzyme (2, 13, 14). Furthermore, either crude or purified preparations of tyrosine hydroxylase can be activated and phosphorylated by purified cAMP-dependent protein kinase (14–16). The phosphorylation of the enzyme correlates well with the activation. This evidence supports the possible role of phosphorylation by cAMP-dependent protein kinase as a mechanism involved in the depolarization-mediated activation of tyrosine hydroxylase. However, recent studies have implicated other protein kinases in this activation. Mestikawy *et al.* (17) and Iuvone (18) have reported the activation of tyrosine hydroxylase by calcium-dependent protein kinases in rat striatal homogenates. Furthermore, tyrosine hydroxylase has been reported to be phosphorylated at multiple sites in intact bovine adrenal chromaffin cells (19) and intact rat pheochromocytoma PC12 cells (20–22). In contrast, treatment of these intact cells with cAMP analogs results in the phosphorylation of only one site on the enzyme (19, 20, 22, 23). In addition, phosphorylation of purified tyrosine hydroxylase is catalyzed by purified calcium/phospholipid-dependent protein kinase (protein kinase C; Ref. 2, 4) and purified calcium/calmodulin-dependent protein kinase (25, 26). It has been reported that protein kinase C phosphorylates the same tryptic peptide derived from tyrosine hydroxylase as cAMP-dependent protein kinase, whereas calcium/calmodulin-dependent protein kinase phosphorylates two phosphopeptides (24–26).

In previous studies we have shown that incubation of rat pheochromocytoma PC12 cells with 56 mM K^+ is associated with an increase in the activity and the phosphorylation of tyrosine hydroxylase (11, 20). We have also demonstrated that tyrosine hydroxylase in PC12 cells treated with 56 mM K^+ is phosphorylated on serine residues at two or more distinct sites (20). Moreover, using two-dimensional thin layer electrophoresis-chromatography to further define the sites on tyrosine hydroxylase phosphorylated in PC12 cells, we have shown that the phosphorylation of three tryptic phosphopeptides derived from the enzyme increases in cells treated with 56 mM K^+, whereas the phosphorylation of only one of these tryptic phosphopeptides increases in cells treated with dibutyryl cAMP (23). In the present study we have characterized further the activation and phosphorylation of tyrosine hydroxylase in PC12 cells treated with high K^+, dibutyryl cAMP, or the calcium ionophore, A23187, and have more fully defined the sites on the enzyme phosphorylated by these treatments by separation of the tryptic phosphopeptides derived from the enzyme using HPLC. Finally, we have compared the tryptic peptides phosphorylated in the intact cells to those phosphorylated *in vitro* using purified rat pheochromocytoma tyrosine hydroxylase and purified preparations of cAMP-dependent and calcium/calmodulin-dependent protein kinases.

Experimental Procedures

Materials. Tissue culture flasks and dishes were obtained from Falcon Plastics Co. (Los Angeles, CA). RPMI 1640 medium was obtained from GIBCO Laboratories (Grand Island, NY). Horse serum was from KC Biologicals, Inc. (Lenexa, KS). Fetal bovine serum was from Sterile Systems, Inc. (Logan, UT). L-[1-^{14}C]Tyrosine and ^{32}P-inorganic phosphate (carrier-free) were purchased from New England Nuclear Corp. (Boston, MA). Poly-L-lysine hydrobromide and A23187 (calcium ionophore) were from Sigma Chemical Co. (St. Louis, MO). Trypsin-TPCK was obtained from Worthington Diagnostic System, Inc. [γ-^{32}P]ATP was synthesized from ^{32}P-phosphate by the method of Johnson and Walseth (27). All other chemicals were of the highest purity available from commercial sources.

Cell culture. Rat pheochromocytoma PC12 cells (28) were kindly provided by Dr. Rosanne Goodman (University of Pennsylvania, Philadelphia, PA). Maintenance and subculture of the PC12 cells have been described in detail elsewhere (11). Briefly, the PC12 cells were subcultured on 35-mm polylysine-treated dishes at a density of 10^6 cells/dish. After 3–4 days in culture, when there were approximately 2×10^6 cells/dish, the cells were washed twice with oxygenated 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 subsequently incubated for 15 min at 37°. The cells were washed once more with warm KRH buffer and then incubated for 60 min at 37° in KRH buffer. The cells were again washed with warm KRH buffer and incubated with or without test compounds. When 56 mM KCl was used, the NaCl content was decreased to maintain the tonicity of the KRH buffer.

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. The supernatant was collected and subjected to gel filtration on a Sephadex G-25 column 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 (29). The standard incubation mixture (final volume, 100 μl) consisted of 100 mM potassium phosphate buffer (pH 6.8), 5 mM ascorbic acid, 6500 units of catalase, 5 mM EDTA, 0.1–1.0 mM 6-MePtH$_4$ (Calbiochem-Behring), and 0.1 mM L-[1-^{14}C]tyrosine. The reaction was allowed to proceed at 30° for 10 min. The reaction was terminated by the addition of 5 mM 3-iodotyrosine in 50 mM potassium phosphate buffer (pH 6.8). The [1-^{14}C]dihydroxyphenylalanine produced in the above reaction was decarboxylated by the addition of partially purified hog kidney L-aromatic amino acid decarboxylase and pyridoxal phosphate. The reaction mixture was incubated at 37° for 30 min, and the reaction was then terminated by the addition of 0.1 ml of 0.8 M perchloric acid. The liberated $^{14}CO_2$ was collected in wells containing 0.2 ml of NCS tissue solubilizer (Amersham), and the wells were then transferred to counting vials. Radioactivity was determined by liquid scintillation spectrometry. Protein was measured by the method of Bradford (30), using bovine serum albumin as standard. The specific activity of tyrosine hydroxylase was expressed as nmol of $^{14}CO_2$ formed/min × mg of protein.

Measurement of phosphorylation of tyrosine hydroxylase. After incubation for 15 min in KRH buffer, the cells were incubated with ^{32}P-phosphate (carrier-free 0.5 mCi/ml) for 60 min at 37°. The cells were then washed with warm KRH buffer and incubated with or without test compounds. After incubation, the cells were immediately frozen on dry ice, 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 (29) in the presence of 5 mM NaF, 10 mM EDTA, 50 mM sodium pyrophosphate, and 50 mM potassium phosphate (pH 7.6) in a final volume of 300 μl. This mixture was allowed to stand at room temperature for 30 min and then incubated at 4° overnight. In preliminary experiments this volume of antiserum was found to precipitate the maximal amount of ^{32}P-phosphate-labeled tyrosine hydroxylase. The mixture was layered over a discontinuous

sucrose gradient by a modification of the method of Taylor and Schimke (31). Both layers of the gradient contained 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate and 20 mM Tris-HCl buffer (pH 7.8). The lower layer (0.5 ml) contained 1.0 M sucrose and the upper layer (0.25 ml) contained 0.5 M sucrose. The tubes were centrifuged at 12,000 × g for 15 min. The resulting pellet was suspended in the following solution: 0.15 M KCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 20 mM Tris-HCl buffer (pH 7.8), and centrifuged at 12,000 × *g* for 15 min. This washing procedure was performed twice. The final pellet was then dissolved in 15 μl of 500 mM dithiothreitol and 45 μl of an SDS solution containing 3% SDS, 5% glycerol, 0.02% bromophenol blue, and 10 mM Tris-HCl (pH 7.8). The solution was placed in a boiling water bath for 10 min and centrifuged at 12,000 × *g* for 5 min. The supernatants were applied to SDS-polyacrylamide slab gels as previously described (11). After electrophoresis at 180 V for 5 hr, the ^{32}P-phosphate incorporated into tyrosine hydroxylase was assessed both from the density of the autoradiographic band on the gel corresponding to purified [^{32}P]tyrosine hydroxylase standard and by cutting out the [^{32}P]tyrosine hydroxylase band on the gel and counting the radioactivity in the band by Cerenkov analysis.

Phosphopeptide maps of tyrosine hydroxylase. After cutting out the gel band (M_r = 57,000) corresponding to [^{32}P]tyrosine hydroxylase, the radiolabeled enzyme was extracted with 0.05 M NH_4HCO_3 and 0.025% SDS at 37° for 36 hr by changing the extraction medium three times (32). The extract of [^{32}P]tyrosine hydroxylase from the gels was filtered through glass wool, and then 0.25 mg of calf thymus histone (Sigma, type IIA) and trichloroacetic acid (25%, v/v, final concentration) were added. This solution was kept at 4° for 15 min and centrifuged at 4000 × *g* for 10 min at 4°. The pellet was suspended in 2 ml of acidified acetone (50 μl of concentrated HCl in 10 ml of acetone) and centrifuged at 4000 × *g* for 10 min at room temperature. After removing the supernatant, the pellet was suspended in 2 ml of acetone and centrifuged at 4000 × *g* for 10 min at room temperature. The final pellet was dissolved in 50 μl of 10 mM dithiothreitol and incubated at 65° for 15 min. The reduced sample was incubated with 10 μl of 1 M ammonium bicarbonate and 5 μl of 5 mg/ml trypsin for 12 hr at 37°, and an additional 5 μl of 5 mg/ml trypsin was added to the solution twice at intervals of 6 hr. After digestion of the phosphorylated tyrosine hydroxylase by trypsin, the ^{32}P-peptides were separated by HPLC. For HPLC analysis the peptides were applied to a Beckman HPLC system equipped with a Hibar II column (10 μm, 250 mm × 4.6 mm) packed with LiChrosorb RP-18. The peptides were eluted using an *n*-propanol gradient (0–20%) in 0.05% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min; 1-ml fractions were collected into tubes and radioactivity was measured by Cerenkov counting.

To verify that the proteolytic digestion was complete and the phosphopeptides eluted from the HPLC column were not partial degradation products, [^{32}P]tyrosine hydroxylase eluted from the gel was subjected to trypsin treatment as described above for 12, 24, 36, and 48 hr. Identical phosphopeptide patterns were obtained under all these conditions. We routinely used a 24-hr trypsin treatment in our subsequent studies.

***In vitro* phosphorylation of purified tyrosine hydroxylase.** Tyrosine hydroxylase was purified from the transplantable rat pheochromocytoma tumor through the hydroxylapatite step as described by Vulliet *et al.* (15). The final specific activity of the purified tyrosine hydroxylase preparation was between 100 and 200 nmol of $^{14}CO_2$ produced/min × mg of protein. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart by the method of Bechtel *et al.* (33). The final specific activity of this preparation was 3.8 μmol of ^{32}P-phosphate transferred to whole histone/min × mg of protein. Calcium/calmodulin-dependent protein kinase was partially purified from rat cerebral cortex through a calmodulin-Sepharose affinity column by the method of Yamauchi and Fujisawa (34, 35). This purification step resulted in a preparation which consisted of approximately six or seven polypeptides visible by silver staining after SDS-polyacrylamide gel electrophoresis. Two of these polypeptides (M_r = 50,000 and 60,000) were presumably the subunits of calcium/calmodulin-dependent protein kinase (36, 37). No attempt was made to resolve the rat brain isozymes of calcium/calmodulin-dependent protein kinase.

Tyrosine hydroxylase was subjected to phosphorylating conditions with either protein kinase preparation for 10 min at 30°. When cAMP-dependent protein kinase was employed, the reaction mixtures contained 50 mM potassium phosphate buffer (pH 6.8), 5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (specific activity 1–3 Ci/mmol; in experiments on enzyme activation unlabeled ATP was employed), 2–5 μg of purified tyrosine hydroxylase, and 4 μg of purified catalytic subunit of cAMP-dependent protein kinase. When calcium/calmodulin-dependent protein kinase was employed, the reaction mixture contained 50 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$, 0.1 mM [^{32}P]ATP (specific activity 1–3 Ci/mmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 12.5 μg/ml of calmodulin, 10 mM NaF, 2–5 μg of purified tyrosine hydroxylase, and 0.2 unit of partially purified calcium/calmodulin-dependent protein kinase (1 unit is defined as 1 nmol of ^{32}P-phosphate transferred to casein/min). The final reaction volumes were 50 μl. The reactions were stopped by the addition of 5 μl of 0.1 M EDTA, and either tyrosine hydroxylase activity was assayed or the solution was prepared for SDS-polyacrylamide gel electrophoresis as described above.

Results

Activation and enhanced phosphorylation of tyrosine hydroxylase in PC12 cells by A23187 and dibutyryl cAMP. In previous studies (11) we showed that tyrosine hydroxylase activity increased in PC12 cells treated with 56 mM K^+. When the cells were preincubated for 30–60 min with ^{32}P-phosphate to label intracellular ATP pools, and then treated with 56 mM K^+, the increase in enzyme activity was shown to be associated with an increase in the ^{32}P-phosphorylation of the enzyme (11). Both the activation and the increased phosphorylation of tyrosine hydroxylase were dependent on the presence of extracellular Ca^{2+} (11). In the present studies we tested the effects of dibutyryl cAMP and the calcium ionophore, A23187, on the activation and phosphorylation of tyrosine hydroxylase in PC12 cells.

Fig. 1B shows an autoradiogram of an SDS-polyacrylamide gel depicting ^{32}P-phosphate incorporated into tyrosine hydroxylase protein in PC12 cells. As shown previously (11), incubation of PC12 cells with ^{32}P-phosphate for 1 hr resulted in a modest degree of incorporation of ^{32}P-phosphate into a prominent band at M_r ~ 57,000, which was isolated from PC12 cell supernatants by immunoprecipitation with antiserum to the enzyme. This band migrated to a position coincident with that of tyrosine hydroxylase purified from rat pheochromocytoma tumor (Fig. 1, A and B) (11).

Treatment of the cells with 10^{-5} M A23187 for 10 min at 37° resulted in a 5-fold increase in the incorporation of ^{32}P-phosphate into this band (Fig. 1, B and C). Treatment of the cells with 10^{-5} M A23187 also increased tyrosine hydroxylase activity about 3-fold, when the enzyme was assayed in the presence of either 0.1 mM or 1 mM 6-MePtH$_4$ (Fig. 2). Deletion of Ca^{2+} from the medium did not affect either the basal activity or the phosphorylation of the enzyme in the absence of A23187. However, the stimulatory effects of A23187 on activation and phosphorylation of tyrosine hydroxylase were abolished when Ca^{2+} was omitted from the incubation medium (Figs. 1 and 2).

Incubation of PC12 cells with 2 mM dibutyryl cAMP (Figs. 1 and 2) was also associated with activation and enhanced phosphorylation of tyrosine hydroxylase. The stimulatory effects of dibutyryl cAMP on both activation and phosphoryla-

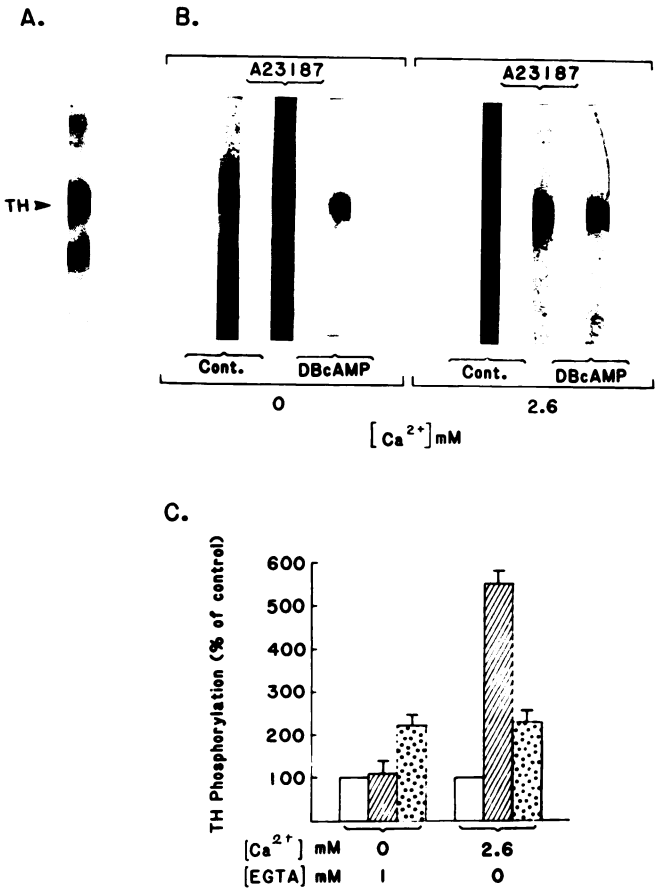

Fig. 1. Effect of external Ca^{++} on the phosphorylation of tyrosine hydroxylase (*TH*) by A23187 and dibutyryl cAMP. The PC12 cells were incubated for 15 min at 37° and washed with prewarmed KRH buffer. The cells were incubated for 1 hr at 37° with ^{32}P-phosphate (0.5 mCi/ml). After washing the cells with prewarmed KRH buffer, the cells were incubated for 10 min without (□), or with 10^{-5} M A23187 (▨) or 2 mM dibutyryl cAMP (▩) in Ca^{++}-free (+1 mM EGTA), or 2.6 mM Ca^{++}-containing medium. Supernatants from these cells were treated with antiserum to tyrosine hydroxylase and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis as described under Experimental Procedures. ^{32}P incorporated into tyrosine hydroxylase was determined by liquid scintillation spectrometry after identifying the gel band corresponding to tyrosine hydroxylase by autoradiography (C). Autoradiograms of SDS-polyacrylamide gels depict phosphorylated, partially purified pheochromocytoma tyrosine hydroxylase (A) and phosphorylated tyrosine hydroxylase immunoprecipitated from PC12 cells (B). The increase in the ^{32}P phosphorylation of tyrosine hydroxylase is expressed as the percentage of control (means ± standard deviation) (n = 4 experiments).

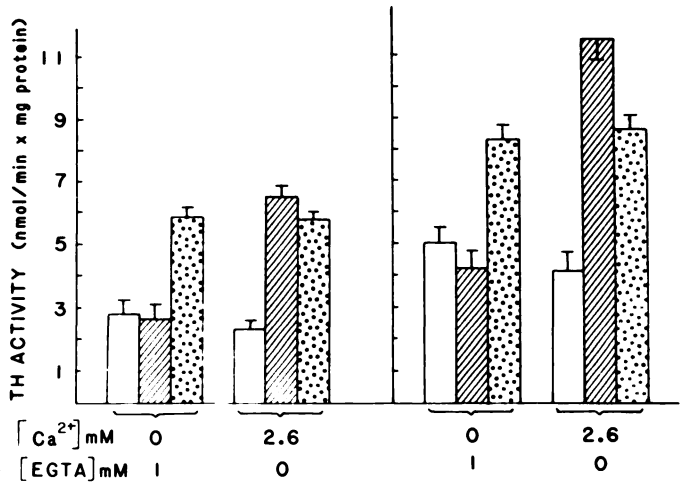

Fig. 2. Effect of external Ca^{++} on tyrosine hydroxylase (*TH*) activation by A23187 and dibutyryl cAMP. PC12 cells were incubated for 15 min at 37° and washed with prewarmed KRH buffer. The cells were incubated at 37° for 10 min without (▨), or with 10^{-5} M A23187 (▨) or 2 mM dibutyryl cAMP (□) in Ca^{++}-free (+1 mM EGTA) or 2.6 mM Ca^{++}-containing medium. Tyrosine hydroxylase activity was determined as described under Experimental Procedures. *Left.* Tyrosine hydroxylase activity using 0.1 mM 6-MePtH$_4$ as cofactor. *Right.* Tyrosine hydroxylase activity using 1 mM 6-MePtH$_4$. Results are the mean ± standard deviation (n = 4–6 experiments).

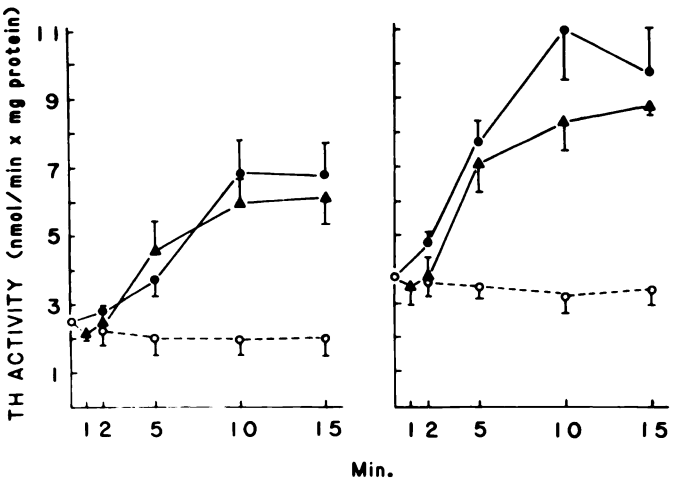

Fig. 3. Time courses of tyrosine hydroxylase (*TH*) activation by A23187 and dibutyryl cAMP. The PC12 cells were incubated at 37° for 15 min and washed with prewarmed KRH buffer. The cells were then incubated at 37° for different periods of time in the absence (○) or presence of 10^{-5} M A23187 (●) or 2 mM dibutyryl cAMP (▲). Tyrosine hydroxylase activity was determined as described under Experimental Procedures. *Left.* Tyrosine hydroxylase activity using 0.1 mM 6-MePtH$_4$. *Right.* Tyrosine hydroxylase activity using 1 mM 6-MePtH$_4$. Results are given as the mean ± standard deviation (n = 4 experiments).

tion of the enzyme persisted in the absence of external Ca^{2+} (Figs. 1, B and C, and 2).

Time courses of activation and phosphorylation of tyrosine hydroxylase by A23187 or dibutyryl cAMP. When PC12 cells were incubated with 10^{-5} M A23187 for 5 min, a significant increase in tyrosine hydroxylase activity was observed. The extent of this increase was identical when the enzyme was assayed using either 0.1 mM or 1 mM 6-MePtH$_4$. Maximal activation of the enzyme was reached after 10 min (Fig. 3). Basal activity of tyrosine hydroxylase did not change significantly during incubation of the cells for at least 15 min. In agreement with the activation time course, a maximal degree of phosphorylation of tyrosine hydroxylase in the cells treated with A23187 was observed after 10 min of incubation. However, a 3–4-fold increase in phosphorylation was achieved after only 2 min of incubation (Fig. 4). In contrast, only a small increase in activation was observed at this time point. Thus, the time courses of activation and phosphorylation of the enzyme by A23187 did not correlate at early time points.

When PC12 cells were incubated with 2 mM dibutyryl cAMP, the time courses of phosphorylation and activation of the enzyme were found to be similar, with the maximal degrees of phosphorylation and activation achieved after 10 min of incubation (Figs. 3 and 4). The degree of enzyme activation by

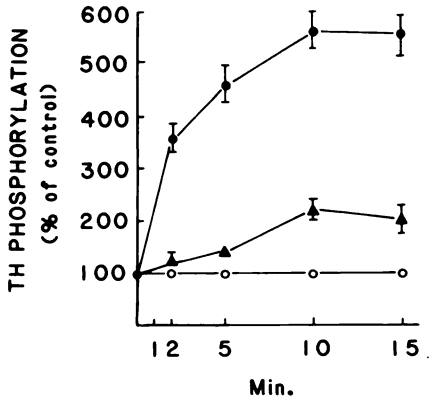

Fig. 4. Time courses of tyrosine hydroxylase (*TH*) phosphorylation by A23187 and dibutyryl cAMP. The PC12 cells were incubated for 1 hr at 37° with ^{32}P-phosphate (0.5 mCi/ml). After washing the cells with prewarmed KRH buffer, the cells were incubated for different periods of time without (○) or with 10^{-5} M A23187 (●) or 2 mM dibutyryl cAMP (▲). The supernatants of incubated cells were treated with antiserum to tyrosine hydroxylase and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis. ^{32}P incorporated into tyrosine hydroxylase was determined as described in Experimental Procedures. The increase in the ^{32}P phosphorylation of tyrosine hydroxylase is expressed as the percentage of control (mean ± standard deviation) (n = 4 or 5 experiments).

dibutyryl cAMP was identical when the enzyme activity was assayed at either 0.1 mM or 1 mM 6-MePtH$_4$.

Comparison of phosphopeptide maps of tyrosine hydroxylase phosphorylated in PC12 cells treated with 56 mM K$^+$, A23187, or dibutyryl cAMP. The sites on tyrosine hydroxylase phosphorylated after treatment of the cells with 56 mM K$^+$, A23187, or dibutyryl cAMP was investigated. After incubation of the cells with ^{32}P$_i$, and exposure of the cells to these agents, tyrosine hydroxylase was isolated, subjected to SDS-polyacrylamide gel electrophoresis, eluted from the gels, and digested with trypsin for 24 hr at 37°. The ^{32}P-phosphopeptides derived from tyrosine hydroxylase were then separated by HPLC. As shown in Fig. 5A, four distinct ^{32}P-peptide peaks from tyrosine hydroxylase were eluted from the column using a 0–20% n-propanol linear gradient (control). For convenience, these ^{32}P-peptide peaks were referred to as peptides 1 (retention time: 18 min), 2 (22 min), 3 (24 min), and 4 (32 min). Other ^{32}P-peptide peaks were not detected between 20 and 100% n-propanol (data not shown). Peak 3 was observed in some, but not all, experiments. When PC12 cells were incubated with 56 mM K$^+$ for 10 min, the phosphorylation of three (peptides 1, 2, and 4) of these peptides markedly increased (Fig. 5B). Treatment of the cells with A23187 for 10 min also enhanced the phosphorylation of these three peptides (peptides 1, 2, and 4) (Fig. 5C). In contrast, the phosphorylation of only one (peptide 4) of these peptides increased after exposure of cells to 2 mM dibutyryl cAMP for 10 min (Fig. 5D).

Phosphorylation of purified tyrosine hydroxylase by cAMP-dependent and calcium/calmodulin-dependent protein kinases. When tyrosine hydroxylase purified from rat pheochromocytoma tumor (15) was incubated with either the catalytic subunit of cAMP-dependent protein kinase (33) or with rat brain calcium/calmodulin-dependent protein kinase (34, 35) in the presence of magnesium and [γ-^{32}P]ATP, increased phosphorylation of the M_r = 57,000 subunit of tyrosine hydroxylase was observed on SDS-polyacrylamide gels (Fig. 6). When calcium/calmodulin-dependent protein kinase was incubated in the presence of calmodulin, but in the absence of calcium, only a minimal amount of protein phosphorylation was observed (Fig. 6, *lanes 1* and *3*), whether or not tyrosine hydroxylase was present in the incubation. When calcium/calmodulin-dependent protein kinase was incubated by itself in the presence of both calcium and calmodulin, ^{32}P-phosphate was incorporated into several protein bands (Fig. 6, *lane 2*). Since calcium/calmodulin-dependent protein kinase is known to be susceptible to autophosphorylation (36, 37), several of these phosphoprotein bands could represent either the subunits of this kinase (particularly the M_r = 50,000 and 60,000 bands) or other protein contaminants. When calcium/calmodulin-dependent protein kinase was incubated with tyrosine hydroxylase in the presence of both calcium and calmodulin, a large increase in the phosphorylation of an M_r = 57,000 protein band, presumably representing the subunit of tyrosine hydroxylase, was observed (Fig. 6, *lane 4*). When the catalytic subunit of cAMP-dependent protein kinase was incubated by itself in the presence of magnesium and [γ-^{32}P]ATP, only minimal protein phosphorylation was observed (Fig. 6, *lane 5*). However, when this kinase was incubated with tyrosine hydroxylase under phosphorylating conditions, an increase in the phosphorylation of the M_r = 57,000 subunit of the enzyme was observed (Fig. 6, *lane 6*). In these *in vitro* experiments, the stoichiometric relationships describing the level of phosphorylation of the enzyme by cAMP-dependent and calcium/calmodulin-dependent protein kinases ranged from 0.5 to 1.1 and 0.4 to 0.9 mol of ^{32}P-phosphate incorporated per mol subunit of tyrosine hydroxylase, respectively.

The increase in the phosphorylation of the enzyme was associated with an increase in the activity of the enzyme when cAMP-dependent protein kinase was employed (Table 1). However, there was no change in the activity of tyrosine hydroxylase incubated with calcium/calmodulin-dependent protein kinase (Table 1). Treatment of the enzyme simultaneously with both cAMP-dependent and calcium/calmodulin-dependent protein kinases resulted in an activation of the enzyme which was equal to that produced by treatment with cAMP-dependent protein kinase alone.

HPLC separation of tryptic ^{32}P-phosphopeptides of tyrosine hydroxylase incubated with cAMP-dependent and calcium/calmodulin-dependent protein kinases. When tyrosine hydroxylase phosphorylated by cAMP-dependent protein kinase was digested with trypsin, there was an increase in the phosphorylation of one phosphopeptide (retention time: 32 min) (Fig. 7A). This phosphopeptide comigrated with peptide 4 derived from tyrosine hydroxylase phosphorylated in the intact cell experiments. When the enzyme phosphorylated by calcium/calmodulin-dependent protein kinase was digested with trypsin, there was an increase in the phosphorylation of three phosphopeptides (retention times: 18, 22, and 32 min), which were chromatographically identical with peptides 1, 2, and 4, derived from the digestion of tyrosine hydroxylase from intact PC12 cells (Fig. 7B). Experiments were performed using numerous preparations of partially purified rat brain calcium/calmodulin-dependent protein kinase. In most of these experiments, results identical to those described in Fig. 7B were obtained. However, with some preparations of this kinase, only phosphopeptides 1 and 4 (retention times: 18 and 32 min) were identified.

Incubation of tyrosine hydroxylase with either cAMP-de-

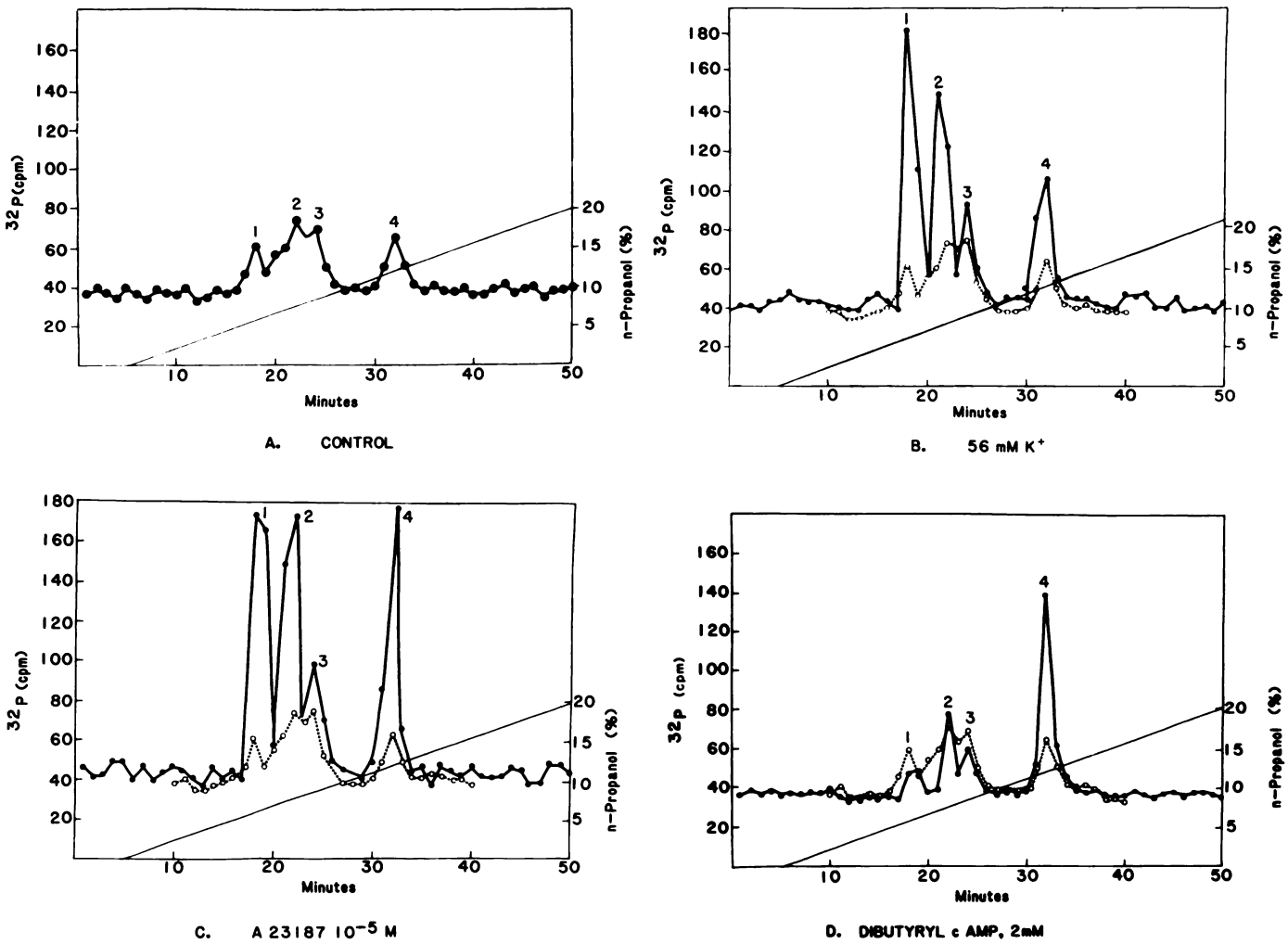

Fig. 5. Separation of tryptic phosphopeptides from tyrosine hydroxylase by HPLC. After preincubation for 60 min at 37° with ^{32}P-phosphate (0.5 mCi/ml), the cells were washed with KRH buffer. The cells were then incubated for 10 min at 37° in the absence (A) or presence of 56 mM K^+ (B), 10^{-5} M A23187 (C), or 2 mM dibutyryl cAMP (D). After immunoprecipitation with tyrosine hydroxylase antiserum and separation by SDS-polyacrylamide gel electrophoresis, phosphorylated tyrosine hydroxylase was eluted from the gel by incubation in 0.05 M ammonium bicarbonate (pH 8.5) and 0.025% SDS for 36 hr at 37°. The eluted [^{32}P]tyrosine hydroxylase was concentrated by precipitation with 25% trichloroacetic acid. The final pellet was dissolved by heating at 65° in 10 mM dithiothreitol and was subjected to tryptic digestion for 24 hr at 37° at pH 8.0. After digestion, the ^{32}P-tryptic peptides of tyrosine hydroxylase were separated by HPLC using a reverse phase RP-18 column. The phosphopeptides were eluted with an *n*-propanol gradient from 0 to 20% as described in Experimental Procedures. Peaks derived from the untreated cells (O) are also shown in B–D.

pendent or calcium/calmodulin-dependent protein kinase results in an increased phosphorylation of phosphopeptide peak 4 (retention time: 32 min). In order to determine whether these two protein kinases catalyzed the phosphorylation of two different tryptic peptides present in peak 4, we digested tyrosine hydroxylase phosphorylated by either cAMP-dependent or calcium/calmodulin-dependent protein kinase with trypsin, and isolated the fractions containing peptide peak 4 after separation of the tryptic phosphopeptides by HPLC. These fractions were then pooled and subjected to either thin layer cellulose (Polygram cel 300 cellulose sheets, Nacherey-Nagel Co.) ascending chromatography using *sec*-butanol:*n*-propanol:isoamylalcohol:pyridine:water (1:1:1:3:3) as a solvent system, or thin layer cellulose electrophoresis at 2000 V for 1 hr using a Pharmacia FBE 3000 flatbed apparatus and formic acid:acetic acid:H_2O (50:150:800) (pH 1.5) as the electrophoresis buffer. Using both these techniques we identified a single, identical ^{32}P-peptide present in peptide peak 4 derived from tyrosine hydroxylase phosphorylated by either cAMP-dependent or calcium/calmodulin-dependent protein kinase (data not shown).

Comparison of the activation and phosphorylation of tyrosine hydroxylase in PC12 cells treated with different combinations of A23187, 56 mM K^+, and dibutyryl cAMP. The stimulatory effect of dibutyryl cAMP on tyrosine hydroxylase activity in PC12 cells was maximal at 1 mM. When PC12 cells were incubated for 10 min with 56 mM K^+ plus either 1 or 2 mM dibutyryl cAMP, an approximately additive increase in tyrosine hydroxylase activity was observed (Table 2). Identical results were obtained when the cells were incubated with 10^{-5} M A23187 plus either 1 or 2 mM dibutyryl cAMP (Table 2). In contrast, the effect of 56 mM K^+ plus 10^{-5} M A23187 on enzyme activation was not additive but was equal to that observed with either treatment alone.

A concentration of 1 mM dibutyryl cAMP also elicited maximal phosphorylation of tyrosine hydroxylase. Incubation of PC12 cells with 56 mM K^+ plus 2 mM dibutyryl cAMP or with 10^{-5} M A23187 plus 2 mM dibutyryl cAMP for 10 min led to additive increases in phosphorylation of tyrosine hydroxylase (Table 3). However, the extent of phosphorylation of the en-

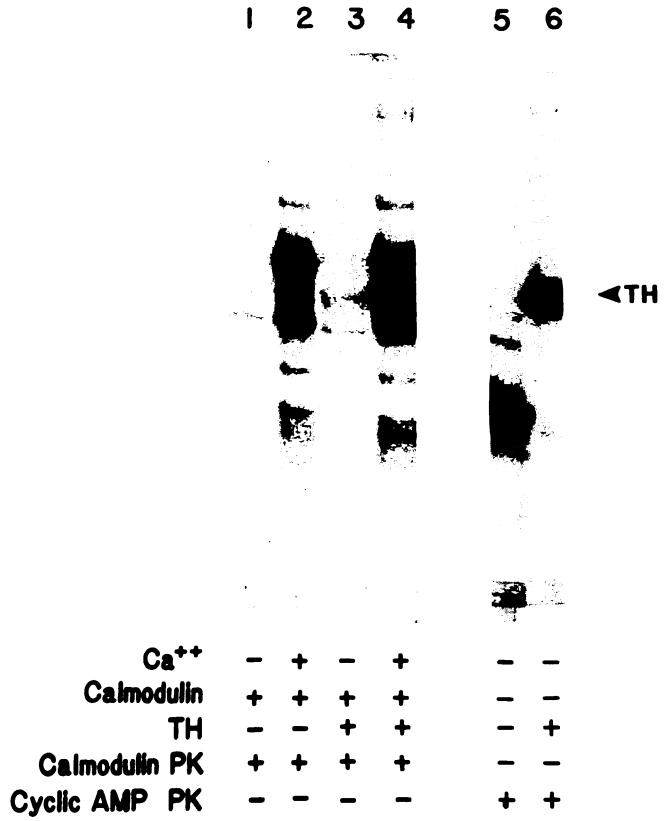

Fig. 6. Autoradiogram of an SDS-polyacrylamide gel depicting phosphorylation of purified tyrosine hydroxylase with either cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase. Tyrosine hydroxylase (*TH*) (40 μg/ml) purified from rat pheochromocytoma tumor was incubated for 10 min at 30° under phosphorylating conditions with either calcium/calmodulin-dependent protein kinase (4 units/ml), or the catalytic subunit of cAMP-dependent protein kinase (80 μg/ml). The reaction was stopped by the addition of SDS and dithiothreitol as described in Experimental Procedures, and the solutions were placed on 10% SDS-polyacrylamide gels. After electrophoresis, the gels were stained for proteins and then dried onto Whatman 3MM paper under heat and vacuum. The dried gels were then exposed to X-ray film.

TABLE 1

Effects of cAMP-dependent and calcium/calmodulin-dependent protein kinases on purified tyrosine hydroxylase activity

Tyrosine hydroxylase (40 μg/ml) purified from rat pheochromocytoma was incubated for 10 min at 37° under phosphorylating conditions with either calcium/calmodulin-dependent kinase (Ca/CaM PK) (4 units/ml), or the catalytic subunit of cAMP-dependent protein kinase (cAMP PK) (80 μg/ml). The reaction mixtures contained 50 mM Hepes buffer (pH 7.0), 0.1 mM ATP, 10 mM NaF, and 5 mM $MgCl_2$ (representing the "No additions" condition in line 1 of the table). The incubations noted in line 2 of the table contained 0.1 mM EGTA and 12.5 μg/ml of calmodulin in addition to the components contained in the incubations noted in line 1. $CaCl_2$ (0.2 mM) was added to the incubations noted in line 3 of the table. The reaction was stopped by the addition of 10 mM EDTA, and aliquots were assayed for tyrosine hydroxylase activity, using 0.1 mM 6-MePtH4. Results represent the mean ± standard error (n = 3 experiments).

Additions	No Added PK	Tyrosine hydroxylase activity		Ca/CaM PK + cAMP PK
		Ca/CaM PK	cAMP PK	
	pmol $^{14}CO_2$ formed/min			
No additions	4.8 ± 0.7	4.8 ± 0.5	27.0 ± 5.8	27.0 ± 8.9
EGTA + calmodulin	4.2 ± 0.7	5.7 ± 1.0	30.0 ± 1.2	31.0 ± 6.6
Ca^{2+} + calmodulin	6.7 ± 2.2	5.7 ± 0.7	28.0 ± 5.7	30.0 ± 7.5

zyme isolated from cells treated with 56 mM K^+ plus 10^{-5} M A23187 was the same as that observed for the enzyme isolated from cells treated with either agent alone.

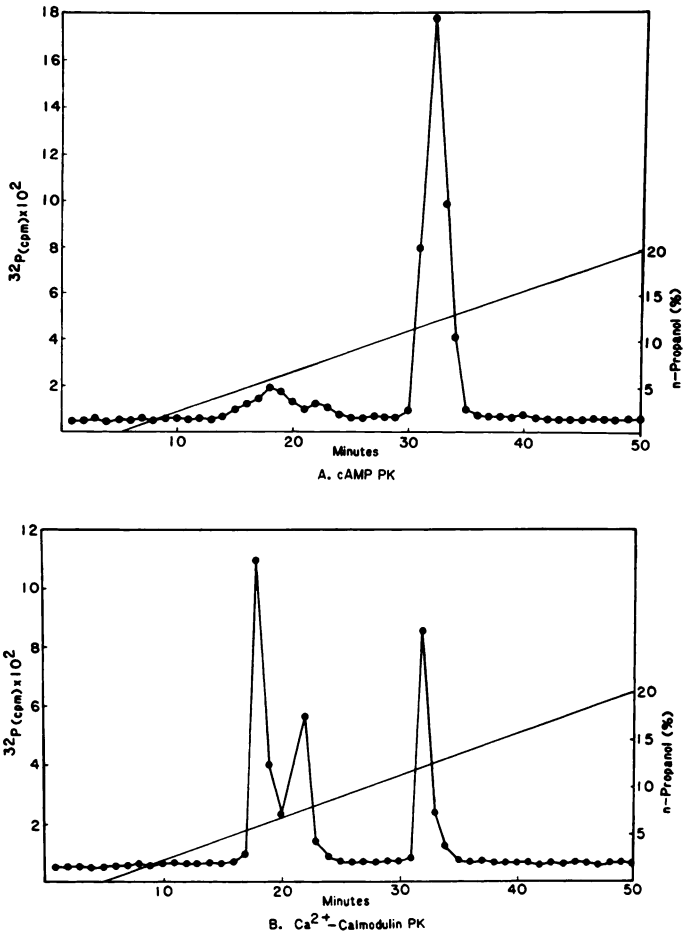

Fig. 7. Separation of ^{32}P-phosphopeptides derived from the tryptic digestion of purified tyrosine hydroxylase phosphorylated by cAMP-dependent and calcium/calmodulin-dependent protein kinases. Tyrosine hydroxylase was phosphorylated as described in the legend to Fig. 6, and the ^{32}P-labeled enzyme was subjected to SDS-polyacrylamide gel electrophoresis. The enzyme was eluted from the gel and proteolytically digested for 24 hr at 37° with 0.5 mg/ml of trypsin. A. ^{32}P-Phosphopeptides from tyrosine hydroxylase phosphorylated by cAMP-dependent protein kinase. B. ^{32}P-Phosphopeptides from tyrosine hydroxylase phosphorylated by calcium/calmodulin-dependent protein kinase.

Discussion

The activity of tyrosine hydroxylase is enhanced in PC12 cells treated with a number of different stimuli, including high K^+, cyclic AMP analogs, phorbol esters, and nerve growth factor (11, 20–22). These increases in activity are associated with increases in the phosphorylation of the enzyme. In the present study we have further characterized the relationship between activation and phosphorylation of tyrosine hydroxylase in PC12 cells treated with high K^+, dibutyryl cAMP, and the calcium ionophore, A23187.

Incubation of PC12 cells with A23187 stimulates the activity of tyrosine hydroxylase and the incorporation of $^{32}P_i$ into the enzyme. The time courses of activation and phosphorylation of tyrosine hydroxylase by A23187 are similar, but not identical, because the phosphorylation of the enzyme slightly precedes the activation of the enzyme. A similar result has been observed for the high K^+-induced activation and phosphorylation of the enzyme (11). The reason for this temporal discrepancy is presently obscure but may indicate that mechanisms not involving phosphorylation of the enzyme are involved in enzyme activa-

TABLE 2

Effects of 56 mM K^+, A23187, and dibutyryl cAMP on activation of tyrosine hydroxylase

The PC12 cells were incubated for 15 min at 37° and washed with prewarmed KRH buffer. The cells were incubated at 37° for 10 min with or without 56 mM K^+, 10^{-5} M A23187, or dibutyryl cAMP. Tyrosine hydroxylase activity was assayed at 0.1 or 1 mM 6-MePtH$_4$ as described under Experimental Procedures. Results are given as the mean ± standard deviation (n = 5 experiments).

Treatments	Tyrosine hydroxylase activity	
	0.1 mM 6-MePtH$_4$	1 mM 6-MePtH$_4$
	nmol/min × mg protein	
Control	2.13 ± 0.23	3.74 ± 0.25
56 mM K^+	7.02 ± 0.09	11.46 ± 0.71
A23187 (10^{-5} M)	7.11 ± 0.13	10.41 ± 0.47
Dibutyryl cAMP		
0.5 mM	3.46 ± 0.03	6.48 ± 0.10
1.0 mM	4.97 ± 0.45	8.07 ± 0.48
2.0 mM	5.50 ± 0.47	8.09 ± 0.53
56 mM K^+ + dibutyryl cAMP		
1.0 mM	10.37 ± 0.18	14.92 ± 0.78
2.0 mM	11.47 ± 1.16	15.01 ± 0.35
A23187 (10^{-5}M) + dibutyryl cAMP		
1.0 mM	10.05 ± 0.14	15.78 ± 1.10
2.0 mM	11.40 ± 0.71	15.54 ± 0.29
+ 56 mM K^+	7.38 ± 0.12	11.02 ± 0.35

TABLE 3

Effects of 56 mM K^+, A23187, and dibutyryl cAMP on phosphorylation of tyrosine hydroxylase

The PC12 cells were incubated for 1 hr at 37° with ^{32}P-phosphate (0.5 mCi/ml). After washing the cells with prewarmed KRH buffer, the cells were incubated for 10 min with or without 56 mM K^+, 10^{-5}M A23187, or dibutyryl cAMP. The supernatants of incubated cells were treated with antiserum to tyrosine hydroxylase and the immunoprecipitates were subject to SDS-polyacrylamide gel electrophoresis. ^{32}P incorporated into tyrosine hydroxylase was determined as described in Experimental Procedures. The increase in the ^{32}P phosphorylation of tyrosine hydroxylase is expressed as the percentage of control (mean ± standard deviation) (n = 4 experiments).

Treatments	Tyrosine hydroxylase phosphorylation
	% of control
Control	100
56 mM K^+	527 ± 29
A23187 (10^{-5} M)	576 ± 32
Dibutyryl cAMP	
1.0 mM	219 ± 12
2.0 mM	231 ± 21
56 mM K^+ + 2.0 mM dibutyryl cAMP	672 ± 33
A23187 (10^{-5}M)	
+ 2.0 mM dibutyryl cAMP	711 ± 27
+ 56 mM K^+	541 ± 28

tion. Since the effects of high K^+ and A23187 are dependent on extracellular Ca^{2+}, the results suggest that an increase in Ca^{2+} concentration in the PC12 cells initiates the activation and enhanced phosphorylation of tyrosine hydroxylase, possibly by Ca^{2+}-dependent protein kinases.

To investigate further the protein kinases involved in the phosphorylation of tyrosine hydroxylase in the intact PC12 cells, we have studied the sites of phosphorylation on the enzyme by tryptic phosphopeptide analysis. Using this technique, Haycock *et al.* (19) have reported that tyrosine hydroxylase is phosphorylated on two distinct peptides *in situ* following stimulation of bovine adrenal chromaffin cells with acetylcholine and only one of these peptides is phosphorylated by 8-bromo-cAMP. Niggli *et al.* (38) have reported that when "leaky" adrenal medullary cells are incubated with Ca^{2+} or cAMP, tyrosine hydroxylase is phosphorylated at several sites. One of these sites is phosphorylated when Ca^{2+} is added to the medium, whereas the other sites are phosphorylated in the presence of Ca^{2+} and cAMP. In previous studies in our laboratory we have shown an increased phosphorylation of at least two distinct phosphopeptides derived from tyrosine hydroxylase isolated from PC12 cells treated with 56 mM K^+ (20). Both peptides are phosphorylated on serine residues, and the rates of phosphorylation of these two peptides are similar up to 3 min of exposure to 56 mM K^+. Lee *et al.* (21) have also reported the phosphorylation of tyrosine hydroxylase by high K^+ depolarization of PC12 cells. These workers have identified phosphorylation sites on three proteolytic peptides derived from the enzyme. McTigue *et al.* (22) have shown that PC12 cell tyrosine hydroxylase is phosphorylated on at least four sites and that the phosphorylation of these sites is regulated by a number of different compounds.

In the present study, using HPLC, we have also identified four distinct ^{32}P-peptide peaks derived from tyrosine hydroxylase, following incubation of the cells with $^{32}P_i$ for 1 hr. Stimulation of the cells with 56 mM K^+ or 10^{-5} M A23187 increases the amount of ^{32}P-phosphate incorporation into three (peptides 1, 2, and 4) of these phosphopeptide peaks, whereas treatment of the cells with dibutyryl cAMP increases the incorporation of ^{32}P-phosphate into only one phosphopeptide peak (peptide 4). Presumably, peptide 4 is identical to the A peptide previously described by Yanagihara *et al.* (20) and Vulliet *et al.* (26), and the E peptide described by Haycock *et al.* (19). Peptide 1 appears to be identical to the C peptide described by Vulliet *et al.* (26). The I peptide described by Yanagihara *et al.* (20) is composed of both peptides 1 and 2.

Since 56 mM K^+ and A23187 increase the level of cAMP in PC12 cells (39, 40), it is possible that the K^+-evoked increase in cAMP contributes to the phosphorylation and activation of tyrosine hydroxylase in the cells via activation of cAMP-dependent protein kinase and subsequent phosphorylation of a site or sites on peptide 4 derived from tyrosine hydroxylase. Analogs of cAMP increase tyrosine hydroxylase activity and phosphorylation in PC12 cells (20, 22), and the time courses of activation and phosphorylation of the enzyme by dibutyryl cAMP are identical (see Figs. 3 and 4). However, the results summarized in Tables 2 and 3 suggest that 56 mM K^+ and A23187 enhance the phosphorylation and activity of tyrosine hydroxylase by a mechanism different from that produced by dibutyryl cAMP. This conclusion is based upon the fact that the simultaneous treatment of the cells with 56 mM K^+ plus dibutyryl cAMP or A23187 plus dibutyryl cAMP results in additive effects on activation and phosphorylation of tyrosine hydroxylase. In contrast, simultaneous treatment with both 56 mM K^+ plus A23187 does not produce additive effects, suggesting that these two agents act by a common mechanism. This common mechanism is presumably due to the influx of extracellular Ca^{2+} into the cells. These conclusions are supported by the analysis of the phosphopeptides derived from tyrosine hydroxylase. Both 56 mM K^+ and A23187 increase the phosphorylation of the same three phosphopeptides (peptide peaks 1, 2, and 4) separated by HPLC. The phosphorylation of only peptide 4 increases after dibutyryl cAMP treatment of the cells. The latter result is in agreement with that obtained when

purified tyrosine hydroxylase is phosphorylated by purified cAMP-dependent protein kinase. The phosphorylation of only peptide 4 is enhanced by cAMP-dependent protein kinase. Thus, the phosphorylation of the sites on peptides 1 and 2 are presumably catalyzed by Ca^{2+}-dependent protein kinase(s).

There are two well characterized classes of Ca^{2+}-dependent protein kinases: calcium/phospholipid-dependent (protein kinase C), and calcium/calmodulin-dependent protein kinases (35–37, 41). Albert *et al.* (24) have presented evidence demonstrating the activation and phosphorylation of tyrosine hydroxylase by calcium/phospholipid-dependent protein kinase *in vitro*. This kinase phosphorylates the enzyme on the same phosphopeptide as that phosphorylated by cAMP-dependent protein kinase. We have confirmed this observation in our laboratory using HPLC to separate the phosphopeptides (data not shown). Thus, the phosphorylation of peptides 1 and 2 derived from tyrosine hydroxylase must be catalyzed by another Ca^{2+}-dependent protein kinase. Yamauchi and Fujisawa (34) have shown that tyrosine hydroxylase can be phosphorylated by calcium/calmodulin-dependent protein kinase purified from rat brain. Vulliet *et al.* (25) have also reported that purified tyrosine hydroxylase is phosphorylated by calcium/calmodulin-dependent protein kinase purified from rabbit skeletal muscle, and have shown that this phosphorylation occurs on a tryptic peptide different from that phosphorylated by cAMP-dependent protein kinase. This tryptic peptide, which they labeled the C peptide, appears to be chromatographically similar to tryptic peptide 1, which we have identified in the present report. In a more recent study, Vulliet *et al.* (26) have demonstrated that calcium/calmodulin-dependent protein kinase, at higher concentrations than those employed in the first study, catalyzes the phosphorylation of a second tryptic peptide derived from tyrosine hydroxylase. This second peptide is identical to the one phosphorylated by cAMP-dependent and calcium/phospholipid-dependent protein kinases, and appears to be chromatographically similar to peptide 4 identified in the present report.

In general agreement with these data, we have shown in the present studies that calcium/calmodulin-dependent protein kinase purified from rat brain phosphorylates rat pheochromocytoma tyrosine hydroxylase on at least three sites, only one of which is phosphorylated by cAMP-dependent protein kinase. These three sites occur on tryptic peptides which are chromatographically identical to those which are phosphorylated by either 56 mM K^+ or A23187 in intact PC12 cells. In these studies we have not attempted to separate the isozymes of calcium/calmodulin-dependent protein kinase which have been shown to exist in rat brain (35–37). It is possible that different isozymes of this protein kinase may catalyze the phosphorylation of different sites on tyrosine hydroxylase. This possibility is supported by the fact that, in some experiments using different preparations of rat brain calcium/calmodulin-dependent protein kinase, tyrosine hydroxylase is phosphorylated only on sites present on peptides 1 and 4. Further work is required to resolve this issue and also to determine whether calcium/calmodulin-dependent protein kinase present in PC12 cells phosphorylates identical sites on tyrosine hydroxylase as that isolated from rat brain.

Even though the evidence suggests that phosphorylation of tyrosine hydroxylase by calcium/calmodulin-dependent protein kinase plays a key role in the activation of tyrosine hydroxylase in PC12 cells treated with high K^+ or A23187, phosphorylation by this protein kinase *in vitro* does not activate the enzyme (Refs. 25 and 34, Table 1 of this paper). Furthermore, we have shown that the phosphorylation of the sites on peptides 1 and 2 does not inhibit the activation of tyrosine hydroxylase by phosphorylation of the sites on peptide 4 by cAMP-dependent protein kinase (see Table 1). It is possible that cAMP-dependent and calcium/calmodulin-dependent protein kinases phosphorylate different peptides or different sites on the same peptide present in peptide peak 4. However, the available evidence argues against this possibility. When peptide peak 4 derived from tyrosine hydroxylase phosphorylated by either protein kinase was subjected to either thin layer electrophoresis or thin layer chromatography, we resolved only one identical phosphopeptide. Vulliet *et al.* (26) reported similar results with respect to the A peptide, which is phosphorylated by both protein kinases and which appears to be chromatographically similar to peptide 4 on HPLC. Furthermore, these workers have presented evidence suggesting that the same serine residue is phosphorylated on the A peptide by cAMP-dependent, calcium/phospholipid-dependent, and calcium/calmodulin-dependent protein kinases. Thus, the evidence suggests that, even though calcium/calmodulin-dependent protein kinase may be involved in the phosphorylation of tyrosine hydroxylase in intact PC12 cells treated with high K^+ or A23187, mechanisms other than phosphorylation, such as activation of phospholipids (42), polyanions (43), or the activator protein described by Yamauchi and Fujisawa (34), may also play a role in the activation of tyrosine hydroxylase in the cells. Alternatively, the phosphorylation by calcium/calmodulin-dependent protein kinase on peptides 1 and/or 2 may affect the stability or some other property of the enzyme activated by cAMP-dependent or calcium/phospholipid-dependent protein kinases.

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