Ca^{2+}-dependent Phosphorylation of Tyrosine Hydroxylase in PC12 Cells

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ABSTRACT Ca^{2+}-dependent protein phosphorylation has been detected in numerous tissues and may mediate some of the effects of hormones and other extracellular stimuli on cell function. In this paper we demonstrate that a Ca^{2+}/calmodulin-dependent protein kinase similar to the enzyme previously purified and characterized from rat brain is present in PC12, a rat pheochromocytoma cell line. We show that Ca^{2+} influx elicited by various forms of cell stimulation leads to increased ^{32}P incorporation into tyrosine hydroxylase (TH), a major phosphoprotein in these cells. Several other unidentified proteins are either phosphorylated or dephosphorylated as a result of Ca^{2+} influx. Acetylcholine stimulates TH phosphorylation by activation of nicotinic receptors. K^+-induced depolarization stimulates TH phosphorylation in a Ca^{2+}-dependent manner, presumably by opening voltage-dependent Ca^{2+} channels. Ca^{2+} influx that results from the direct effects of the ionophore A23187 also leads to TH phosphorylation. Phosphorylation of TH is accompanied by an activation of the enzyme. These Ca^{2+}-dependent effects are independent of cyclic AMP and thus implicate a Ca^{2+}-dependent protein kinase as a mediator of both hormonal and electrical stimulation of PC12 cells.

There is increasing evidence that Ca^{2+} may affect many cellular processes by activation of distinct Ca^{2+}-dependent protein kinases. An elaborate documentation of Ca^{2+}-stimulated phosphorylation in primary hepatocytes suggests that phosphorylase kinase, a Ca^{2+}/calmodulin-dependent kinase with a very limited substrate specificity, and protein kinase C, a Ca^{2+}/diglyceride-dependent kinase with a broad substrate specificity, cannot account for phosphorylation of many of the Ca^{2+}-regulated proteins (1) and suggests the involvement of other Ca^{2+}-dependent enzymes. A similar approach, aimed at studying the action of secretagogues, resulted in the elegant demonstration of Ca^{2+}-stimulated phosphorylation of ribosomal protein S6 in primary cultures of pancreatic acinar cells (2, 3) and an in vitro demonstration of a Ca^{2+}/calmodulin-dependent kinase in cytosolic extracts from these cells (4). Recently, Ca^{2+}-dependent phosphorylation was shown to accompany thyrotropin-releasing hormone stimulation of GH3 cells (5, 6), although the substrates and protein kinases involved have not been identified. Thus, the notion of Ca^{2+}-stimulated phosphorylation in response to hormones is gaining experimental verification.

To elucidate the molecular mechanism of Ca^{2+} action we have begun to investigate the presence of Ca^{2+}-dependent protein kinases. A Ca^{2+}/calmodulin-dependent protein kinase(s) has been identified in membranes from various rat tissues (7, 8). A similar enzyme present on presynaptic vesicles may be involved in neurotransmitter release (9). We identified a soluble Ca^{2+}/calmodulin-dependent protein kinase in bovine brain cytosol (10) and purified the enzyme from rat brain cytosol (11, 12). This enzyme was demonstrated to be the major Ca^{2+}/calmodulin-dependent kinase phosphorylating microtubule-associated protein 2 (MAP-2)[1] and tau in vitro. A similar enzyme has been purified from rat brain using other proteins as substrates (13–16). This enzyme and a similar enzyme or isozyme have a broad substrate specificity and are common in nature (17). Thus, this class of enzyme may be a multifunctional Ca^{2+}/calmodulin-dependent protein kinase. By analogy with cAMP and the cAMP-dependent protein kinase, this enzyme may coordinate many complex cellular processes that are regulated by extracellular stimuli that use Ca^{2+} as a second messenger (18). To test this scenario in situ, we have set up a cell culture system amenable to manipulation.

We describe here the use of PC12, the clonal pheochromocytoma cell line (19), to examine the role of Ca^{2+}-dependent protein kinases in mediating the intracellular effects of

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; MAP-2, microtubule-associated protein 2; NGF, nerve growth factor; TH, tyrosine hydroxylase.

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Ca^{2+}. This cell line has been widely used as a neuronal model system and offers many advantages over other in situ systems (19–21). These cells synthesize and store neurotransmitters and exhibit depolarization-induced secretion of catecholamines and acetylcholine. Moreover, these cells have voltage-dependent Ca^{2+} channels that open after either electrical (high extracellular K^+) or chemical (nicotinic cholinergic agonist) depolarization. As a correlate of their ability to synthesize catecholamines, these cells contain very high levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis (22).

There is increasing evidence for the role of Ca^{2+} in the regulation of catecholamine biosynthesis. For example, it has been demonstrated that in a synaptosomal preparation, dopamine biosynthesis is increased by membrane depolarization in a Ca^{2+}-dependent manner (23). Similarly, in bovine adrenal chromaffin cells, acetylcholine causes a Ca^{2+}-dependent increase in TH activity and TH phosphorylation (22). Regulation of TH by the cAMP-dependent protein kinase in vitro (24–27) and in situ (28, 29) is well established. The recent finding that cAMP and acetylcholine stimulate differential phosphorylation of TH in adrenal chromaffin cells (30) suggests that Ca^{2+}-dependent kinases may function in concert with the cAMP-dependent protein kinase in regulating TH activity in vivo. Thus, TH may be a good model substrate with which to examine cAMP and Ca^{2+}-dependent phosphorylation.

In the present studies, we demonstrate that Ca^{2+} influxes elicited by high K^+, by stimulation of nicotinic receptors, and by the ionophore A23187 lead to an increased phosphorylation of TH. In addition, we demonstrate that a Ca^{2+}/calmodulin-dependent protein kinase similar to the one found in brain is present in PC12 cells.

MATERIALS AND METHODS

Cell Culture Materials: PC12 cells were kindly provided by Dr. Eric Shooter (Stanford University, CA). Fetal calf serum and horse serum were purchased from HyClone Laboratories, Sterile Systems Inc. (Logan, UT). Poly-D-lysine was purchased from Sigma Chemical Co. (St. Louis, MO).

Biochemicals and Radiochemicals: Nerve growth factor (NGF) was purified from mouse submaxillary glands by the method of Smith et al. (31) and was the generous gift of Drs. Stu Feinstein and Eric Shooter (Stanford University). Bovine thyroglobulin, catalase, aldolase, ovalbumin, chymotrypsinogen A, ribonuclease A, and blue dextran 2000 standards were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Rabbit muscle phosphorylase b, bovine serum albumin, egg ovalbumin, trypsinogen, β-lactoglobulin, and lysozyme were purchased from Sigma Chemical Co. Acetyl-β-methylcholine chloride (methacholine) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Nitrendipine was generously provided by Dr. L. Toll (Life Sciences Div., SRI International, Menlo Park, CA). Bisacrylamide, TEMED, Coomassie Brilliant Blue R-250 stain, protein assay reagent dye, and tracking dyes pyronin Y and bromphenol blue were purchased from Bio-Rad Laboratories (Richmond, CA). Ultrapure SDS was obtained from BDH Chemicals Ltd. (Poole, England). Ammonium persulfate was purchased from Eastman Kodak (Rochester, NY). Ampholines were purchased from LKB Producter AB (Bromma, Sweden). Ultrapure urea was obtained from Schwarz-Mann (Spring Valley, NY). Glass fiber filters (934-AH) were purchased from Whatman Chemical Separation Inc. (Clifton, NJ). Amino acid and vitamin supplements for cell culture media were purchased from Gibco Laboratories Inc. (Grand Island, NY). *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories Inc. (Elkhart, IN). Trypsin was purchased from Sigma Chemicals Co. Pansorbin (*S. aureus* cells) was purchased from Calbiochem-Behring Corp. (San Diego, CA). $Na^{125}I$ (17 Ci/mg, carrier-free in 0.1 M NaOH) was purchased from New England Nuclear (Boston, MA). [γ-^{32}P]ATP was purchased from ICN K & K Laboratories Inc. (Plainview, NY) (6,000 Ci/mmol) and Amersham Corp. (Arlington Heights, IL) (>2,000 Ci/mmol, triethylammonium salt in 5 mM 2-mercaptoethanol). H_3PO_4 (carrier-free in H_2O) was purchased from ICN K & K Laboratories Inc. L-[3,5-^{3}H]Tyrosine (40 Ci/mmol) was purchased from Amersham Corp. All other reagents were of the best reagent grade available.

Microtubules were prepared from bovine brain through two cycles of polymerization (32). MAP-2 was prepared by the following protocol: twice-polymerized microtubules were made 250 mM NaCl and 20 mM 2-mercaptoethanol, boiled for 3 min, and centrifuged (20,000 *g*) in a Sorvall SS-34 rotor (DuPont Instruments–Sorvall Biomedical Div., Wilmington, DE) for 15 min. Heat-stable MAP-2 (soluble fraction) was decanted off the tubulin pellet and stored at −20°C. Calmodulin was prepared from bovine cerebral cortex as described (8). Calmodulin-substituted Sepharose 4B was prepared by published procedures (33). Polyclonal antibody to TH was kindly provided by Dr. J. Barchas (Stanford University). Antibody to cAMP was obtained from Dr. G. Brooker (Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA). [^{125}I]Tyrosylsuccinyl-cAMP was prepared by the chloramine T method as described by Steiner et al. (34).

Ca^{2+}/Calmodulin-dependent Protein Kinase Assay: Ca^{2+}/calmodulin-dependent protein kinase was assayed by a modification of a published procedure using SDS PAGE (11). For quantitation of ^{32}P incorporation, ^{32}P-labeled bands were excised from the gel and Cerenkov radiation was measured in a Packard Tricarb scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, IL) at 60% efficiency.

TH Assay: TH was assayed by the method of Shiman et al. (35) using 167 μM 6-methyltetrahydropteridine.

Two-dimensional Gel Electrophoresis: Samples for two-dimensional gel electrophoresis were prepared by a modification of the method of Anderson and Anderson (36). Cytosolic fractions (50 μl) of ^{32}P-labeled cells were boiled for 2 min in 1% SDS, 0.5% glycerol, and 4% 2-mercaptoethanol. After cooling, samples were made 9 M urea, 4% ampholines (1:1 ratio of pH 3–10 to 6–8 ampholines), and 2% Nonidet P-40. 10 μL-samples were then loaded onto isoelectric focusing gels (1.75-mm diam cast in 3% acrylamide (17.8:1 ratio of acrylamide to bisacrylamide) and overlaid with 20 μL of 4% ampholines in 8 M urea containing a trace amount of Evan's blue dye. Isoelectric focusing was performed as described by O'Farrell (37). Gels were run for 17 h at 400 V and then 2 h at 800 V (8,400 V·h). After electrophoresis, the isoelectric focusing gels were removed from their glass tubes and equilibrated with 5 ml equilibration buffer (50 mM Tris HCl, pH 6.7, 2% SDS, 5% glycerol, 5% 2-mercaptoethanol, and a trace amount of bromphenol blue) for 15 min. Equilibrated tube gels were then loaded onto 9% SDS PAGE gels, sealed with agarose, and electrophoresed as described (11).

Cell Culture: PC12 cells were plated on poly-D-lysine-coated tissue culture dishes at a density of 2×10^6/150-mm dish. Cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum and 5% horse serum, in a water saturated atmosphere of 88% air and 12% CO_2. After cells were allowed to attach to the plate (24 h), they were refed either with or without NGF (50 ng/ml). Cells were used for experiments after 5–7 d in culture. Extracts from NGF-treated cells contained 80–100% more Ca^{2+}/calmodulin-dependent MAP-2 kinase activity per milligram protein than did extracts from non-NGF-treated cells. For this reason, all in situ ^{32}Pi labeling experiments were performed using cells treated with NGF. To reduce effects of NGF on TH phosphorylation, cells were rinsed and incubated for 24 h with NGF-free media before ^{32}Pi-labeling experiments.

Phosphate-free Media: Phosphate-free medium consisted of 2 mM $CaCl_2$, 5 mM KCl, 0.8 mM $MgSO_4$–$7H_2O$, 110 mM NaCl, 25 mM $NaHCO_3$, 4.5 mg/ml glucose, 10 μg/ml phenol red, 30 μg/ml glycine, 42 μg/ml serine, supplemented with amino acids and vitamins. The medium was brought to pH 7.2 with NaOH and filter sterilized. A high K^+ medium was prepared as above, using 50 mM KCl instead of 5 mM KCl and replacing NaCl with 240 mM sucrose.

Pi Labeling: Cells were washed once with phosphate-free media and then preincubated for 30 min in 10 ml/150-mm dish phosphate-free media. ^{32}Pi in 250 μM K_2HPO_4 was then added for a final concentration of 1 μM PO_4 and 50–500 μCi ^{32}Pi/10^6 cells. After cells were incubated (37°C) with ^{32}Pi for 0.5–2 h, drugs (in phosphate-buffered saline [PBS] or phosphate-free media) were added. Immediately after the treatment period, cells were placed on ice and treatment medium was aspirated. Cells were harvested in 10 ml of ice-cold PBS. A cell pellet was obtained by spinning this suspension at 200 *g* for 5 min. Cells were then resuspended in 0.3 ml of a homogenizing buffer containing phosphatase and protease inhibitors: 50 mM Tris-Cl, pH 7.4, 1 mM EGTA, 20 mM benzamidine, 50 mM NaF, 10 mM nitrilotriacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. Cells were homogenized by hand using a Teflon/glass homogenizer, and the suspension was spun at 150,000 *g* for 45 min. The resulting supernatant is referred to as the cytosolic extract. An aliquot of this extract was added to 2 vol of SDS "stop" solution I (4.5% SDS, 0.093 M Tris HCl, pH 6.7, 12.5% glycerol, 3% 2-mercaptoethanol, and trace amounts of the tracking dye

bromphenol blue). Samples were heated at 100°C for 2 min and then analyzed by SDS PAGE and autoradiography as described.

Preparation of Unlabeled Cytosol: Cells were harvested and pelleted as described above. Cells were then either homogenized in a motor driven Teflon/glass homogenizer (14 strokes at 1,200 rpm) or sonicated using a cell disrupter setting 5, 50% cycle, 14 pulses; model W-225R, Heat Systems-Ultrasonics, Inc., Plainview, NY). The cytosol fraction was obtained by centrifugation at 150,000 *g* for 60 min. The homogenizing buffer consisted of 50 mM Tris-Cl, pH 7.4, 1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin.

Immunoprecipitation of TH: All manipulations were performed at 4°C. Cytosol (100 μl) from ^{32}P-labeled cells was incubated with 10 μL polyclonal antibody prepared against TH from rat pheochromocytoma. After 30 min, a 10% pansorbin suspension (15 μl) was added and incubated for an additional 20 min. This material was pelleted by centrifugation at 6,000 *g* for 10 min. The resulting pellet was washed twice with a buffer containing 0.5% Nonidet P-40, 2 mM KI, and 0.02% sodium azide in PBS. Pellets were resuspended in 250 μL of SDS stop solution II (3% SDS, 0.062 M Tris HCl, pH 6.7, 8.3% glycerol, 2% 2-mercaptoethanol, and trace amounts of bromphenol blue), boiled for 2 min, and centrifuged at 6,000 g for 10 min. The supernatant was analyzed by SDS PAGE.

Assay of cAMP Levels: Cells were cultured and prepared for experiments using a protocol identical to that used for ^{32}P-labeled cells, with the exception that 60-mm rather than 150-mm dishes of cells were used. Cells were rinsed with 5 ml phosphate-free DME and then incubated for 1–2 h with 4.5 ml phosphate-free DME. Cells were then stimulated with 0.5 ml of 10× concentrations of drug. Stimulation was terminated by the addition of 0.55 ml 1 N HCl. Aliquots of this acid extract (450 μl) were then neutralized with 50 μL 1 N NaOH, acetylated, and assayed for cAMP using the radioimmunoassay procedure of Brooker et al. (38).

Protein Assay: Protein was routinely determined by the method of Bradford (39) as modified by Spector (40), using bovine γ-globulin as protein standard.

Statistics and Calculations: Within a given experiment, equal aliquots of the cytosolic extract were spotted on glass fiber filters which were then washed with 3–5-ml rinses of 10% trichloroacetic acid (TCA), 100 mM NaPPi using a manifold filtration apparatus. Treated filters were dried under a heat lamp and Cerenkov radiation was counted as described above. The extent of ^{32}P incorporation into TH (as determined by Cerenkov radiation of the TH band on SDS PAGE of equal aliquots of ^{32}P-labeled cytosol) was then normalized for equal amounts of TCA-precipitable radioactivity. Data are expressed as the mean (±SEM), unless otherwise noted. In Table I, the significance of the magnitude of TH phosphorylation was determined by student's *t*-test for paired observations (using values expressed as precentage of control). In Table III, the significance of the difference between groups was assessed by student's *t*-test for two means.

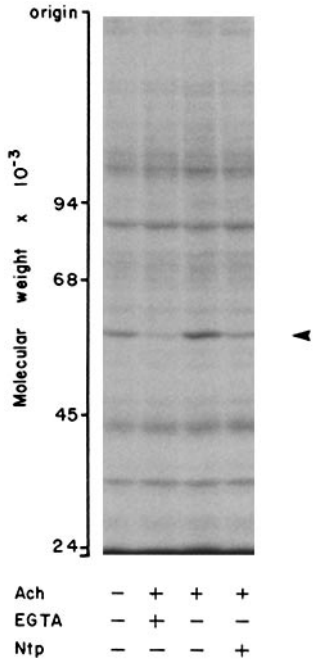

FIGURE 1 Autoradiogram showing the effect of acetylcholine on phosphorylation of cytosolic proteins in PC12 cells. Cells were prelabeled with ^{32}Pi and then treated with 100 μM acetylcholine (*Ach*) for 5 min as described in Materials and Methods. Some cells were pretreated with 5 mM EGTA or 10 μM nitrendipine (*Ntp*) for 1 min before the addition of acetylcholine. Cytosol proteins were resolved on SDS gels as described. The arrowhead indicates the major protein whose phosphorylation is influenced by acetylcholine treatment.

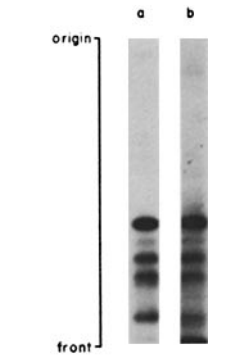

FIGURE 2 Comparison of immunoprecipitated TH with putative TH band by partial proteolysis. PC12 cells were prelabeled with ^{32}Pi and then stimulated by depolarization with 56 mM extracellular K^+ in the presence of 2 mM Ca^{2+}. Cytosolic extracts were prepared and aliquots were immunoprecipitated with a polyclonal antibody to TH. Cytosolic proteins and the immunoprecipitated TH were then resolved on SDS gels. Approximately 30% of the ^{32}P migrating as the 60K band was immunoprecipitated. The ^{32}P-labeled 60-kD polypeptide from crude cytosol (lane *a*) and the ^{32}P-labeled immunoprecipitate (*b*) were excised from gels and subjected to partial proteolysis with 3 μg *S. aureus* V8 protease as described in Materials and Methods. The gel of the immunoprecipitated sample (lane *b*) was exposed longer than the 60K band because it contained less ^{32}P.

RESULTS

Regulation of Protein Phosphorylation in PC12 Cells

To examine the role of Ca^{2+} in the regulation of TH phosphorylation, we labeled PC12 cells with [^{32}P]orthophosphate and analyzed those cytosolic proteins that were phosphorylated after Ca^{2+} elevation. Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels was stimulated by either high extracellular K^+ or by nicotinic cholinergic agonists. As shown in Fig. 1, ^{32}Pi-labeled PC12 cells contain numerous cytosolic phosphoproteins, including a major one with an M_r of 60,000. Stimulation of these cells with 100 μM acetylcholine results in a 33% increase in phosphate incorporation into this protein. Pretreatment of the cells with EGTA or the Ca^{2+} channel blocker nitrendipine blocked the ability of acetylcholine to stimulate ^{32}P-incorporation. Although the protein at M_r of 60,000 is the most conspicuous protein that shows increased phosphorylation, numerous other proteins were also phosphorylated by this treatment (see below).

It has previously been shown that TH, a 60,000-D protein, is a major phosphoprotein in PC12 cells (41). To determine whether the 60,000-D protein whose phosphorylation was stimulated by acetylcholine was TH, it was analyzed with a polyclonal antibody prepared against rat pheochromocytoma TH. Cytosolic extracts of ^{32}P-labeled cells were analyzed either directly by SDS PAGE or first immunoprecipitated with the antibody and then analyzed by SDS PAGE. Indeed, most of the ^{32}P-protein migrating in the 60,000-D region of the gel was selectively immunoprecipitated by the antibody (data not shown). A further comparison was made by excising the putative TH (60K) band and immunoprecipitated TH band from an SDS gel and subjecting them to partial proteolysis using *S. aureus* V8 protease (42). As shown in Fig. 2, the phosphopeptide patterns generated are nearly identical. The major difference, the ^{32}P migrating at the dye front, is found variably in different experiments and can also be seen in digests of the 60K band. Partial proteolysis with chymotrypsin also generates nearly identical patterns for the 60K and immunoprecipitated bands (data not shown). The absence of any novel phosphopeptide generated from the putative TH band indicates that no contaminating phosphoproteins comigrate with TH upon SDS PAGE. Thus, ^{32}P incorporation

into TH can be easily quantified by excising this band from the SDS gels and counting the radioactivity.

The identity of the 60,000-D phosphoprotein was further analyzed by two-dimensional SDS/isoelectric focusing gels. Cells prelabeled with $^{32}PO_4$ were incubated under control and K^+-depolarized conditions, and then cytosolic proteins were analyzed by two-dimensional gels as described in Materials and Methods. Duplicate gels run in parallel were electrophoretically transferred to nitrocellulose filters and immunostained to localize TH (data not shown). The antibody selectively stained three major isoelectric forms of TH in control preparations, corresponding to TH with zero, one, and two phosphates. The autoradiograph of control cells, Fig. 3*A*, shows that TH is a major phosphoprotein. Most of the ^{32}P is present in the monophosphorylated form. Upon depolarization there is phosphorylation of all forms of TH, accounting for a 32% increase in ^{32}P incorporation. This is detected as an increased ^{32}P incorporation into the isoelectric forms with one and two phosphates (Fig. 3*B*, arrows) as well as the appearance of ^{32}P in more highly acidic spots. A commensurate shift in the position of TH is detectable by immunostaining (data not shown). We have seen up to five isoelectric forms of TH in depolarized or carbachol-treated cells. Thus, the major protein responsive to both acetylcholine- and K^+-induced depolarization is TH.

Examination of the two dimensional gels (Fig. 3) also reveals a limited array of other proteins whose phosphorylation is affected by K^+-depolarization. Although ^{32}P incorporation into several other phosphoproteins is increased, a number of other phosphoproteins demonstrate a net loss of ^{32}P.

The significance of TH phosphorylation is apparent when one examines TH activity. K^+-induced depolarization increased TH activity by 86 ± 19% ($n = 9$). The effect depended on extracellular Ca^{2+}. This activation is not due to a direct effect of Ca^{2+}-calmodulin on TH since the increase in activity can be detected when TH is assayed in the presence of either Ca^{2+} or EGTA. Thus, TH is both phosphorylated and activated by Ca^{2+} influx in PC12 cells.

Various other potential agonists were examined for their ability to stimulate ^{32}P incorporation into TH. The results are illustrated in Table I. Acetylcholine and its more stable analogue carbachol both stimulated TH phosphorylation in situ by ~35% as compared with PBS-treated cells. These agonists are presumably acting through the well-characterized nico-

TABLE I. *Effect of Various Stimuli on TH Phosphorylation*

Agent	TH phosphorylation, % PBS Control
	mean ± SD
100 μM acetylcholine (5 min)	135.7 ± 10.0*
100 μM carbachol (5 min)	132.7 ± 5.0*
50 μM 2-chloroadenosine (11 min)	113.5 ± 3.8*
400 μM methacholine (5 min)	102.7 ± 2.4
100 μM phenylephrine (5 min)	97.8 ± 4.3
10 μM A23187 (12 min)	129.2 ± 8.4*

PC12 cells were prelabeled with ^{32}Pi and then treated with PBS or the agents indicated above. Cytosol proteins were resolved on SDS gels and ^{32}P incorporation into TH was determined by Cerenkov radiation, as described in Materials and Methods. 100% represents percent ^{32}P incorporated into TH after treatment with PBS alone. Data are presented as the mean ± SD for the following *n* experiments: acetylcholine ($n = 3$), carbachol ($n = 7$), 2-chloroadenosine ($n = 3$), methacholine ($n = 3$), phenylephrine ($n = 2$), and A23187 ($n = 5$).

* Value significantly greater than control value, $0.001 < P < 0.05$.

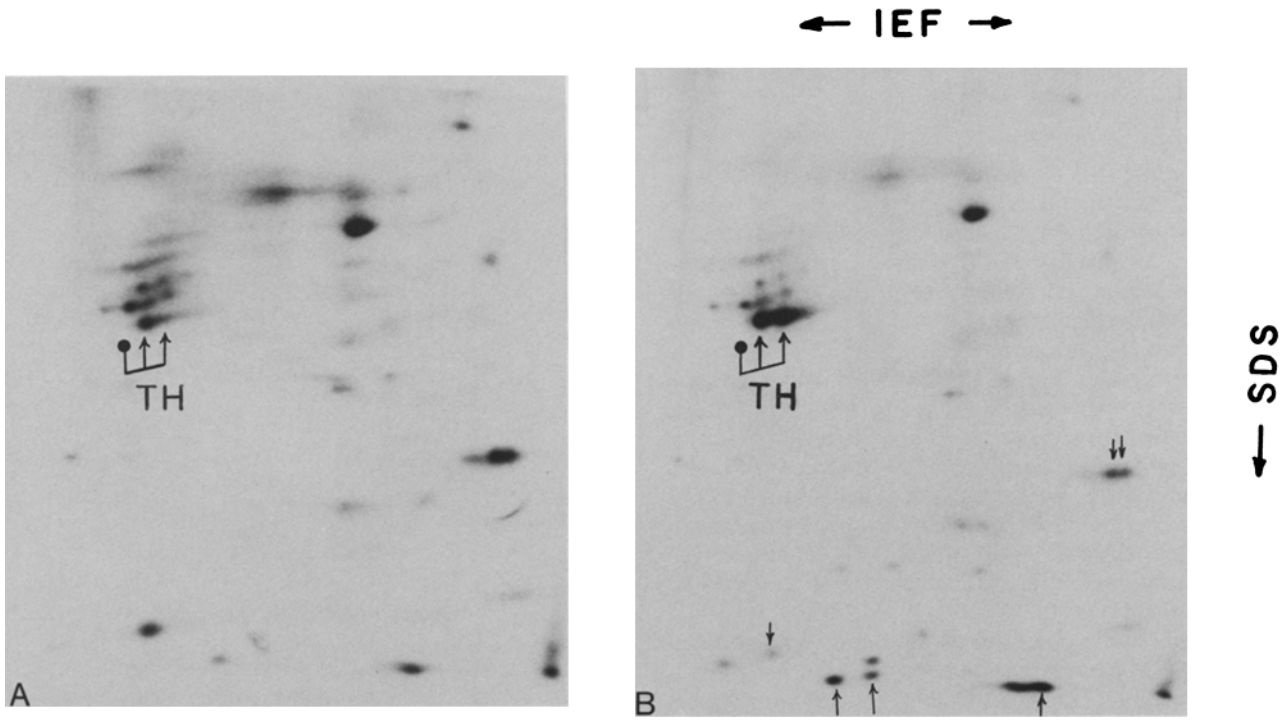

FIGURE 3 Two-dimensional gel electrophoresis of ^{32}P-labeled PC12 cell cytosols. PC12 cells were prelabeled with ^{32}Pi and then stimulated by depolarization using 56 mM K^+ in the presence of 2 mM $CaCl_2$ plus 5 mM EGTA (*A*, control) or 2 mM $CaCl_2$ (*B*, depolarized). Cytosolic proteins were resolved by two-dimensional gel electrophoresis as described in Materials and Methods. The acidic end of the isoelectric focusing (*IEF*) dimension is on the right of each panel. Polypeptides that undergo an increase in ^{32}P incorporation are indicated by an upward arrow, polypeptides that undergo a decrease in ^{32}P incorporation by a downward arrow. The position of TH is indicated; the unphosphorylated species with a pI of 6.3 is indicated by a filled circle.

TABLE II. *Effect of d-Tubocurarine on Carbachol- and A23187-induced TH Phosphorylation*

	% Maximal phosphorylation
100 μM carbachol	100
100 μM carbachol + 10 μM *d*-tubocurarine	34 ± 6
10 μM *d*-tubocurarine	22 ± 13
10 μM A23187	100
10 μM A23187 + 10 μM *d*-tubocurarine	92 ± 18

Cells prelabeled with ^{32}Pi were incubated with either 100 μM carbachol for 5 min or 10 μM A23187 for 12 min. 10 μM *d*-tubocurarine was added 10 min before the addition of carbachol, A23187, or PBS. Data are presented as the mean ± SE for $n = 3$ or 4.

tinic channels on PC12 cells, since *d*-tubocurarine, a nicotinic receptor antagonist, blocked the effect (see Table II) and neither methacholine (which shows preferential muscarinic agonist activity) nor phenylephrine (which shows preferential α-adrenergic activity) was effective. The Ca^{2+} ionophore A23187 (10 μM) could mimic the effect of either chemical or electrical depolarization of these cells. In these studies, 2-chloroadenosine (50 μM) was not as effective as the cholinergic agonists in stimulating TH phosphorylation. 2-Chloroadenosine presumably acts through its ability to increase cAMP levels and thereby activate the cAMP-dependent protein kinase. The apparently small percentage change in ^{32}P incorporation into TH elicited by this and other agonists may be a function of relatively high basal levels of ^{32}P incorporation in TH in control cells grown in the presence of NGF, an agent known to increase TH phosphorylation (41). The shift to more acidic isoelectric forms of TH does indicate, however, that this change in ^{32}P incorporation represents stoichiometric amounts of phosphate.

Dose Response and Time Course of Carbachol Effects

Dose response and time course of carbachol action were examined. Cells were preincubated with ^{32}Pi and stimulated with 100 μM carbachol for 1, 4, 6, 12, and 24 min. Cytosolic proteins were separated by SDS PAGE, and ^{32}P incorporation into TH was determined. An increase in TH phosphorylation was observable within 1 min of stimulation, with ^{32}P incorporation increasing linearly for the next 5 min (Fig. 4). Beyond the 6 min incubation time, TH phosphorylation decreased, virtually returning to basal levels after 24 min of incubation. This decline in TH phosphorylation may result from a combination of desensitization of the nicotinic receptor, coupled with the time-dependent closing of Ca^{2+} channels, and the action of phosphoprotein phosphatases known to exist in these cells (unpublished observations). This dephosphorylation is consistent with a regulatory role for TH phosphorylation.

Concentration–response studies were performed in a manner analogous to the time-course experiments, using a 4-min stimulation period and carbachol concentrations ranging between 1 and 500 μM (Fig. 5). The half-maximal stimulation occurred at 30 μM carbachol and maximal TH phosphorylation occurred at 100 μM carbachol. When TH activity was examined in a similar experiment, it was found to be equally sensitive to stimulation by carbachol. At a concentration above 100 μM (e.g., 500 μM), TH phosphorylation reached only 74% of its maximal value. Again, this decrease may be a function of desensitization of the cholinergic receptor. Note that stimulation-induced release of norepinephrine from PC12 cells shows a similar decrease with agonist concentrations above a certain maximal value (43).

Dependence of TH Phosphorylation on Ca^{2+}

Because carbachol, acting at either muscarinic or nicotinic receptors, is known to increase Ca^{2+} influx, it was of interest to determine whether extracellular Ca^{2+} was essential for TH phosphorylation. As shown in Fig. 6, pretreatment of cells with EGTA actually reduced control levels of ^{32}P incorporation in TH by ~40%. Chelation of extracellular Ca^{2+} results in a relatively specific decrease in TH phosphorylation (compare with Fig. 1), with few other cytosolic phosphoproteins being affected. The effect of EGTA was apparent within 1 min. As shown in Fig. 6, pretreatment of cells with EGTA

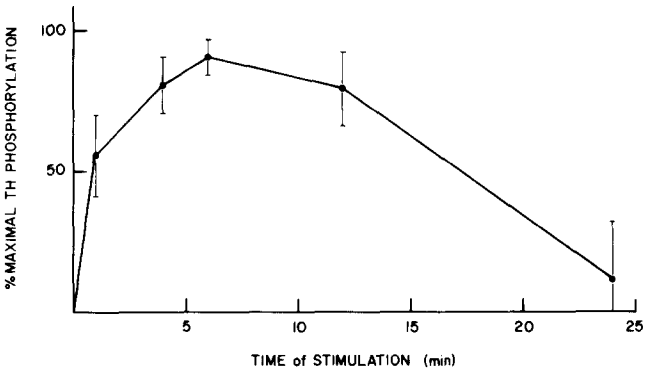

FIGURE 4 Time course of carbachol-stimulated phosphorylation of TH. PC12 cells were prelabeled with ^{32}Pi and then incubated in the presence of 100 μM carbachol. Treatment was terminated at the times indicated, and ^{32}P incorporation into TH was determined as described in the legend to Table I. Phosphorylation of TH is expressed as the percentage of maximal ^{32}P incorporation within a given experiment, where maximal ^{32}P incorporation equals 100%. Data are presented as the mean ± SE for $n = 3$ or 4.

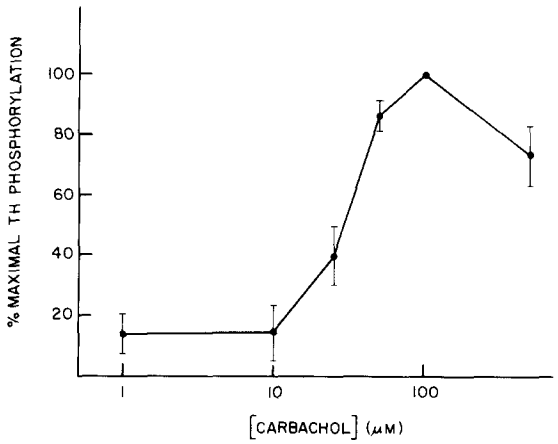

FIGURE 5 The effect of various concentrations of carbachol on TH phosphorylation. PC12 cells were prelabeled with ^{32}Pi and then incubated for 4 min with from 1 to 500 μM carbachol. ^{32}P incorporation into TH was determined as described in the legend to Table I. Phosphorylation of TH is expressed as the percentage of maximal ^{32}P incorporation within a given experiment, where maximal ^{32}P incorporation equals 100%. Data are presented as the mean ± SE for $n = 3$ or 4.

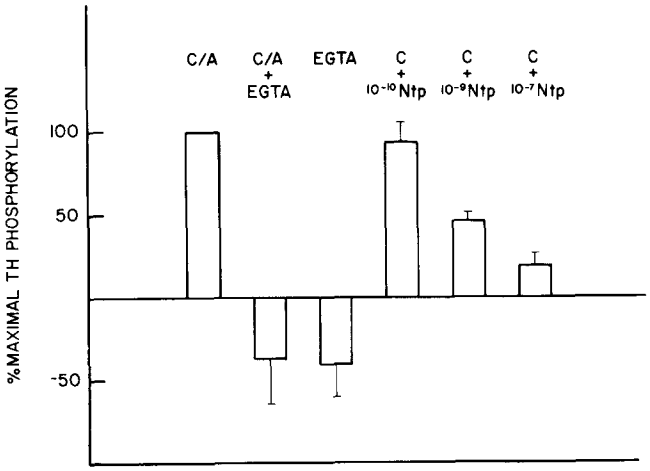

FIGURE 6 Ca^{2+} dependence of depolarization-induced TH phosphorylation. PC12 cells, prelabeled with ^{32}Pi, were incubated with either 100 μM carbachol (C) or 100 μM acetylcholine (A) for 5 min. EGTA and nitrendipine (Ntp) were added 1 min before the addition of either agonist. Data for carbachol and acetylcholine have been combined into one column (C/A) since they are not statistically different. ^{32}P incorporation into TH was determined as described. Results are expressed as the percentage of maximal ^{32}P incorporation within a given experiment, where maximal ^{32}P incorporation equals 100%. Data are presented as the mean ± SE for $n = 3$ or 4.

completely abolished the ability of carbachol to increase TH phosphorylation beyond the level seen with EGTA alone.

Nitrendipine has been shown to be a potent blocker of voltage-dependent Ca^{2+} channels in PC12 cells (44). This compound was examined for its ability to antagonize the carbachol-dependent phosphorylation of TH. As shown in Fig. 6, nitrendipine was able to inhibit the carbachol-induced phosphorylation of TH in a concentration-dependent manner. Moreover, the concentration of nitrendipine required to inhibit depolarization-induced ^{45}Ca uptake by 50% (5.5 nM) is approximated by the concentration of nitrendipine (1 nM) needed to reduce carbachol-induced TH phosphorylation by 50%.

d-Tubocurarine was used to further probe the mechanism by which carbachol induces TH phosphorylation (Table II). Although *d*-tubocurarine appeared to have slight activity of its own in enhancing TH phosphorylation, the subsequent addition of carbachol produced no effects greater than those of *d*-tubocurarine alone. *d*-Tubocurarine is, in fact, a partial agonist of the nicotinic receptor of the neuromuscular junction (45, 46). The overall effect of *d*-tubocurarine, however, was to inhibit carbachol-induced TH phosphorylation by >60%. As mentioned earlier, 10 μM A23187 was able to mimic the action of carbachol in the presence of extracellular Ca^{2+}. *d*-Tubocurarine at 10 μM had no effect in decreasing TH phosphorylation induced by A23187. From these results, it appears that carbachol effects are mediated through the nicotinic cholinergic receptor in these cells. Kinase activation and TH phosphorylation may thus be a consequence of receptor stimulation, causing a depolarization of the cells and subsequent opening of Ca^{2+} channels. The ability of a Ca^{2+} ionophore to mimic the effects of carbachol without being affected by the presence of *d*-tubocurarine supports this model.

cAMP Levels after Stimulation

Since the cAMP-dependent protein kinase is present in PC12 cells, the possibility remained that the various treatments used to stimulate TH phosphorylation were mediated by an increase in cytosolic cAMP and consequent stimulation of the cAMP-dependent protein kinase. To examine this possibility, cAMP levels were measured in PC12 cell cytosol after stimulation by A23187, carbachol, 2-chloroadenosine, and methacholine (Table III). Only 2-chloroadenosine was effective in eliciting an increase in cAMP levels; the concentration of cAMP after treatment with the other agents remained at control levels. Thus, no correlation exists between those compounds that can induce TH phosphorylation (carbachol and A23187) and the one compound that can increase cAMP levels (2-chloroadenosine).

Ca^{2+}/Calmodulin-dependent Protein Kinase in PC12 Cells

The rat brain Ca^{2+}/calmodulin-dependent kinase has been shown to phosphorylate TH in vitro (47). Thus it was important to determine whether this kinase existed in PC12 cells. The cultures of PC12 cells were grown in the presence or absence of NGF and harvested, and cytosolic extracts were prepared as described in Materials and Methods. To achieve a rapid purification of any Ca^{2+}/calmodulin-dependent enzymes, the extracts were fractionated on a calmodulin-Sepharose affinity column. Fractions were assayed for Ca^{2+}/calmodulin-dependent protein kinase activity using MAP-2 as a substrate, since it has been identified as one of the best substrates for this enzyme in brain (11, 17). Indeed, a Ca^{2+}/calmodulin-dependent MAP-2 kinase activity was retained by the calmodulin-Sepharose column after a high salt wash in the presence of Ca^{2+} but was eluted from the column with a buffer that contained EGTA. Using this rapid fractionation procedure and MAP-2 as substrate, we detected Ca^{2+}/calmodulin-dependent kinase activity in both − and + NGF-treated PC12 cells (data not shown). It is interesting that extracts from NGF-treated cells contained 80–100% more kinase activity per milligram protein than did extracts from non-NGF-treated cells. This kinase exhibits an absolute requirement for both Ca^{2+} and calmodulin. In addition, the kinase is inhibited by fluphenazine, consistent with other calmodulin-dependent enzymes.

To compare further this enzyme to that isolated from rat brain, the site specificity of MAP-2 phosphorylation was compared by the method of partial proteolysis using *S. aureus* V8 protease (42). Previous studies from this lab have shown

TABLE III. *cAMP Levels as a Function of Various Agents*

Agent	fmol cAMP/μg protein
DME (media)	81 ± 3
10 μM A23187	87 ± 8
50 μM 2-chloroadenosine	2,268 ± 376*
100 μM carbachol	91 ± 8
400 μM methacholine	75 ± 9

PC 12 cells were cultured and prepared for experiments in a manner identical to that used for ^{32}Pi labeling. Cells were then incubated with the indicated concentrations of A23187 for 12 min, 2-chloroadenosine for 11 min, or DME, carbachol, or methacholine for 5 min. cAMP levels were subsequently analyzed by radioimmunoassay as described in Materials and Methods. Data are presented as the mean ± SE for $n = 6$.

* Value significantly greater than DME control value, $P < 0.001$.

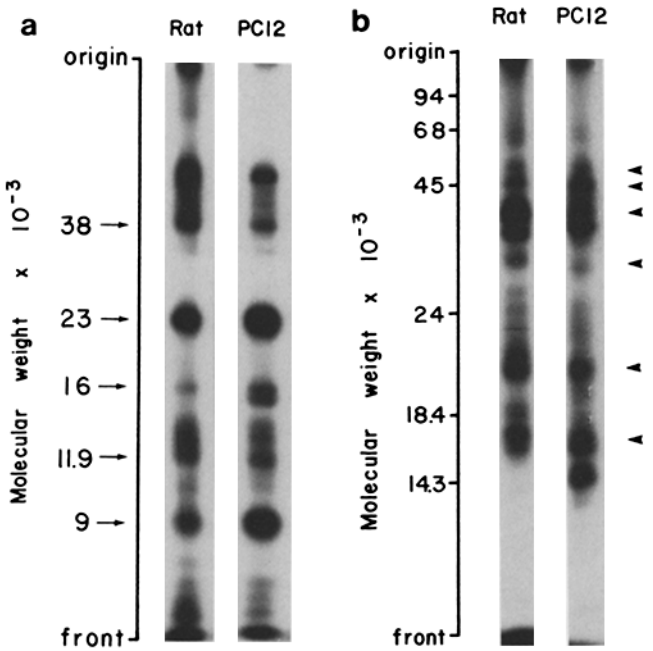

FIGURE 7 Comparison of Ca^{2+}/calmodulin-dependent protein kinase from PC12 and rat brain. (*a*) Site specificity of MAP-2 phosphorylation. MAP-2 was phosphorylated using purified rat brain kinase or PC12 kinase fractionated on calmodulin-Sepharose 4B. MAP-2 was resolved on SDS gels, ^{32}P-labeled MAP-2 was excised and subjected to partial proteolysis using 3 μg *S. aureus* V8 protease. Molecular weight ($\times 10^{-3}$) of MAP-2 phosphopeptides are indicated. (*b*) Comparison of phosphopeptides generated from autophosphorylated rat brain kinase and putative autophosphorylated PC12 kinase. Purified rat brain enzyme and partially purified PC12 cell kinase (calmodulin-Sepharose 4B fraction) were incubated under standard reaction conditions in the absence of substrate. SDS PAGE was used to resolve rat brain autophosphorylated 51-kD subunit and a phosphorylated 53-kD polypeptide from PC12 cells. These ^{32}P-labeled polypeptides were excised from the gels and subjected to partial proteolysis with 2 μg *S. aureus* V8 protease. Homologous phosphopeptides are indicated by arrowheads.

that the rat brain Ca^{2+} kinase phosphorylates MAP-2 at distinct sites different from those phosphorylated by the cAMP-dependent protein kinase (48). As shown in Fig. 7*a*, MAP-2 phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase from either rat brain or PC12 yields identical phosphopeptides when analyzed by partial proteolysis.

One of the properties of the rat brain Ca^{2+}/calmodulin-dependent protein kinase is its ability to autophosphorylate subunits of 51- and 60-kD (11, 12, 15, 16). The Ca^{2+}-dependent kinase from PC12 cells similarly exhibited putative autophosphorylation of polypeptides of M_r 47,000–60,000. These putative autophosphorylated subunits were compared with authentic rat brain autophosphorylated subunits by partial proteolysis, as shown in Fig. 7*b*. Homologous phosphopeptides are indicated by arrows. This analysis reveals considerable homology between autophosphorylated rat brain and PC12 kinases.

DISCUSSION

Ca^{2+}/calmodulin-dependent protein kinases have been purified from several sources, using a variety of substrates (11–16, 49–51). Although only two of these have been shown to be identical (52), many of the others, based on their detailed characteristics, appear to be very similar enzymes or isozymes. They are regulated by physiological levels of Ca^{2+} and calmodulin and can phosphorylate a wide variety of substrates in vitro (17, 52). These characteristics suggest a role for the Ca^{2+}/calmodulin-dependent kinase as a multifunctional mediator of Ca^{2+} action in vivo, analogous to the role of the cAMP-dependent protein kinase in mediating cAMP effects in the cell. Functional correlates of Ca^{2+}/calmodulin kinase activity in vivo are only now being examined.

The purpose of the present study was to investigate the in vivo action of this Ca^{2+}/calmodulin-dependent protein kinase by testing whether physiological stimuli can activate Ca^{2+}-dependent phosphorylation with resultant functional consequences. Five conclusions can be drawn from this study. First, a Ca^{2+}/calmodulin-dependent protein kinase exists in PC12 cells that resembles the kinase purified from rat brain. Based on its Ca^{2+} and calmodulin dependence, its site specificity for MAP-2 phosphorylation, and the site specificity of its autophosphorylation, the kinase from PC12 cells appears functionally and structurally very similar to that from brain tissue. Nonetheless, the presence of several distinct autophosphorylated peptides suggests that the enzymes are homologous but not identical. Small differences among the enzymes from rat brain, heart, spleen, and lung, and from rabbit skeletal muscle have previously been noted (17, 52). However, because the PC12 enzyme is not pure, it is possible that either some or all of the phosphopeptides that are distinct from the rat brain kinase are generated from a contaminating phosphoprotein rather than an autophosphorylated PC12 kinase. In light of their similar size, subunit composition, and capacity for autophosphorylation, these enzymes may belong to a family of multifunctional Ca^{2+}/calmodulin-dependent kinases. Recently, Vulliet et al. (53) partially purified a Ca^{2+}/calmodulin kinase from rat pheochromocytoma cells and showed that the substrate specificity of this enzyme is similar to that of a Ca^{2+}/calmodulin kinase from rabbit skeletal muscle. These findings support our own, suggesting the presence of a Ca^{2+}/calmodulin-dependent protein kinase with a broad substrate specificity in PC12 cells.

Second, incubation of PC12 cells with carbachol in the presence of extracellular Ca^{2+} does indeed lead to relatively specific changes in ^{32}P incorporation into a number of cytosolic proteins. TH, the major phosphoprotein in these cells, is heavily phosphorylated. The effect of carbachol is via the nicotinic acetylcholine receptor since it can be blocked by *d*-tubocurarine. In fact, *d*-tubocurarine has a small effect of its own, consistent with its being a partial agonist of the nicotinic receptor. Moreover, TH phosphorylation after depolarization of PC12 cells is dependent on voltage-dependent Ca^{2+} channels, since nitrendipine blocks the effect. The lack of an effect by 10 μM phenylephrine suggests that the effect of carbachol is not mediated by the catecholamines released after carbachol treatment. The phosphorylation of TH appears to be a reversible process since extended incubation with a depolarizing concentration of carbamylcholine leads to dephosphorylation of TH to control levels. This is not surprising since extracts of PC12 cells contain active phosphoprotein phosphatases that can dephosphorylate Ca^{2+} kinase-phosphorylated MAP-2 (unpublished observations).

Third, the effect of carbachol is probably mediated by Ca^{2+} acting as a second messenger since an increase in intracellular Ca^{2+} is sufficient to increase TH phosphorylation in these cells. Ca^{2+} influx caused by membrane depolarization using

high K^+ leads to an increase in TH phosphorylation. Two-dimensional gel analysis of cytosolic protein after depolarization indicates that this is not a general increase in total ^{32}P incorporation; only TH and a few other proteins undergo an increase in phosphorylation, whereas several others actually undergo a decrease. It is possible that an enzyme similar to calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, may mediate the dephosphorylation (54). This effect of K^+ is not a direct consequence of depolarization since it is dependent on extracellular Ca^{2+}. Chelation of Ca^{2+} with EGTA completely abolishes the phosphorylation changes. Moreover, TH phosphorylation after depolarization of PC12 cells is dependent on voltage-dependent Ca^{2+} channels since nitrendipine blocks it at concentrations known to inhibit Ca^{2+} uptake. A similar effect of K^+ on TH phosphorylation has recently been demonstrated (55–57). Direct entry of Ca^{2+}, facilitated by the ionophore A23187 also leads to the same degree of TH phosphorylation.

Fourth, Ca^{2+}-dependent TH phosphorylation is not mediated by elevation of cAMP and activation of the cAMP-dependent protein kinase. Carbachol and A23187 had no effect on intracellular levels of cAMP yet had a greater effect on TH phosphorylation than did 2-chloroadenosine, which markedly elevates cAMP. This conclusion is an important one since most studies thus far have focused on the role of cAMP in regulating TH.

Fifth, Ca^{2+}-dependent phosphorylation of TH is accompanied by an activation of the enzyme. Thus, both cAMP and Ca^{2+} stimulate phosphorylation and activation of TH. In recent studies we have found that the effects of cAMP and Ca^{2+} on TH activity are, in fact, additive (unpublished observation). In adrenal chromaffin cells, differential phosphorylation of TH is observed. TH is phosphorylated at two sites; cAMP increases phosphorylation at only one of these whereas acetylcholine stimulates phosphate incorporation at both these sites (22, 30). Vulliet et al. have also demonstrated differential phosphorylation of rat pheochromocytoma TH in vitro after treatment with either cAMP or a Ca^{2+}/calmodulin-dependent kinase (53). Thus, acetylcholine-induced TH phosphorylation appears to be mediated by a kinase distinct from and/or in addition to the cAMP-dependent protein kinase.

There are two cAMP-independent protein kinases that may fulfill such a role. Raese et al. (58) used a proteolytically activated Ca^{2+}/diglyceride-dependent protein kinase (kinase C) to phosphorylate and activate TH. Kinase C is widely distributed in mammalian tissues and is thought to mediate signal transduction of hormones that elicit the phosphatidylinositol response (59). However, in preliminary studies, we have been unable to consistently stimulate TH phosphorylation by activation of kinase C in PC12 cells using phorbol esters. In addition, this enzyme is not stimulated by activation of nicotinic receptors. Yamauchi and Fujisawa have used a Ca^{2+}/calmodulin-dependent kinase to activate TH (14). Given the similarity of the Ca^{2+}/calmodulin-dependent protein kinase from PC12 cells and that from rat brain, we suggest that this is the enzyme responsible for mediating depolarization-induced TH phosphorylation in PC12 cells.

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REFERENCES

1. Garrison, J. C., D. E. Johnsen, and C. P. Campanile. 1984. Evidence for the role of phosphorylase kinase, protein kinase C, and other calcium-sensitive protein kinases in the response of hepatocytes to angiotensin II and vasopressin. *J. Biol. Chem.* 255:11063–11066.
2. Freedman, S. D., and J. D. Jamieson. 1982. Hormone-induced protein phosphorylation. I. Relationship between secretagogue action and endogenous protein phosphorylation in intact cells from the exocrine pancreas and parotid. *J. Cell Biol.* 95:903–908.
3. Freedman, S. D., and J. D. Jamieson. 1982. Hormone-induced protein phosphorylation. II. Localization to the ribosome fraction from rat exocrine pancreas and parotid of a 29,000-dalton protein phosphorylated *in situ* in response to secretagogues. *J. Biol. Chem.* 95:909–917.
4. Gorelick, F. S., J. A. Cohn, S. D. Freedman, N. G. Delahunt, J. M. Gershoni, and J. D. Jamieson. 1983. Calmodulin-stimulated protein kinase activity from rat pancreas. *J. Cell Biol.* 97:1294–1298.
5. Drust, D. S., and T. F. J. Martin. 1982. Thyrotropin-releasing hormone rapidly and transiently stimulates cytosolic calcium-dependent protein phosphorylation in GH3 pituitary cells. *J. Biol. Chem.* 257:7566–7573.
6. Sobel, A., and A. H. Tashjian, Jr. 1983. Distinct patterns of cytoplasmic protein phosphorylation related to regulation of synthesis and release of prolactin by GH cells. *J. Biol. Chem.* 258:10312–10324.
7. Schulman, H., and P. Greengard. 1978. Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein. *Nature (Lond.).* 271:478–479.
8. Schulman, H., and P. Greengard. 1978. Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues and its activation by "calcium-dependent regulator." *Proc. Natl. Acad. Sci. USA.* 75:5432–5436.
9. DeLorenzo, R. J., S. D. Freedman, W. B. Yohe, and S. C. Maurer. 1979. Stimulation of Ca^{2+}-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 76:1838–1842.
10. Nose, P., and H. Schulman. 1982. Protein phosphorylation system in bovine brain cytosol dependent on calcium and calmodulin. *Biochem. Biophys. Res. Commun.* 107:1082–1090.
11. Schulman, H. 1984. Phosphorylation of microtubule-associated protein 2 by a Ca^{2+}/calmodulin-dependent protein kinase. *J. Cell Biol.* 99:11–19.
12. Kuret, J. A., and H. Schulman. 1984. Purification and characterization of a Ca^{2+}/calmodulin-dependent protein kinase from rat brain. *Biochemistry.* 23:5495–5504.
13. Fukunaga, K. H., H. Yamamoto, K. Matsui, K. Higashi, and E. Miyamoto. 1982. Purification and characterization of a Ca^{2+}- and calmodulin-dependent protein kinase from rat brain. *J. Neurochem.* 39:1607–1617.
14. Yamauchi, T., and H. Fujisawa. 1983. Purification and characterization of the brain calmodulin-dependent protein kinase (kinase II), which is involved in the activation of tryptophan 5-monooxygenase. *Eur. J. Biochem.* 132:15–21.
15. Goldenring, J. R., B. Gonzales, J. S. McGuire, Jr., and R. J. DeLorenzo. 1983. Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associated proteins. *J. Biol. Chem.* 258:12632–12640.
16. Bennett, M. K., N. E. Erondu, and M. B. Kennedy. 1983. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* 258:12735–12744.
17. Schulman, H., J. Kuret, A. B. Jefferson, P. Nose, and K. H. Spitzer. 1984. Ca^{2+}/calmodulin-dependent MAP-2 kinase: broad substrate specificity and multifunctional potential in diverse tissues. *Biochemistry.* In press.
18. Schulman, H. 1982. Calcium-dependent protein phosphorylation. *In* Handbook of Experimental Pharmacology. Vol. 58. J. A. Nathanson and J. W. Kebabian, editors. Springer-Verlag, Inc., New York. 425–478.
19. Green, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.
20. Chalfie, M., and R. L. Perlman 1977. Regulation of catecholamine biosynthesis in a transplantable rat pheochromocytoma. *J. Pharmacol. Exp. Ther.* 200:588–597.
21. Greene, L. A., and G. Rein. 1978. Short-term regulation of catecholamine biosynthesis in a nerve growth factor responsive clonal line of rat pheochromocytoma cells. *J. Neurochem.* 30:549–555.
22. Haycock, J. W., J. A. Medigeni, W. F. Bennett, and J. C. Waymire. 1982. Phosphorylation and activation of tyrosine hydroxylase mediate the acetylcholine-induced increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Cell. Biol.* 257:12641–12648.
23. Patrick, R. L., and J. D. Brachas. 1974. Stimulation of synaptosomal dopamine synthesis by veratridine. *Nature (Lond.).* 250:737–739.
24. Morgenroth, V. H., III, L. R. Hegstrand, R. H. Roth, and P. Greengard. 1975. 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.
25. Joh, T. H., D. H. Park, and D. J. Reis. 1978. Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: mechanism of enzyme activation. *Proc. Natl. Acad. Sci. USA.* 75:4744–4748.
26. Vulliet, P. R., T. A. Langan, and N. Weinger. 1980. Tyrosine hydroxylase: a substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 77:92–96.
27. Edelman, A. M., J. D. Raese, M. A. Lazar, and J. D. Barchas. 1981. Tyrosine hydroxylase: studies on the phosphorylation of a purified preparation of the brain enzyme by the cyclic AMP-dependent protein kinase. *J. Pharmacol. Exp. Ther.* 216:647–653.
28. El Mestikawy, S., J. Glowinski, and M. Hamon. 1983. Tyrosine hydroxylase activation in depolarized dopaminergic terminals—involvement of calcium-dependent phosphorylation. *Nature (Lond.).* 302:830–832.
29. Meligeni, J. A., J. W. Haycock, W. F. Bennett, and J. C. Waymire. 1982. Phosphorylation

and activation of tyrosine hydroxylase mediate the cAMP-induced increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.* 257:12632–12640.
30. Haycock, J. W., W. F. Bennett, R. J. George, and J. C. Waymire. 1982. Multiple site phosphorylation of tyrosine hydroxylase. *J. Biol. Chem.* 257:13699–13703.
31. Smith, A. P., S. Varon, and E. M. Shooter. 1968. Multiple forms of the nerve growth factor protein and its subunits. *Biochemistry.* 7:3259–3268.
32. Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA.* 70:765–768.
33. Klee, C. B., and M. H. Krinks. 1978. Purification of 3′:5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to Sepharose. *Biochemistry.* 17:120–126.
34. Steiner, A. L., C. W. Parker, and D. N. Kipnis. 1972. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* 247:1106–1113.
35. Shiman, R., M. Alcino, and S. Kaufman. 1971. Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J. Biol. Chem.* 246:1330–1340.
36. Anderson, L., and N. G. Anderson. 1977. High resolution two-dimensional electrophoresis of human plasma proteins. *Proc. Natl. Acad. Sci. USA.* 74:5421–5425.
37. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
38. Brooker, G., J. F. Harper, W. C. Terasaki, and R. D. Moylan. 1979. Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotide Res.* 1–32.
39. Bradford, M. M. 1976. 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.
40. Spector, T. 1978. Refinement of the Coomassie blue method of protein quantitation. *Anal. Biochem.* 86:142–146.
41. Halegoua, S., and J. Patrick. 1980. Nerve growth factor mediates phosphorylation of specific proteins. *Cell.* 22:571–581.
42. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
43. Green, L. A., and G. Rein. 1977. Release of [^{3}H]norepinephrine from a clonal line of pheochromocytoma cells (PC12) by nicotinic cholinergic stimulation. *Brain Res.* 138:521–528.
44. Toll, L. 1982. Calcium antagonists. *J. Biol. Chem.* 257:13189–13192.
45. Ziskind, L., and M. J. Dennis. 1978. Depolarizing effect of curare on embryonic rat muscle. *Nature (Lond.).* 276:622–623.
46. Takeda, K., and A. Trautman. 1984. A patch-clamp study of the partial agonist actions of tubocurarine on rat myotubes. *J. Physiol. (Lond.).* 349:353–374.
47. Yamauchi, T., H. Nakata, and H. Fujisawa. 1981. A new activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of calcium-calmodulin-dependent protein kinase. *J. Biol. Chem.* 256:5404–5409.
48. Schulman, H. 1984. Differential phosphorylation of MAP-2 stimulated by calcium/calmodulin and cyclic AMP. *Mol. Cell. Biol.* 4:1175–1178.
49. Ahmad, Z., A. A. DePaoli-Roach, and P. J. Roach. 1982. Purification and characterization of a rabbit liver calmodulin-dependent protein kinase able to phosphorylate glycogen synthase. *J. Biol. Chem.* 257:8384–8355.
50. Payne, M. E., C. M. Schworer, and T. R. Soderling. 1983. Purification and characterization of rabbit liver calmodulin-dependent glycogen synthase kinase. *J. Biol. Chem.* 268:2376–2382.
51. Woodgett, J. R., M. T. Davison, and P. Cohen. 1981. The calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle. *Eur. J. Biochem.* 136:481–487.
52. McGuiness, T. L., Y. Lai, P. Greengard, J. R. Woodgett, and P. Cohen. 1983. A multifunctional calmodulin-dependent protein kinase. Similarities between skeletal muscle glycogen synthase kinase and a brain synapsin I kinase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 163:329–334.
53. Vulliet, P. R., J. R. Woodgett, and P. Cohen. 1984. Phosphorylation of tyrosine hydroxylase by calmodulin-dependent multiprotein kinase. *J. Biol. Chem.* 259:13680–13683.
54. Stewart, A. A., T. S. Ingebritsen, A. Manalan, C. B. Klee, and P. Cohen. 1982. Discovery of a calcium- and calmodulin-dependent protein phosphatase, probable identity with calcineurin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 137:80–84.
55. Lee, K., E. Sabban, M. Goldstein, P. J. Seeley, and L. A. Greene. 1982. Phosphorylation of tyrosine hydroxylase in cultured PC12 cells. *Soc. Neurosci. Symp.* 8:886. (Abstr.)
56. Lee, K., E. L. Sabban, M. Goldstein, P. J. Seeley, and L. A. Greene. 1983. Effect of the calmodulin antagonist trifluoperazine (TFP) on phosphorylation of tyrosine hydroxylase (TH). *Fed. Proc.* 42:879. (Abstr.)
57. Yanagihara, N., A. W. Tank, and N. Weiner. 1984. Relationship between activation and phosphorylation of tyrosine hydroxylase by 56 mM K^+ in PC12 cells in culture. *Mol. Pharmacol.* 26:141–147.
58. Raese, J. D., A. M. Edelman, G. Makk, E. A. Bruckwick, W. Lovenberg, and J. D. Barchas. 1979. Brain striatal tyrosine hydroxylase: activation of the enzyme by cyclic AMP-independent phosphorylation. *Commun. Psychopharmacol.* 3:295–301.
59. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693–698.