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Regulation of Tyrosine Hydroxylase Activity in Pheochromocytoma PC-12 Cells by Bradykinin

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SUMMARY

Tyrosine hydroxylase is activated and phosphorylated following treatment of PC-12 cells with bradykinin. In order to determine the mechanisms by which this occurs, we have evaluated the second messenger systems that may be responsible for this activation and phosphorylation. Inositol phosphates appear to play an important role in the activation and phosphorylation of tyrosine hydroxylase because bradykinin treatment significantly increased the formation of [^{3}H]inositol phosphates and the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in PC-12 cells. The uptake of extracellular $^{45}Ca^{2+}$ into PC-12 cells at 1 min was significantly increased (107%) by bradykinin treatment and this increase was blocked by La^{3+}, an inorganic calcium channel inhibitor, but not by nifedipine, an inhibitor of voltage-dependent calcium channels. The activation of tyrosine hydroxylase in PC-12 cells following bradykinin treatment was partially inhibited by La^{3+}. Additivity experiments were performed to evaluate whether the activation and phosphorylation of tyrosine hydroxylase in PC-12 cells following treatment with bradykinin (10 μM) was similar to the activation and phosphorylation of tyrosine hydroxylase in PC-12 cells following treatment with dibutyryl cAMP (2 mM), 4β-phorbol-12β-myristate-13α-acetate (PMA) (2 μM), and high K^+ (56 mM). The combination of bradykinin and PMA produced additive effects, indicating that the activation of tyrosine hydroxylase by treatment with these two compounds was through different mechanisms. Furthermore, exposure of PC-12 cells to bradykinin did not increase intracellular cAMP levels. The combination of bradykinin and PMA treatments produced only partial additivity in tyrosine hydroxylase activity and phosphorylation. No additivity was produced with bradykinin and high K^+ treatment. Phosphopeptide analysis was performed on tyrosine hydroxylase obtained from PC-12 cells treated with bradykinin. Bradykinin treatment produced a significant incorporation of [^{32}P]-phosphate into two phosphopeptides of tryptically digested tyrosine hydroxylase. One of these peptides corresponds to a peptide obtained by trypsinization of purified tyrosine hydroxylase that is phosphorylated by purified calcium/calmodulin-dependent protein kinase. The other ^{32}P-tyrosine hydroxylase-peptide obtained from PC-12 cells treated with bradykinin corresponds to the phosphorylation site obtained during PMA stimulation of PC-12 cells. These results indicate that bradykinin treatment increases intracellular inositol phosphates, calcium, and possibly diacylglycerol levels in PC-12 cells. These effects could then increase calcium/calmodulin-dependent protein kinase activity and possibly calcium/phospholipid-dependent protein (protein kinase C) activity, resulting in increased phosphorylation and activity of tyrosine hydroxylase.

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of the catecholamines. Purified tyrosine hydroxylase has been shown to be phosphorylated and activated by cAMP-dependent protein kinase and calcium/phospholipid-dependent protein kinase (protein kinase C) *in vitro* (1–4). In addition, calcium/calmodulin-dependent protein kinase also phosphorylates tyrosine hydroxylase, but an activator protein is necessary for this phosphorylated form of tyrosine hydroxylase to exhibit increased activity (5, 6). Tyrosine hydroxylase can be activated in a number of systems *in situ*, including bovine adrenal chromaffin cells (7, 8), rat superior cervical ganglion (9, 10), and rat pheochromocytoma cells (11–15). In these tissues, tyrosine hydroxylase is activated following incubation with cAMP analogs or forskolin (an activator of adenylate cyclase), phorbol esters (activators of protein kinase C) or compounds that elevate cytoplasmic calcium concentrations (nicotinic receptor stimulation, K^+-induced depolarization, Ca^{2+} ionophores). In rat pheochromocytoma PC-12 cells, we have reported that the activity and phosphorylation of tyrosine hydroxylase can be increased by depolarization with high K^+ (56 mM) or treatments with calcium ionophores, dibutyryl cAMP, and phorbol esters (13, 14).

Recently, we have reported that tyrosine hydroxylase is activated in rat pheochromocytoma PC-12 cells following bradykinin treatment and that this activation is receptor mediated (16). In the present study we have investigated the mechanisms

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ABBREVIATIONS: HEPES, *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; HPLC, high pressure liquid chromatography; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; PMA, phorbol myristate acetate.

responsible for the activation of tyrosine hydroxylase in PC-12 cells following bradykinin treatment. Bradykinin treatment has been shown to increase phosphatidylinositol turnover and intracellular calcium levels in PC-12 cells (17–19) as well as a number of other cell types (20–29). We anticipated that tyrosine hydroxylase may be activated by bradykinin treatment in PC-12 cells by increasing phosphatidylinositol turnover and intracellular calcium levels in these cells, which would in turn affect the activity of calcium/calmodulin-dependent protein kinase and protein kinase C.

Experimental Procedures

Materials. Tissue culture flasks and dishes were obtained from Falcon Plastics Co. (Los Angeles CA); RPMI 1640 medium was obtained from GIBCO (Grand Island NY). Horse serum was obtained from KC Biologicals, Inc. (Lenexa KS). Fetal bovine serum was obtained from Sterile Systems, Inc. (Logan UT). L-[1-^{14}C]Tyrosine, [^{32}P]-P_i, myo-[2-^{3}H]inositol, and cAMP ^{125}I-radioimmunoassay kit were purchased from New England Nuclear Corp. (Boston MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis MO).

Cell culture. The PC-12 cells were subcultured on 35-mm polylysine-treated dishes at a density of 1×10^6 cells/dish in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, streptomycin (50 μg/ml), and penicillin (50 units/ml). After 3 days in culture, cells were washed three times with oxygenated prewarmed (37°) Krebs-Ringer HEPES buffer (KRH buffer), pH 7.4, containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, and 5.6 mM glucose. The cells were then incubated in KRH buffer (37°) with or without test compounds.

Assay of tyrosine hydroxylase activity. Each experiment was terminated by removing the KRH buffer from the dishes and immediately freezing the cells on dry ice. The cells were scraped into a solution containing 30 mM potassium phosphate buffer (pH 6.8), 50 mM NaF, and 1 mM EDTA. The suspension was homogenized, and the homogenate was centrifuged at 10,000 × *g* for 10 min. Tyrosine hydroxylase activity was determined by a modification of the coupled decarboxylase assay (30). The standard incubation medium consisted of 100 mM potassium phosphate buffer (pH 6.8), 5 mM ascorbic acid, 6500 units of catalase, 5 mM EDTA, 0.2 mM L-2-amino-4-hydroxy-6-methy-5,6,7,8-tetrahydropteridine-HCl (Calbiochem-Behring), 0.1 mM (0.1 μCi) L-[1-^{14}C]tyrosine, and 65 μl of supernatant enzyme (final volume, 100 μl). This mixture was incubated for 10 min at 30° and the reaction was terminated by the addition of 3-iodotyrosine (5 mM final concentration) in potassium phosphate buffer (50 mM final concentration, pH 6.8). The [1-^{14}C]dihydroxyphenylalanine produced in the above reaction was decarboxylated by further incubation of the assay tubes for 30 min at 37° following addition of 20 μl of partially purified hog kidney L-aromatic amino acid decarboxylase and pyridoxal phosphate (0.5 mM final concentration). The reaction was terminated by addition of 0.1 ml of 0.8 N perchloric acid and the tubes were incubated at 37° for 60 min. The $^{14}CO_2$ liberated was collected in plastic wells containing 0.2 ml of NCS tissue solubilizer (Amersham), and the wells were transferred to counting vials. Radioactivity was determined by liquid scintillation counting. Protein content was measured by the method of Bradford (31), with bovine serum albumin as the standard. Tyrosine hydroxylase activity was expressed as nmol of $^{14}CO_2$ formed/min/mg of protein.

Measurement of cyclic AMP levels. For the measurement of cAMP levels, 3-isobutyl-α-methylxanthine (0.5 mM) was added to all tissue culture dishes to prevent the breakdown of cAMP by cyclic nucleotide phosphodiesterase. After incubation with or without test compounds, the medium was removed, and the cells were immediately frozen on dry ice. The cells were scraped from the dish into a 10% TCA solution, and the samples were centrifuged at 20,000 × *g* for 10 min. Cyclic AMP was isolated from the acid supernatant by chromatography on Dowex-50 columns by the method of Su *et al.* (32). The eluate fractions containing cAMP were pooled, and the cAMP was acetylated with triethylamine and acetic anhydride. The cAMP levels were measured by the radioimmunoassay procedure of Harper and Brooker (33). cAMP levels are expressed as pmol/mg of protein.

Measurement of $^{45}Ca^{2+}$ uptake. KRH buffer containing 6 μCi of $^{45}Ca^{2+}$ was added to PC-12 cells for 1, 2, 5, and 10 min. After incubation, the dishes were immediately chilled on ice and washed three times with Ca^{2+}-free KRH buffer containing 1 mM EGTA. The $^{45}Ca^{2+}$ taken up into the cells was extracted with 0.4 N perchloric acid and counted in a liquid scintillation counter. $^{45}Ca^{2+}$ uptake was expressed as nmol/mg of protein.

Measurement of intracellular calcium by fura-2. Intracellular Ca^{2+} levels in PC-12 cells were measured using the fluorescent Ca^{2+} indicator fura-2. PC-12 cells were incubated at 37° for 30 min with KRH buffer containing 2 μM fura-2/acetoxy methyl ester. After the incubation, the cells were rinsed twice with KRH buffer and removed from the plates. Intracellular fura-2 fluorescence was measured in 1-ml samples of continuously stirred cell suspensions (1×10^6 cells) in 1-cm quartz cuvettes maintained at 37°. Measurements were obtained at 340 and 380 nm. Test compounds were added from concentrated stock solutions (100 ×). Intracellular Ca^{2+} concentrations were determined using the equation previously described by Grynkiewicz *et al.* (34).

$$[Ca^{2+}]_i \text{ (nM)} = 225 \text{ nM} \times \frac{(F - F_{min})}{(F_{max} - F)}$$

where F was the measured fluorescence intensity, F_{max} was the fluorescence intensity after lysis and F_{min} was the fluorescence intensity in the presence of EGTA (10 mM final concentration).

Measurement of inositol phosphate formation. PC-12 cells were incubated for 48 to 72 h with 5 μCi/ml [^{3}H]inositol in inositol-free Dulbecco's modified Eagle's serum-free medium, to label phosphatidylinositols. The labeling medium was replaced by KRH buffer containing 10 mM LiCl and preincubated for 10 min at 37°. The cells were then incubated for 5 min in the presence or absence of test compounds. The reaction was stopped by addition of ice-cold TCA (10%, v/v, final concentration). The plates were scraped and the samples were centrifuged in a Beckman microfuge for 10 min. The TCA was extracted from each sample by five washes with 5 ml of diethyl ether saturated with distilled water per wash. After the extraction of TCA, the solution was neutralized to pH 7 and subjected to anion-exchange chromatography as described previously (35, 36).

Measurement of phosphorylation of tyrosine hydroxylase. PC-12 cells were incubated with [^{32}P]phosphate (carrier-free; 1.5 mCi/ml) for 60 min at 37° in KRH buffer. The cells were washed with prewarmed KRH buffer (37°) and incubated with or without test compounds for 5 min. Following this 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 60 μl of antiserum specific for tyrosine hydroxylase in a solution containing 5 mM NaF, 10 mM EDTA, 50 mM sodium pyrophosphate, and 50 mM potassium phosphate buffer (pH 7.6) (final volume of 300 μl). This mixture was allowed to stand at room temperature for 30 min and was then incubated at 4° overnight. The mixture was layered over a discontinuous sucrose gradient by a modification of the method of Taylor and Schimke (37). 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 a solution of 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 repeated once. The final pellet was dissolved in 15 μl of 500 mM dithiothreitol and 45 μl of a solution containing 3% SDS, 5% glycerol, 0.2% bromophenol blue, and 10 mM

Tris·HCl buffer (pH 7.8). The solution was placed in boiling water 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 analyzed by measuring 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. Following electrophoresis of tyrosine hydroxylase, the gel band corresponding to [^{32}P]-tyrosine hydroxylase (≈ 56,000) was cut out and the radiolabeled band was extracted with 0.05 M NH_4HCO_3 and 0.25% SDS at 37° for 36 hr. The extraction medium was changed three times during this period (38). The extracted [^{32}P]tyrosine hydroxylase was filtered through glass wool and was mixed with 0.25 mg of calf thymus histone (type IIA) in TCA (25% v/v, final concentration). 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 HCl-acetone mixture (99.5% acetone) and centrifuged at 4000 × *g* for 10 min at room temperature. After the supernatant was removed, the pellet was suspended in 2 ml of acetone and centrifuged at 4000 × *g* for 10 min. The pellet was dissolved in 50 μl of 10 mM dithiothreitol and incubated at 65° for 15 min. The sample was incubated with 10 μl of 1 M ammonium bicarbonate and 25 μg of trypsin in 5 μl for 12 to 18 hr.

After digestion of the phosphorylated tyrosine hydroxylase by trypsin, the ^{32}P-labeled peptides were separated by HPLC. The peptides were applied to a Beckman HPLC system equipped with a Hibar II column packed with LiChrosorb RP-18 (pore size, 10 μm; 250 × 4.6 mm). Mobile phase consisted of an *n*-propanol gradient (0–20%) in 0.5% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min and 1-ml fractions were collected and measured by Cerenkov counting in a Beckman scintillation counter.

Results

In a previous paper (16), we reported that tyrosine hydroxylase is rapidly activated in PC-12 cells by bradykinin (10^{-6} to 10^{-8} M) and this activation was receptor mediated. In an attempt to establish the intracellular mechanisms responsible for the activation of tyrosine hydroxylase by bradykinin treatment, we have evaluated the effects of bradykinin treatment on cAMP levels, phosphoinositide turnover, and Ca^{2+} levels in the PC-12 cells.

Fig. 1 illustrates the effects of bradykinin, forskolin, and high K^+ on cAMP levels and tyrosine hydroxylase activity in PC-12 cells. Forskolin, an activator of adenylate cyclase, dose-dependently increased cAMP levels in these cells. Bradykinin and high K^+, however, had no effect on cAMP levels. In contrast, all three treatments significantly activated tyrosine hydroxylase activity. These data indicate that the activation of tyrosine hydroxylase following bradykinin treatment is independent of cAMP-dependent mechanisms.

Next, a series of experiments were performed to evaluate the effects of bradykinin on intracellular calcium levels. Fig. 2 illustrates the effect of bradykinin on $^{45}Ca^{2+}$ uptake into PC-12 cells. Depolarization of PC-12 cells with high K^+ significantly increased $^{45}Ca^{2+}$ uptake into PC-12 cells by approximately 553%. Bradykinin treatment also significantly increased $^{45}Ca^{2+}$ accumulation in PC-12 cells within 1 min by approximately 107%, but this effect was negligible by 5 min. We evaluated the effects of nifedipine, a voltage-dependent calcium channel inhibitor, and La^{3+}, an inorganic calcium inhibitor, on bradykinin-stimulated $^{45}Ca^{2+}$ uptake and on tyrosine hydroxylase activity (Table 1). Nifedipine (10 μM) completely inhibited the effects of high K^+ (56 mM) on $^{45}Ca^{2+}$ accumulation and tyrosine hydroxylase activity. However, nifedipine had no effect on bradykinin (1 μM)-stimulated $^{45}Ca^{2+}$ accumulation or tyrosine hydroxylase activation, indicating that the increase in $^{45}Ca^{2+}$ accumulation produced by bradykinin in PC-12 cells is not through the voltage-dependent calcium channel. La^{3+} (1 mM) completely inhibited the accumulation of $^{45}Ca^{2+}$ produced by bradykinin and high K^+. In contrast, La^{3+} (1 mM) completely blocked the activation of tyrosine hydroxylase by high K^+ but only partially inhibited the activation of tyrosine hydroxylase by bradykinin (25%).

It has been reported that bradykinin treatment produces a breakdown of PIP_2 in PC-12 cells (17, 18). The breakdown

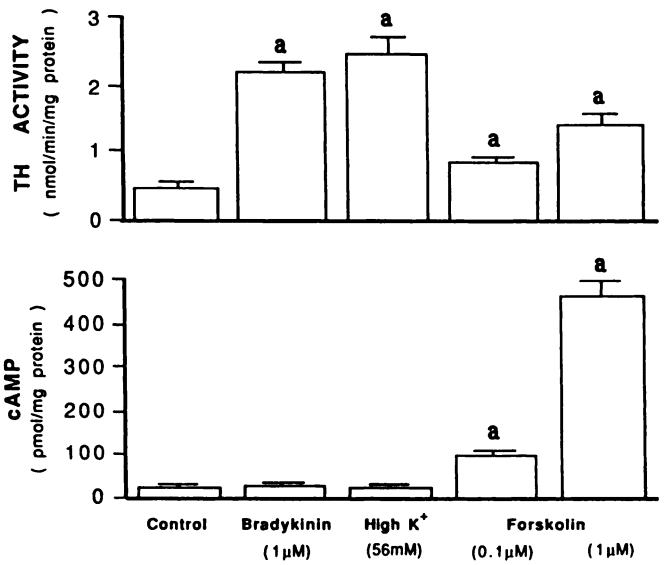

Fig. 1. Effects of bradykinin, high K^+, and forskolin on cyclic AMP levels and tyrosine hydroxylase activity in PC-12 cells. PC-12 cells were preincubated for 10 min with 0.5 mM isobutylmethylxanthine and exposed to various compounds for 10 min in the presence of 0.5 mM isobutylmethylxanthine. cAMP levels were determined by radioimmunoassay and tyrosine hydroxylase activity (*TH*) was determined by the [^{14}C] decarboxylase assay, as described in Experimental Procedures. Data are means ± standard errors from five to seven experiments. $^a p < 0.01$, significantly greater than control.

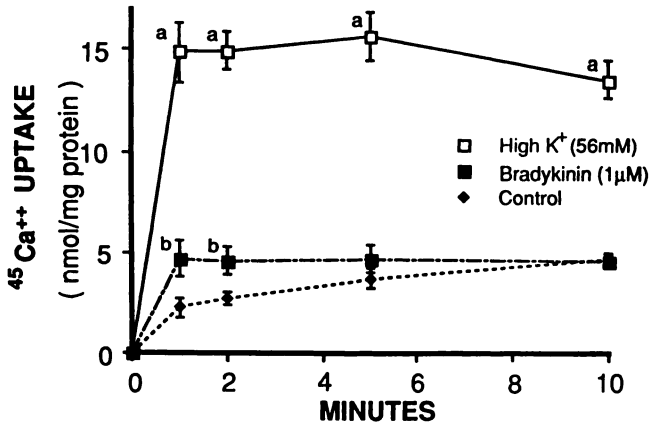

Fig. 2. Effects of bradykinin and high K^+ on $^{45}Ca^{2+}$ accumulation in PC-12 cells. PC-12 cells were incubated for 1, 2, 5, or 10 min with high K^+ (56 mM) or bradykinin (1 μM). $^{45}Ca^{2+}$ uptake into the PC-12 cells was determined as described in Experimental Procedures. Data are means ± standard errors from seven to nine experiments. $^a p < 0.01$; $^b p < 0.05$, significantly greater than control.

TABLE 1

Calcium uptake and tyrosine hydroxylase activity in PC-12 cells following treatment with bradykinin and high K^+: effects of nifedipine and La^{3+}

PC-12 cells were preincubated for 10 min with or without nifedipine or La^{3+} and then exposed to bradykinin or high K^+ for 1 min (calcium studies) or 5 and 15 min (tyrosine hydroxylase studies) in the presence or absence of nifedipine or La^{3+}. $^{45}Ca^{2+}$ was added to the incubation buffer for 1 min. $^{45}Ca^{2+}$ uptake and tyrosine hydroxylase activity were determined as described in Experimental Procedures. Data are means ± standard errors from five to nine experiments.

	$^{45}Ca^{2+}$ uptake		
	Control	Bradykinin (1 μM)	High K^+ (56 mM)
	nmol/mg of protein		
None	2.24 ± 0.50	4.70 ± 0.96	14.93 ± 1.45
Nifedipine (10 μM)	2.02 ± 0.35	4.62 ± 0.78	3.22 ± 0.48[a]
La^{3+} (1 mM)	2.22 ± 0.39	2.11 ± 0.26[b]	3.56 ± 0.36[a]
	Tyrosine hydroxylase activity		
	Control	Bradykinin (1 μM)	High K^+ (56 mM)
	nmol/min/mg of protein		
None	0.49 ± 0.07	2.17 ± 0.12	2.42 ± 0.26
Nifedipine (2 μM)	0.44 ± 0.05	2.01 ± 0.20	0.59 ± 0.08[a]
La^{3+} (1 mM)	0.51 ± 0.08	1.66 ± 0.18[b]	0.62 ± 0.08[a]

[a] $p < 0.01$, significantly lower than high K^+-stimulated $^{45}Ca^{2+}$ accumulation or tyrosine hydroxylase activity.

[b] $p < 0.05$, significantly lower than bradykinin-stimulated $^{45}Ca^{2+}$ accumulation or tyrosine hydroxylase activity.

products of PIP_2 (IP_3 and diacylglycerol) could potentially regulate tyrosine hydroxylase activity by an effect on intracellular Ca^{2+} and/or protein kinase C. Fig. 3 illustrates the effects of bradykinin treatment on inositol phosphate formation and intracellular Ca^{2+} levels in PC-12 cells. Treatment of PC-12 cells with bradykinin for 5 min increased the accumulation of total [^{3}H]inositol phosphates by 350% (Fig. 3A). This increase was not inhibited by nifedipine but was slightly inhibited by La^{3+}. The concentration of intracellular free Ca^{2+} was measured using fura-2 (Fig. 3B). Bradykinin treatment increased the intracellular free Ca^{2+} concentration by 550% and this increase was not affected by nifedipine.

Fig. 4 compares the activation and phosphorylation of tyrosine hydroxylase produced by bradykinin treatment with the activation and phosphorylation of tyrosine hydroxylase produced by treatment with dibutyryl cAMP, PMA, and high K^+. Concentrations of these compounds were chosen to produce maximal activation and phosphorylation of tyrosine hydroxylase and the additivity of this activation and phosphorylation with that produced by bradykinin treatment was evaluated. The increase in tyrosine hydroxylase activity produced by bradykinin treatment (356%) and dibutyryl cAMP treatment (181%) were additive (544%). Likewise, the enhanced phosphorylation of tyrosine hydroxylase produced by bradykinin treatment (114%) and dibutyryl cAMP treatment (44%) were additive (153%). PMA treatment increased by 19% the activation of tyrosine hydroxylase produced by bradykinin treatment. Likewise, the phosphorylation of tyrosine hydroxylase was increased to a similar degree. High K^+ treatment had no effect on the increase in tyrosine hydroxylase activity or phosphorylation produced by bradykinin treatment. These data suggest that the mechanism of activation and phosphorylation of tyrosine hydroxylase produced by bradykinin treatment is similar to that produced by high K^+ treatment and to a minor extent by PMA treatment.

In order to evaluate this further, the sites on tyrosine hydroxylase that were phosphorylated by bradykinin treatment were determined. PC-12 cells were incubated with $^{32}P_i$ for 1 hr to label the cellular ATP stores. After exposure of the cells to bradykinin, tyrosine hydroxylase was isolated, subjected to SDS-polyacrylamide gel electrophoresis, eluted from the gels, and digested with trypsin for 12 to 18 hr at 37°. The ^{32}P-labeled phosphopeptides derived from tyrosine hydroxylase were separated by HPLC, as described in Experimental Procedures. Fig. 5 illustrates the HPLC elution pattern of three ^{32}P-labeled

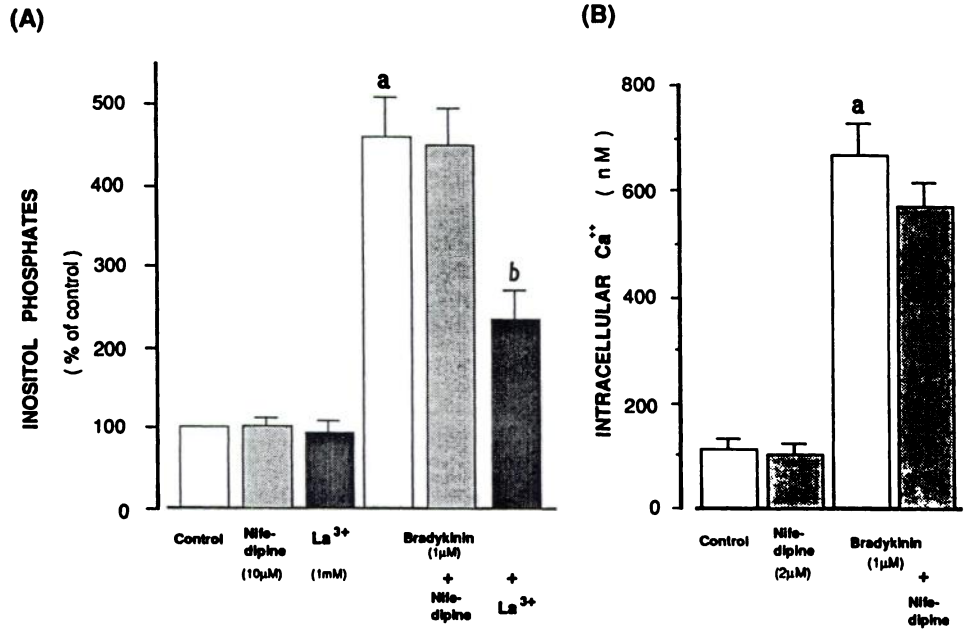

Fig. 3. Effect of bradykinin on [^{3}H]inositol phosphate formation and intracellular free Ca^{2+} concentration in PC-12 cells. A, PC-12 cells were incubated for 2–3 days with 5 μCi/ml [^{3}H]inositol in inositol-free medium in the absence of serum. Cells were incubated in the presence or absence of bradykinin (1 μM) with or without the Ca^{2+} uptake blockers nifedipine or La^{3+}. The accumulation of [^{3}H]inositol phosphates was assayed as described in Experimental Procedures. The control level of radioactivity accumulation was 1356 ± 10 cpm/10^4 cells. Data are means ± standard errors from five to seven experiments. [a] $p < 0.01$, significantly different from control; [b] $p < 0.01$, significantly different from bradykinin-induced inositol phosphate formation. B, PC-12 cells were incubated for 30 min with 2 μM fura-2/acetoxy methyl ester. Concentrations of intracellular free Ca^{2+} were calculated by measuring the peak fura-2 fluorescence, 10–15 sec after the addition of bradykinin. Nifedipine pretreatment was for 10 min. Intracellular free Ca^{2+} was determined as described in Experimental Procedures. Data are means ± standard errors from five to seven experiments. [a] $p < 0.01$, significantly different from control.

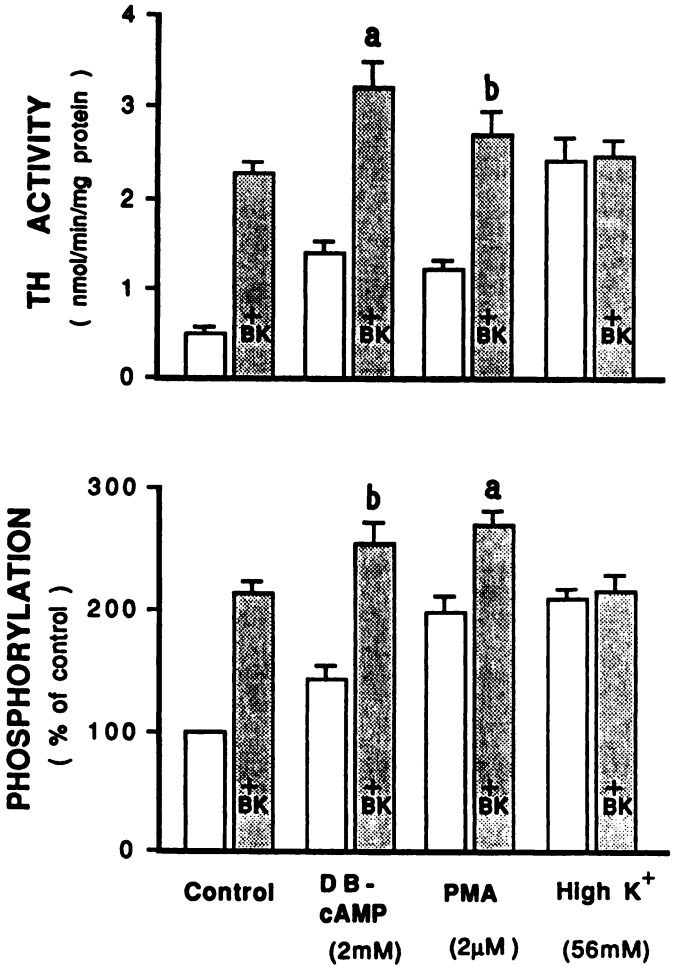

Fig. 4. Effects of bradykinin on dibutyryl cAMP-, PMA-, and high K^+-stimulated activation and phosphorylation of tyrosine hydroxylase in PC-12 cells. The PC-12 cells were incubated for 15 min with concentrations of dibutyryl cAMP (2 mM), PMA (2 μM), or high K^+ (56 mM). These concentrations had previously been shown to produce a maximal activation and phosphorylation of tyrosine hydroxylase. The PC-12 cells were incubated with bradykinin (10 μM) for 5 min. The activity and phosphorylation of tyrosine hydroxylase (*TH*) was determined as described in Experimental Procedures. Dibutyryl cAMP (*DB-cAMP*), PMA, high K^+, and bradykinin (*BK*) all significantly increased tyrosine hydroxylase activity and phosphorylation over control levels. Data are means ± standard errors from five to seven experiments. $^a p < 0.01$; $^b p < 0.05$, significantly greater than bradykinin-stimulated tyrosine hydroxylase activity and tyrosine hydroxylase phosphorylation.

peptide peaks that were produced by bradykinin treatment, peptide peak A (retention time, 16 min), peak B (19 min), and peak C (27 min). The phosphorylation of peaks A and B was increased markedly when PC-12 cells were treated with bradykinin, whereas peak C was unaffected. Previously, we have reported in PC-12 cells that peak B is phosphorylated by activators of protein kinase C (PMA or 1-oleyl-2-acetylglycerol), peak C is phosphorylated by activators of cAMP-dependent protein kinase (dibutyryl cAMP or forskolin), and peak A is phosphorylated by compounds that increase the intracellular Ca^{2+} concentration and possibly activate calcium/calmodulin-dependent protein kinase (high K^+, ionomycin) (12–14) Because bradykinin selectively stimulated the phosphorylation of peaks A and B, the possibility exists that bradykinin may activate tyrosine hydroxylase by an action on both

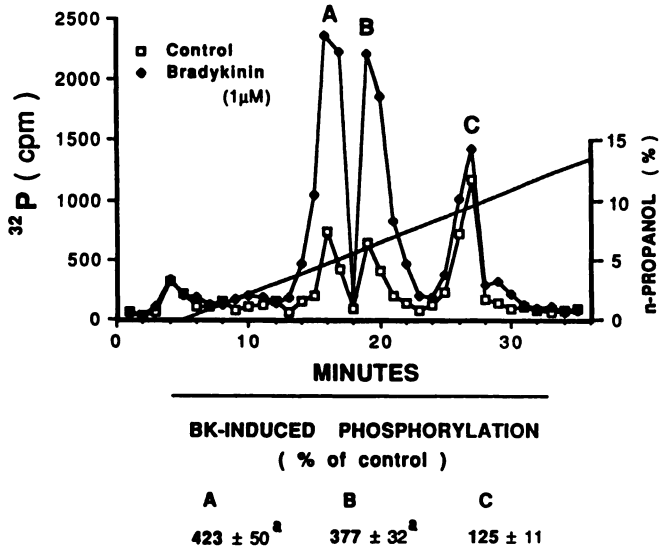

Fig. 5. HPLC analysis of tryptic phosphopeptides isolated from phosphorylated tyrosine hydroxylase obtained from PC-12 cells after treatment with bradykinin. ^{32}P-labeled tryptic peptides from tyrosine hydroxylase were separated by HPLC as described in Experimental Procedures. *Upper*, graph of the phosphopeptide chromatography from a representative sample of five experiments. *Lower*, data represent the numerical expression of the above graph showing the percentage increase in [^{32}P] phosphate incorporated into peaks A, B, and C of tyrosine hydroxylase obtained from PC-12 cells treated with bradykinin. Data are means ± standard errors from five experiments. $^a p < 0.01$, significantly greater than control.

calcium/calmodulin-dependent protein kinase and protein kinase C.

Discussion

In the present paper we have evaluated the intracellular mechanisms responsible for the activation of tyrosine hydroxylase following treatment of rat pheochromocytoma PC-12 cells with bradykinin. We have previously reported that bradykinin treatment rapidly activates tyrosine hydroxylase in PC-12 cells (16). This effect of bradykinin is receptor mediated and is due to an increase in the affinity of tyrosine hydroxylase for its pterin cofactor. As illustrated in Fig. 1, bradykinin treatment had no effect on cAMP levels in PC-12 cells, whereas forskolin treatment produced a profound increase in cAMP levels and tyrosine hydroxylase activity. This implies that the activation of tyrosine hydroxylase by bradykinin treatment is independent of cAMP-dependent mechanisms. It has been reported that treatment of the murine neuroblastoma clone NIE-115 with bradykinin increases cyclic GMP levels (39). Although we did not measure cyclic GMP levels in PC-12 cells following bradykinin treatment, we found that treatment of PC-12 cells with compounds that activate cyclic GMP-dependent protein kinase, dibutyryl cGMP (2 mM) or nitroprusside (1 mM), had no effect on tyrosine hydroxylase activity in these cells (data not shown). This implies that bradykinin is not working through a cyclic GMP-dependent mechanism in the PC-12 cells to activate tyrosine hydroxylase. However, this is in contrast to the data of Roskoski and Roskoski (15), who reported an increase in tyrosine hydroxylase activity in PC-12 cells following treatment with dibutyryl cGMP.

Bradykinin treatment increased $^{45}Ca^{2+}$ uptake into PC-12 cells by approximately 15% of that which occurs following

treatment with 56 mM K^+ (Fig. 2). The increase in Ca^{2+} uptake produced by bradykinin was not inhibited by nifedipine but was completely inhibited by La^{3+} (Table 1). In contrast, the activation of tyrosine hydroxylase produced by bradykinin was inhibited only 25% by La^{3+}.

These data suggest that the uptake of extracellular Ca^{2+} into PC-12 cells following bradykinin treatment may account for approximately 25% of the activation of tyrosine hydroxylase observed. Another interpretation of these data is that the slight but significant increase in external $^{45}Ca^{2+}$ uptake into the PC-12 cells occurs at the time when internal calcium is also increasing; therefore, the increase in external calcium uptake may be due to an exchange of external calcium with internal calcium. This exchange could also be blocked by high concentrations of La^{3+} and would suggest that the activation of tyrosine hydroxylase produced by bradykinin treatment is not dependent upon external Ca^{2+} but could occur entirely through an enhanced breakdown of phosphatidylinositols into diacylglycerol and inositol phosphates in the PC-12 cells. Diacylglycerol is produced concurrently with production of IP_3 from the breakdown of PIP_2 by phospholipase C (40). In addition, diacylglycerol can also be produced from the breakdown of either phosphatidylinositol monophosphate or phosphatidylinositol. Diacylglycerol is thought to activate protein kinase C by increasing the affinity of the enzyme for calcium (40, 41). IP_3 is thought to act by increasing the release of Ca^{2+} intracellularly from endoplasmic reticulum and mitochondria (42). This increase in intracellular Ca^{2+} may then activate calcium/calmodulin-dependent protein kinase. We found that bradykinin treatment produced an increase in inositol phosphate formation and intracellular calcium concentrations, suggesting that these second messengers may be responsible for the activation of tyrosine hydroxylase.

Similar results have been reported in other laboratories. Volonte *et al.* (19) reported that PC-12 cells contain bradykinin receptor binding sites (0.15 pmol/mg of protein). Stimulation of these sites with 1 μM bradykinin produced a 9-fold increase in inositol monophosphate formation, a 2.5-fold increase in inositol bisphosphate formation, and a negligible effect on IP_3 formation. In our study, total inositol phosphates were measured and the increase in PC-12 cells after bradykinin treatment was approximately 4.5-fold (600 nM) (Fig. 3B).

We have reported that tyrosine hydroxylase is activated and phosphorylated following treatment of PC-12 cells with dibutyryl cAMP, high K^+, PMA, or bradykinin (11–14) (Fig. 4). To evaluate whether these treatments act through a similar mechanism, additivity experiments were performed. Bradykinin treatment failed to increase the activation and phosphorylation of tyrosine hydroxylase produced by high K^+ treatment, suggesting that these treatments might be acting through the same mechanisms (Fig. 4). In contrast, treatment of PC-12 cells with bradykinin and dibutyryl cAMP has an additive effect on tyrosine hydroxylase activation and phosphorylation, which implies that these effects of bradykinin are not mediated by cAMP-dependent mechanisms. The activation and phosphorylation of tyrosine hydroxylase following bradykinin and PMA treatment of PC-12 cells were less than additive, suggesting that protein kinase C may play a partial role in the action of bradykinin.

The effects of bradykinin treatment on the phosphorylation of tyrosine hydroxylase in the PC-12 cells were further investigated by tryptic phosphopeptide analysis (Fig. 5). In previous studies in our laboratory, we reported an increased phosphorylation of at least three distinct phosphopeptides (A, B, and C) derived from tyrosine hydroxylase isolated from PC-12 cells treated with 56 mM K^+, cAMP analogs, or calcium ionophores (13, 14). Bradykinin treatment increased [^{32}P]phosphate incorporation mainly into the phosphopeptides A and B (Fig. 5). Previous work in our laboratory indicates that ^{32}P incorporation into peptide C is stimulated by compounds that activate cAMP-dependent protein kinase, such as dibutyryl cAMP or forskolin. ^{32}P incorporation into peptide B is stimulated in PC-12 cells by compounds that activate protein kinase C, such as PMA or 1-oleyl-2-acetylglycerol (14). In contrast, stimulation of PC-12 cells with high K^+ produces an increase in [^{32}P]-phosphate incorporation into peptides A and B, similar to the [^{32}P]phosphate incorporation produced by bradykinin treatment. The activation and phosphorylation of tyrosine hydroxylase by high K^+ depolarization requires extracellular Ca^{2+} and is thought to be mediated by calcium/calmodulin-dependent protein kinase and to a minor extent by protein kinase C. Our data suggest that bradykinin treatment of PC-12 cells produces an activation and phosphorylation of tyrosine hydroxylase in these cells through a combination of two possible mechanisms; 1) an increase in phosphatidylinositol turnover could lead to increased IP_3 formation and increased intracellular Ca^{2+}, which also would activate calcium/calmodulin-dependent protein kinase and 2) the concomitant increase in diacylglycerol produced by PIP_2 breakdown could lead to an increase in the activity of protein kinase C.

Bradykinin is localized in neurons in the central nervous system and may act as a neurotransmitter or neuromodulator in the brain (43). In the peripheral nervous system, bradykinin has been shown to stimulate the release of catecholamines from the adrenal medulla and the superior cervical ganglia (44, 45). The physiological significance of this is not certain, because bradykinin has not been shown to be localized in neurons of the peripheral nervous system. However, the possibility exists that under certain physiological conditions bradykinin could be produced in high concentrations in the blood or locally near adrenal medullary tissue and could regulate the release and synthesis of catecholamines in these neurons (46).

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