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Regulation of Tyrosine Hydroxylase Activity in Rat PC12 Cells by Neuropeptides of the Secretin Family

ROBERT ROSKOSKI, JR., LISA WHITE, ROBERT KNOWLTON, and LAURA M. ROSKOSKI
Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana
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SUMMARY

Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is subject to regulation by the cAMP as well as the calcium and cGMP second messenger systems. Treatment of intact rat PC12 cells with neuropeptides including secretin and vasoactive intestinal polypeptide (VIP) stimulated tyrosine hydroxylase activity 2 to 3-fold *in vitro*. Secretin (EC_{50} = 10 nM) was about 3 orders of magnitude more potent than VIP (EC_{50} = 3 μM). A combination of several protease inhibitors failed to enhance the potency of either peptide. Other members of the secretin family including glucagon and peptide histidine isoleucine (PHI) stimulated tyrosine hydroxylase activity to a lesser extent. Somatostatin, which is not homologous to secretin, was ineffective. The maximal response of tyrosine hydroxylase activation to 1 μM secretin occurred within 6–15 sec. Secretin, VIP, and forskolin also enhanced tyrosine hydroxylase activity (3,4-dihydroxyphenylalanine production) in intact cells, as determined by high performance liquid chromatography and electrochemical detection. Secretin, VIP, PHI, and glucagon increased the levels of cAMP in PC12 cells more than 10-fold, as determined by radioimmunoassay. We also demonstrated that cAMP is released from the cells into the incubation medium following secretin treatment. Secretin and VIP treatment also enhanced the activity of cAMP-dependent protein kinase in a concentration-dependent fashion, as measured subsequently *in vitro*. Based on the greater potency of secretin in comparison with VIP, PHI, and glucagon, we suggest that the PC12 cells contain a secretin-preferring receptor that increases cAMP levels and brings about an activation of tyrosine hydroxylase activity through the stimulation of cAMP-dependent protein kinase.

Tyrosine hydroxylase (EC 1.14.16.2) catalyzes the first and rate-limiting step in the biosynthesis of catecholamines (1). This enzyme is a substrate for cAMP-dependent protein kinase (2, 3) and phosphoprotein phosphatase (4). The cAMP-dependent phosphorylation of tyrosine hydroxylase decreases the K_m for its reducing co-substrate and increases the K_i for end-product inhibitors such as dopamine, as determined by steady state kinetic analysis at pH 6 *in vitro* (5–7). The cAMP second messenger system is activated by a large number of hormones, neurotransmitters, and neuropeptides (cf. Ref. 8).

The rat central nervous system contains receptors for secretin and for VIP (9). These receptors, moreover, are coupled to and stimulate adenylate cyclase activity. The secretin-preferring receptor (a) binds secretin with high affinity and VIP and PHI with lower affinity and (b) is antagonized by secretin (5–27). The brain and pancreas contain a VIP-preferring receptor that binds VIP with high, PHI with intermediate, and secretin with low affinity. Intracerebral ventricular injection of secretin in rats, moreover, increases hypothalamic tyrosine hydroxylase activity (10). Furthermore, Tischler *et al.* (11) showed that VIP increases tyrosine hydroxylase activity in PC12 cells. Ip and co-workers (12) reported that neuropeptides of the secretin family increase the cAMP content and activate tyrosine hydroxylase activity in rat superior cervical ganglia.

The PC12 cell line is active in catecholamine biosynthesis and has provided a convenient system for studying the regulation and phosphorylation of tyrosine hydroxylase (11, 13, 14). The present experiments indicate that PC12 cells responded to neuropeptides of the secretin family and that this response involved the generation of cAMP and activation of its protein kinase. One consequence of this cascade is that tyrosine hydroxylase activity measured *in vitro* and *in situ* is increased.

Materials and Methods

Measurement of tyrosine hydroxylase *in vitro*. Stock cultures of PC12 cells (passages between 50 and 70) were grown as described by Baizer and Weiner (15). Cells were harvested by trituration and incubated at 30° in HEPES-Ringer solution containing the following components, as described by Greene and Rein (16): 125 mM NaCl, 4.8 mM

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ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; BH_4, tetrahydrobiopterin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; HPLC, high performance liquid chromatography; 6-MPH_4, 6-methyltetrahydropterin; PHI, peptide histidine isoleucine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DOPA, 3,4-dihydroxyphenylalanine.

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KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5.6 mM glucose, and 25 mM HEPES, all adjusted to pH 7.4 with NaOH. The medium also contained 100 μM theophylline as an adenosine receptor antagonist, as previously described (17). To measure tyrosine hydroxylase *in vitro* following specific treatments *in situ*, aliquots of PC12 cell suspensions (about 2 mg of protein/ml) were dispensed following gentle vortexing into 1.5-ml centrifuge tubes. Specific additions were made (2-μl portions) to give the final specified concentration and the incubations were continued for 20 min at 30° unless otherwise noted. Then, 1 ml of ice-cold HEPES-Ringer solution was added and the cells were harvested by centrifugation (13,000 × *g*, 30 sec). The supernatants were aspirated, and cells were either stored at −70° (for up to 2 months) or taken for immediate tyrosine hydroxylase activity measurements. After 100 μl of a chilled solution of 30 mM potassium phosphate, 50 mM NaF, 0.25 mM EDTA, all at pH 7.2, was added, the cells were disrupted by sonication for 10 sec with a Kontes Micro-Ultrasonic cell disrupter. Portions (10 μl) were taken for tyrosine hydroxylase activity determination by a coupled decarboxylase procedure, as previously described (6), unless noted otherwise. Incubations were performed with 125 μM 6-MPH_4 (unless noted otherwise) at pH 7.2, with 25 mM 3-(*N*-morpholino)propanesulfonic acid as buffer, at 30° for 15 min. A 5 mM stock solution of 6-MPH_4 was prepared in ice-cold 5 mM HCl; the concentration was determined spectrophotometrically in 100 mM HCl (ambient temperature) at 265 nm, using an extinction coefficient of 14.7 mM^{-1}. BH_4 was prepared in a similar fashion, using a wavelength of 263 nm and an extinction coefficient of 15.9 mM^{-1}. Protein was measured by the procedure of Bradford (18) using bovine γ-globulin as standard.

Measurement of cAMP. PC12 cells (200-μl portions) were treated as described above for measurement of tyrosine hydroxylase *in vitro*. At the end of the incubation, the tubes were placed in a boiling water bath for 5 min and then stored at −70° until assay. After thawing, the cells were subjected to brief (10-sec) sonication. Protein was measured by the Bradford procedure (18) and cAMP or cGMP was measured in the 13,000 × *g* supernatant (5 min) by radioimmunoassay (19). In one group of experiments, as specified later, the cells were centrifuged for 30 sec at the end of the incubation. The resolved supernatants and cell pellets were placed in a boiling water bath, and cAMP was subsequently measured in each fraction.

Measurement of tyrosine. PC12 cells were harvested and collected by centrifugation at 3000 × *g* for 2 min. To remove the culture medium containing tyrosine, the cells were resuspended in HEPES-Ringer solution (37°) by gentle vortexing and recentrifuged. This was repeated once. Triplicates were taken for each tyrosine determination, which was by the fluorescence procedure of Waalkes and Udenfriend (20), and for each protein determination, as described by Bradford (18).

Determination of the cAMP-dependent protein kinase activity ratio. PC12 cells (38-μl portions) were treated as described above for measurement of tyrosine hydroxylase activity *in vitro*. At the end of the incubation, the cells were centrifuged at 13,000 × *g* for 30 sec, at ambient temperature. The supernatants were aspirated and 0.15 ml of ice-cold buffer was added, which contained 10 mM potassium phosphate, 5 mM disodium EDTA, 0.1 mM isomethylbutylxanthine, and 0.4 M NaCl, all at pH 7.0 (21). The cells were disrupted by brief (10-sec) sonication and 25-μl portions were taken for duplicate protein kinase activity determinations (5 min, 30°) without and with 10 μM cAMP, as previously described (22). Ser-peptide (LeuArgArgAlaSerLeuGly) (100 μM) was used as acceptor. Results are expressed as the activity ratio (−cAMP/+10 μM cAMP). Assays were performed immediately after sonication. Experiments indicated, however, that the activity ratios remained essentially constant when the sonicated samples were allowed to stand on ice for 90 min.

Materials. Forskolin was obtained from Calbiochem (La Jolla, CA). A 10 mM stock solution was stored at 0° in 95% ethanol for up to 6 months without loss of activity. Secretin, secretin(5–27), VIP, and Ser-peptide were purchased from Bachem (Torrance, CA). Brocresine was a gift from Lederle Laboratories (Pearl River, NY). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin and (6*R,S*)-6-methyl-5,6,7,8-tetrahydropterin were purchased from Dr. B. Schirks Laboratories (Jona, Switzerland). Bovine γ-globulin was purchased from Bio-Rad Laboratories (Richmond, CA). Lanthanum chloride was obtained from Fischer Scientific Co. (Fairlawn, NJ). All other chemicals, peptides, and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Peptides were stored as 1 mM stock solutions in water at −20° for periods up to 1 month without loss of potency. Dilutions were made into Ringer's solution before addition of 2-μl portions to PC12 cell suspensions. A 20 mM theophylline stock solution in water was stable at ambient temperature for more than 1 year. The cAMP and cGMP radioimmunoassay kits were purchased from New England Nuclear Corp (Boston, MA). [^{14}C]Tyrosine, uniformly labeled, was purchased from ICN (Irvine, CA) and L-[3,5-^{3}H]tyrosine was purchased from NEN Research Products (Boston, MA). The PC12 cells were kindly provided by Dr. James Byrd (National Institute of Mental Health, Washington, DC) and the PC18 cells by Dr. A. William Tank (University of Rochester, Rochester, NY).

Results

Effects of theophylline on cAMP levels and tyrosine hydroxylase activity. We used 100 μM theophylline to decrease basal tyrosine hydroxylase activity in PC12 cells (17). Although this agent can function as an adenosine receptor antagonist and as a cyclic nucleotide phosphodiesterase inhibitor (23), we found that 100 μM theophylline decreased both cAMP content and tyrosine hydroxylase activity about 66 and 33%, respectively (Table 1). Even 2.5 mM theophylline treatment for 20 min failed to increase cAMP levels above control values. Concentration-dependence studies on the effect of theophylline indicated that the optimum concentrations for decreasing tyrosine hydroxylase activity extend from 50 to 200 μM (not shown), and we routinely employed 100 μM theophylline in all subsequent studies reported in this paper. These results indicate that blockade of the adenosine receptor from endogenously generated ligands was the predominant action of 100 μM theophylline in PC12 cells.

Effects of various peptides on tyrosine hydroxylase activity and cAMP content in PC12 cells in vitro. Following the lead of Ip and co-workers (12), who examined rat superior cervical ganglion, we tested the possible effects of pretreatment of PC12 cells with neuropeptides of the secretin family on tyrosine hydroxylase activity measured *in vitro*. We found that secretin (10 μM) and VIP (10 μM) produced a 2.2-fold increase in tyrosine hydroxylase activity (Table 2). Moreover, PHI produced a 1.9-fold increase in activity. In contrast to the results of Ip *et al.* (12) using rat superior cervical ganglia, we found that glucagon also increased (60–80%) tyrosine hydroxylase activity in PC12 cells. Somatostatin (10 μM), not a

TABLE 1

Theophylline decreases tyrosine hydroxylase activity and cAMP levels in PC12 cells

Cells were harvested and incubated in Ringer's solution for 20 min at 30° with the specified concentration of theophylline. Tyrosine hydroxylase activity with 6-MPH_4 as cofactor and total cAMP levels (cells and medium) were measured as described in Methods and Materials. The data represent the mean ± standard error of triplicate determinations. Similar results were obtained in three other experiments.

Addition	Tyrosine hydroxylase activity	cAMP
	pmol/min/mg	*pmol/mg*
None	147 ± 12	32.6 ± 2.1
Theophylline, 100 μM	80.9 ± 9[a]	23.9 ± 1.8[a]
Theophylline, 2.5 mM	153 ± 11	36.2 ± 2.4

[a] Significantly different from control, $p < 0.01$.

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TABLE 2

Effects of several neuropeptides on tyrosine hydroxylase activity and cAMP content in PC12 cells

Cells were harvested and incubated in Ringer's solution containing 100 μM theophylline for 20 min at 30°, with 10 μM of the specified compound. Tyrosine hydroxylase activity or total cAMP was measured as described in Materials and Methods. The data represent the mean ± standard error of triplicate determinations. Similar results were obtained in four to six other experiments.

Peptide	Tyrosine hydroxylase activity	cAMP
	pmol/min/mg	*pmol/mg*
Control	179 ± 19	64 ± 9
Secretin	410 ± 32[a]	4190 ± 340[a]
VIP	391 ± 34[a]	3470 ± 310[a]
PHI	342 ± 27[b]	1020 ± 94[a]
Glucagon	323 ± 27[b]	753 ± 174[a]
Secretin + VIP	414 ± 30[a]	4220 ± 340[a]
Secretin + PHI	405 ± 33[a]	3980 ± 330[a]
Secretin + glucagon	392 ± 28[a]	3810 ± 310[a]
Secretin(5–27)	185 ± 13	72 ± 10
Somatostatin	168 ± 19	66 ± 12
Forskolin	416 ± 32[a]	14,200 ± 410[a]

[a] Significantly different from control, $p < 0.001$.
[b] Significantly different from control, $p < 0.01$.

member of the secretin family, failed to alter tyrosine hydroxylase activity. Secretin(5–27), the carboxyl terminal portion of secretin, had no effect on basal tyrosine hydroxylase activity. The efficacy of neuropeptides at 10 μM was secretin = VIP > PHI > glucagon.

We also measured levels of cAMP following treatment of the PC12 cells with neuropeptides of the secretin family. Secretin and VIP produced about a 60-fold increase in cAMP levels (Table 2). PHI (16-fold) and glucagon (12-fold) also produced significant increases in cAMP levels. In contrast, somatostatin had no effect on cAMP content. Forskolin (10 μM), an activator of adenylate cyclase (24), produced greater than a 200-fold increase in cAMP. None of these agents altered cGMP levels (not shown). The activation of tyrosine hydroxylase by secretin and VIP was not attenuated when the experiment was performed in calcium-free medium containing either 0.1 mM EGTA, 1 mM lanthanum chloride, or 4 mM $MnCl_2$ (not shown). Although there was considerable variation in the specific activity of tyrosine hydroxylase activity (10-fold) and cAMP (4-fold) content in different passages of PC12 cells and at different times after splitting, precision within a given experiment was within 5%. All of the experiments reported in this paper were performed with passages in the 50–70 range. At passages greater than 100, the cells exhibited a low specific activity of tyrosine hydroxylase and its activity failed to respond to secretin, forskolin, or theophylline in a consistent fashion.

The predominant effect of cAMP in eukaryotic systems is to activate its cognate cAMP-dependent protein kinase (8). Corbin *et al.* (25) developed a method for assessing cAMP-dependent protein kinase activity *in situ*. The cells were homogenized in high ionic strength buffer, which prevents reassociation of the regulatory and catalytic subunits following dilution of the endogenous cAMP during the homogenization. The enzyme activity was then measured with and without exogenous cAMP added to the assay medium. An increase in the protein kinase activity ratio (−cAMP/+cAMP) reflects an increase in enzyme activation *in situ*. We found that the dependence of the protein kinase activity ratio on secretin and VIP concentrations paralleled the increases in cAMP levels and in tyrosine hydroxylase activity measured *in vitro* (Fig. 1). Secretin was 2–3 orders of

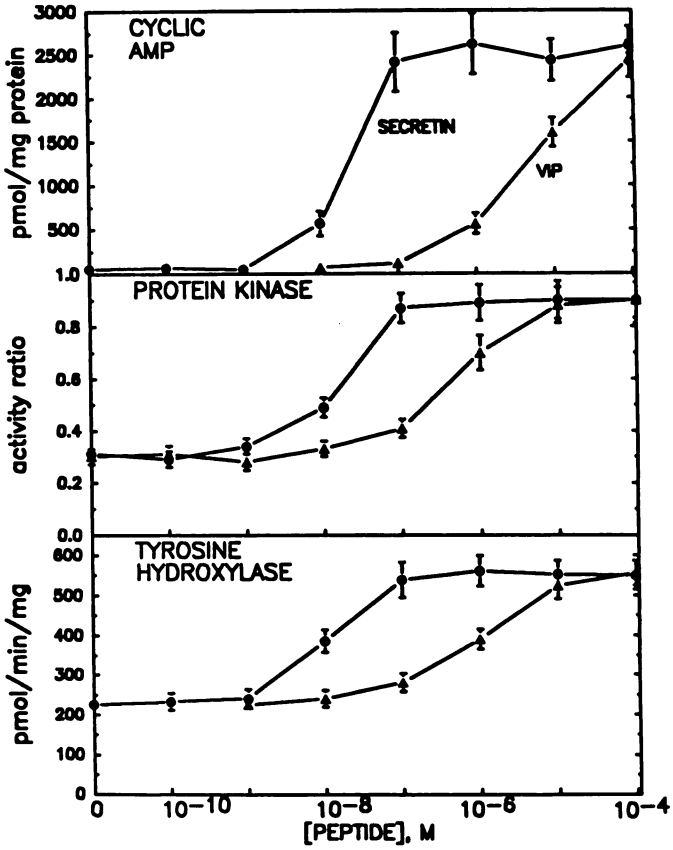

Fig. 1. Concentration dependence of the effects of secretin and VIP on cAMP levels (a), the cAMP-dependent protein kinase activity ratio (b), and tyrosine hydroxylase activity (c). ●, Secretin; ▲, VIP. PC12 cells in Ringer's solution containing 100 μM theophylline were treated with the specified concentrations of secretin or VIP for 20 min at 30°. The determinations were performed as specified in Methods and Materials. The values represent the mean ± standard error of triplicate determinations of one experiment. Similar results were obtained in three other experiments.

magnitude more potent than VIP, whereas the efficacy (maximal response) of these two peptides was the same in PC12 cells. Performing this experiment on clonal cells in culture ensures that the cells that exhibit elevated protein kinase activity are the same as those with enhanced tyrosine hydroxylase activity. This would not necessarily be the situation with tissues such as brain or ganglia, which contain multiple cell types.

Characterization of the nature of the receptors responding to secretin and VIP in PC12 cells. Secretin(5–27) had no effect on basal tyrosine hydroxylase activity in the PC12 cells (Table 1). However, it displaced the dose-response curves of both secretin and VIP to the right (Fig. 2). It thus functions as a competitive antagonist of both secretin and VIP. Secretin(5–27) also attenuated the response of PHI and glucagon in a similar fashion (not shown). Not shown are experiments (three experiments) indicating that the concentration dependence of tyrosine hydroxylase to secretin or VIP was unchanged in the presence of a combination of protease inhibitors dissolved immediately before the experiment. These include chymostatin, pepstatin A, leupeptin, and aprotinin, each at 33 μg/ml, plus bacitracin at 250 μg/ml and phenylmethylsulfonyl fluoride at 50 μM. We also found that the effects of secretin plus VIP, PHI, or glucagon on cAMP content or tyrosine hydroxylase activity were not additive (Table 1). On

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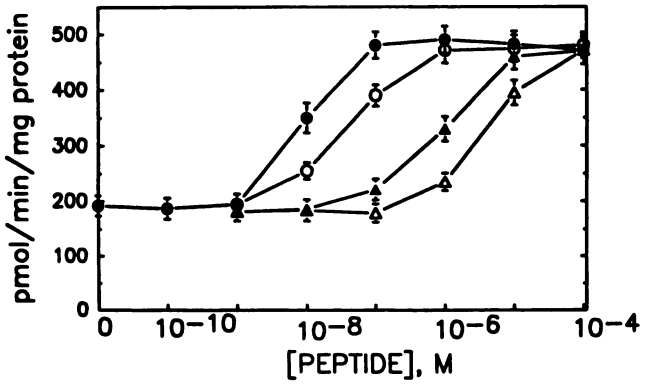

Fig. 2. Effect of secretin(5–27) on the concentration dependence of secretin and VIP activation of tyrosine hydroxylase activity in PC12 cells. After harvesting and suspending cells in Ringer's solution containing 100 μM theophylline, portions (58 μl) were dispensed into 1.5-ml tubes. Secretin(5–27) at a final concentration of 40 μM was added to the tubes 3–5 min before the addition of secretin or VIP to give the final specified concentration. After 18–22 min, the supernatants were aspirated following a 30-sec centrifugation (13,000 × *g*) and the cells were frozen at −70° before tyrosine hydroxylase activity measurements *in vitro*. ●, Secretin alone; ○, secretin plus secretin(5–27); ▲, VIP alone; △, VIP plus secretin(5–27). The points represent the mean ± standard error of triplicate samples. Similar results were observed in two other experiments.

the other hand, Fremeau *et al.* (9) reported that secretin plus VIP or secretin plus PHI treatments elevated cAMP in brain slices to a greater extent than each peptide alone.

Response of tyrosine hydroxylation *in situ* to secretin, VIP, and forskolin. One goal in the study of the regulation of catecholamine biosynthesis is to ascertain the relationship between tyrosine hydroxylase activity measured *in vitro* and the rate of DOPA production *in situ*. We measured the latter by the procedure of Erny *et al.* (26). This involved the addition of a DOPA decarboxylase inhibitor (brocresine) to the cells in Ringer's solution and subsequent treatment by the specified compound. After the incubation, DOPA production was measured by high performance liquid chromatography and electrochemical detection. Our experiments showed that secretin, VIP, and forskolin all increased DOPA production in the intact cells (Table 3). The degree of activation was 50–65%. We also measured tyrosine hydroxylase activity in the corresponding cells *in vitro*. In this group of experiments, we used 4 μM BH_4, the natural cofactor, at pH 7.2 with 60 μM radiotyrosine. The degree of activation *in vitro* was 2–3-fold and, thus, greater than that observed *in situ*. These values were based on the BH_4 content of PC12 cells of 60 ng/mg of protein (29), assuming 200 mg of protein/ml of intracellular water and a measured tyrosine content of 290 ± 42 pmol/mg of protein (three experiments; not shown). These data yield values of 4 and 58 μM for BH_4 and tyrosine, respectively. The activity *in vitro* is only about 10% of the activity *in situ*. The percentage of stimulation in cell-free extracts, however, was consistently greater than that observed *in situ*.

Time course of the response of tyrosine hydroxylase activity and cAMP to secretin and forskolin. We found that tyrosine hydroxylase activity was increased to almost its maximum within 6 sec after the addition of 1 μM secretin (Fig. 3). This was the shortest time after addition of secretin and vortexing that the incubation could be quenched by addition of ice-cold Ringer's solution. The range for attaining maximal activation was 6–15 sec in experiments performed with eight

TABLE 3

Stimulation of tyrosine hydroxylase activity *in situ* and *in vitro* by secretin and forskolin

PC12 cells were harvested and incubated in Ringer's solution in the presence of 150 μM brocresine (a DOPA decarboxylase inhibitor), 100 μM theophylline, and 50 μM tyrosine. After the cells were pipetted into 1.5-ml tubes (200 μl of about 1 mg/ml protein) containing any specified addition in a volume of 5 μl or less, the samples were incubated for 15 min at 30°. The samples were centrifuged at 13,000 × *g* for 30 sec and the supernatants (150 μl) containing DOPA were transferred to a tube containing 200 pmol of dihydroxybenzylamine, as internal standard, in 0.5 M HCl (10 μl) with 1 mg/ml sodium bisulfite and 1 mM EDTA. The samples were stored at −20° until the catecholamines were purified by alumina adsorption, as described by Gauchy *et al.* (27). The remainder of the supernatant was aspirated, and the cell pellets were stored at −70° until tyrosine hydroxylase was measured by the procedure of Reinhard *et al.* (28), using 4 μM BH_4 as cofactor and 60 μM [^{3}H] tyrosine at pH 7.2. DOPA produced by the intact PC12 cells was measured in the alumina eluants by HPLC and electrochemical detection, on a Bioanalytical Systems apparatus, as described by Erny *et al.* (26). The data represent the mean ± standard error of triplicate determinations from one experiment. Similar results were obtained in three other experiments.

Addition to cells	DOPA production *in situ*	Tyrosine hydroxylase activity
	pmol/min/mg	
None	19.8 ± 1.6	2.53 ± 0.19
Secretin, 1 μM	34.4 ± 3.2[a]	9.31 ± 0.77[a]
VIP, 1 μM	28.2 ± 1.6[a]	5.18 ± 0.38[a]
Forskolin, 10 μM	33.2 ± 2.7[a]	7.25 ± 0.64[a]

[a] Significantly different from the control, $p < 0.01$.

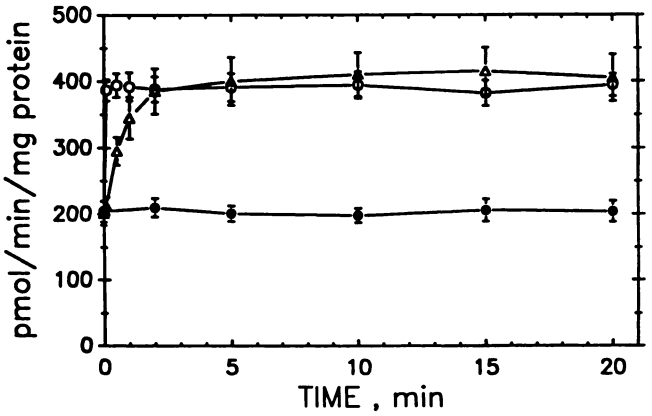

Fig. 3. Time course of the response of tyrosine hydroxylase activity to addition of secretin or forskolin to PC12 cells. Cells were harvested and incubated in Ringer's solution (containing 100 μM theophylline) for 5 min at 30°. Additions (2 μl) of secretin (○) to give a 1 μM final concentration, or forskolin (△) to give a 10 μM final concentration, or Ringer's solution (●) were made and the tubes were vortexed. Ice-cold Ringer's solution (1 ml) was added to the cells to stop metabolism at the indicated times and the cells were collected by a 30-sec centrifugation (13,000 × *g*). After the medium was aspirated, the 1.5-ml tubes were placed in aluminum blocks equilibrated in a −70° freezer before tyrosine hydroxylase activity measurements. Each point represents the mean ± standard error of three samples. The earliest time point is 6 sec. The data are taken from a representative experiment. Similar results were obtained in seven other experiments.

different cell cultures. In contrast, it required between 2 and 5 min for the maximal increase in tyrosine hydroxylase activity to occur following addition of 10 μM forskolin, as determined in six independent experiments. The maximal activity reached in response to both agents was identical. The temporal responses of cAMP to these two agents also were different. Although increases were observed at the earliest possible time that the samples could be processed, the levels continued to increase for the 20-min duration of the experiment (Fig. 4). The magnitude of the response of cAMP to forskolin was considerably greater than that to secretin. Because of the

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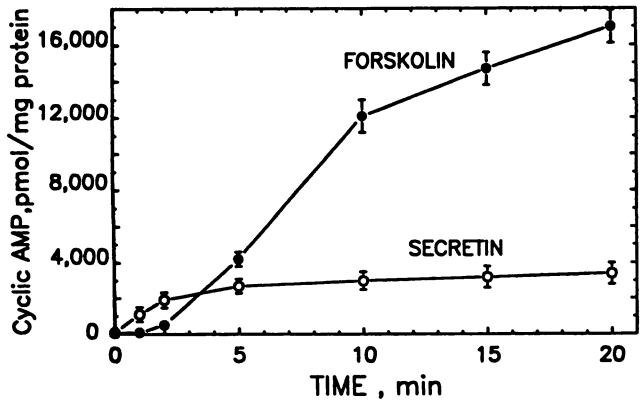

Fig. 4. Time course of the cAMP response to secretin and forskolin. After cells were suspended in Ringer's solution containing 100 μM theophylline for 3–5 min, the cells (98-μl portions) were dispensed into 1.5-ml tubes. Secretin (1 μM final) or forskolin (10 μM final) were added in 2-μl portions and the suspensions were vortexed for about 2 sec. At the time specified, the tubes were transferred to a boiling water bath. Samples were stored at −70° until protein and cAMP were measured. ○, Secretin; ●, forskolin. The points represent the mean ± standard error of triplicate samples. Similar results were obtained in two other experiments.

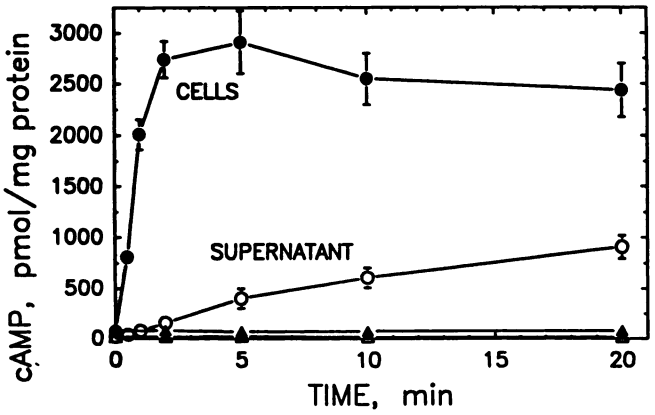

Fig. 5. Time course of the effects of secretin on cAMP levels in PC12 cells and incubation medium. After cells were suspended in Ringer's solution containing 100 μM theophylline for 3–5 min, the cells (98-μl portions) were dispensed into 1.5-ml tubes. Secretin was added to specified samples (2 μl) to give a final concentration of 1 μM. The cells were centrifuged for 30 sec (13,000 × *g*), the supernatant was aspirated from the cell pellet, and each was placed in boiling water for 5 min to inactivate adenylate cyclase and phosphodiesterase. Samples were stored at −70° until protein and cAMP was measured. ●, cAMP content of secretin treated cells; ○, supernatant of secretin-treated cells; △, control cells; ▲, control supernatant. The points represent the mean ± standard error of triplicate samples. The cAMP in the supernatant was based upon protein content of the corresponding cell pellet. Similar results were obtained in two other experiments.

different methods used to quench the samples for measuring tyrosine hydroxylase activity and cAMP content (ice-cold Ringer's versus incubation in a boiling water bath), there was less certainty of the precise termination time in the case of the cAMP measurements.

Fiscus and co-workers (30) reported that PC12 cells release cGMP into the incubation medium following treatment with the atriopeptins. This and studies on cAMP egress in other systems (31) prompted us to measure the accumulation of cAMP in the extracellular medium during treatment with secretin. There was a continuous increase in cAMP in the supernatant over the course of the experiment (Fig. 5). The maximal increase in intracellular levels occurred within 5 min. The incubations were performed in 200 μl at a typical value of 2 mg/ml of protein. If one assumes that 1 mg of protein corresponds to 5 μl of intracellular volume, then the calculated basal intracellular concentration (corresponding to 71 pmol/mg of protein) is 14.2 μM and, after 10 min of secretin treatment, the intracellular concentration of cAMP is 428 μM. The basal extracellular concentration is 38 nM (corresponding to 19 pmol/mg of protein) and, after 10 min of secretin treatment, this increases to 1.2 μM. In either case, the calculated ratio of intracellular to extracellular cAMP is about 340.

Discussion

Theophylline functions both as an adenosine receptor antagonist and as an inhibitor of cyclic nucleotide phosphodiesterase (23). At low concentrations (100 μM) in the PC12 system, the adenosine receptor effects predominate. Although theophylline decreases the basal activity of tyrosine hydroxylase and basal cAMP levels, the activity of cAMP-dependent protein kinase activity is still appreciable (Fig. 1). The basal level of cAMP (in the micromolar range) is consistent with the appreciable protein kinase activity observed in the absence of exogenous cAMP-elevating agents.

The present experiments are consistent with the notion that PC12 cells contain a secretin-preferring receptor that can also be activated by high concentrations of VIP. The potency of secretin in activating cAMP-dependent protein kinase and tyrosine hydroxylase in PC12 cells was near the expected physiological range. In contrast, much higher concentrations of VIP were required to produce a similar response. Thus, it is possible that high concentrations of VIP can activate the secretin receptor. Our concentration dependence of the response of tyrosine hydroxylase activation to VIP in PC12 cells was essentially the same as that reported by Tischler *et al.* (11). Secretin(5–27), furthermore, acted as a competitive antagonist of both secretin and VIP (Fig. 2). An alternative explanation is that VIP interacted with a VIP-preferring receptor in a physiological fashion but was more susceptible to degradation by endogenous proteases. Although this possibility cannot be completely ruled out, it seems less likely for two reasons. First, a combination of several protease inhibitors failed to alter the dose-response curve for VIP. Second, a similar dose-response profile was observed when the duration of incubation was decreased from 20 to 4 min (not shown). A shorter incubation would be expected to decrease the extent of proteolysis, but this had no effect on the potencies of either VIP or secretin. The absence of an additive response to secretin and VIP in elevating cAMP levels is consistent with the notion that the cells contain a single class of receptor and not different receptors for each agent.

The secretin family of neuropeptides increased tyrosine hydroxylase activity (Table 2) in PC12 cells, as it does in the superior cervical ganglia (12). There are, however, differences in the two systems. In the case of the PC12 cells, secretin and VIP stimulated tyrosine hydroxylase activity to the same extent. In the superior cervical ganglia, on the other hand, VIP exhibits a greater efficacy than does secretin in stimulating tyrosine hydroxylase activity and in increasing cAMP levels. Moreover, glucagon was not effective in increasing tyrosine hydroxylase activity in the superior cervical ganglion preparation, but it produced a significant response in PC12 cells. The

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reasons for these differences between the two responsive systems is unknown. One advantage of the PC12 cells, when compared with superior cervical ganglion or other heterogeneous tissues such as brain slices, is that the former constitute a single cell type. In contrast, the latter tissues contain a variety of cells and it is possible that the observed changes in tyrosine hydroxylase activity in response to agents such as secretin may be indirect.

The activation of tyrosine hydroxylase activity in PC12 cells by secretin, VIP, PHI, and glucagon was most likely mediated by the cAMP second messenger system. All of these agents produced an increase in cAMP, as determined by radioimmunoassay. Moreover, all of these agents increased cAMP-dependent protein kinase activity. The concentration dependence of the increase in tyrosine hydroxylase activity, cAMP levels, and activation of protein kinase activity produced by either secretin or VIP was very similar. Following treatment with either 10 μM secretin or VIP, the apparent K_m for 6-MPH$_4$ at pH 6.0 decreased from 640 ± 73 μM (control) to 131 ± 19 μM (secretin) or 147 ± 16 μM (VIP). The K_m values were determined in three independent experiments, as previously described (6). These results are also consistent with the activation of tyrosine hydroxylase by the cAMP second messenger system and by cAMP-dependent protein kinase (3–6, 32). The response to secretin did not require exogenous calcium. Moreover, there was no increase in the levels of cGMP in response to any of the peptides of the secretin family (not shown).

We also performed a series of experiments with PC18 cells. These cells, unlike the PC12 cells from which they were derived, fail to respond to nerve growth factor (33). We found that they failed to respond to 10 μM secretin, VIP, or PHI in terms of cAMP increases or activation of tyrosine hydroxylase. However, they responded to forskolin treatment in terms of these two parameters, as reported here for the parent cell line.

Some nerve fibers in the adrenal medulla of rats (34) and humans (35) contain VIP-like immunoreactivity. Malhotra and Wakade (36) reported that VIP stimulates the secretion of catecholamines from the rat adrenal gland in a dose-dependent fashion (from 0.3 to 3 μM concentrations). Secretion was inhibited by the inclusion of 1 mM EGTA in the medium. Malhotra et al. (37) reported that the phosphoinositides may play a role in this response. Although the concentration dependence of the response is similar to that described here, activation of tyrosine hydroxylase in PC12 cells was not abrogated by EGTA, suggesting that activation is not directly related to calcium-dependent secretion.

The time course of the activation of tyrosine hydroxylase was very rapid in response to secretin. It reached a maximum within 6–15 sec. This rapidity was surprising, when compared with the time course of activation of purified tyrosine hydroxylase by purified catalytic subunit of cAMP-dependent protein kinase. With 1.5 μM tyrosine hydroxylase (as the subunit concentration) and 0.5 μM catalytic subunit, it required 2–5 min to achieve full phosphorylation and activation (32). Perhaps effectors are present within the cell that alter the rate of activation. In addition, the conformation of the tyrosine hydroxylase or protein kinase may change to a less active state as a result of purification, storage, decreased temperature, or perturbations resulting from handling. The temporal response to forskolin was less rapid than that to secretin. Perhaps this reflects the time necessary for forskolin to traverse the plasma membrane and to directly activate adenylate cyclase. This finding emphasizes the rapidity of the transmembrane transduction systems such as that exemplified by secretin.

The secretin receptor in brain is also coupled to adenylate cyclase, as demonstrated by Fremeau *et al.* (9). Using rat brain cerebral cortex slices, they found that 1 μM secretin gave the half-maximal increase. Our system may be more sensitive to secretin because it lacks diffusional barriers. In brain (9), the basal level of cAMP was 54 pmol/mg of protein, which is similar to that found in PC12 cells. The maximal response to secretin, however, amounted to a 3-fold increase (155 pmol/mg of protein), in contrast to the situation in PC12 cells where a robust 65-fold increase was observed (Table 2). Part of this difference may be related to the absence of secretin-preferring receptors on many of the cells in the brain slices, in contrast to clonal PC12 cells. In contrast to Fremeau *et al.* (9), we were readily able to demonstrate an increase in cAMP in the absence of inhibition of cyclic nucleotide phosphodiesterase activity. This suggests that the ratio of activities of adenylate cyclase to phosphodiesterase is greater in the PC12 cells than in cerebral cortex. In contrast to brain slices (9), the absence of temporal changes in basal cAMP levels provides a significant experimental advantage in the use of PC12 cells in studying secretin effects.

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Send reprint requests to: Robert Roskoski, Jr., Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70119.

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