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Vasoactive Intestinal Polypeptide Facilitates Tyrosine Hydroxylase Induction by Cholinergic Agonists in Bovine Adrenal Chromaffin Cells

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SUMMARY

The possibility that vasoactive intestinal polypeptide (VIP) may facilitate the nicotine-mediated induction of adrenal medullary tyrosine hydroxylase (TH) was investigated with primary cultures (5–7 days *in vitro*) of bovine adrenal chromaffin (BAC) cells. Exposure of BAC cells to 100 μM nicotine led to only a marginal increase in the amount of TH mRNA, TH protein, and TH activity. VIP, alone or in the presence of a phosphodiesterase inhibitor, produced a marked increase in TH mRNA, TH protein, and TH activity. Moreover, VIP together with nicotine, at concentrations that alone were devoid of effect, increased the amount of TH mRNA and TH activity. A synergistic effect of VIP and nicotine on cAMP accumulation in BAC cells was also apparent. The marginal effects of large doses of nicotine on both cAMP accumulation and TH induction were blocked completely by hexamethonium but were also partially inhibited by the VIP antagonist [*p*-chloro-D-Phe^6,Leu^{17}]-VIP. Nicotine may, therefore, stimulate the release of VIP from cultured BAC cells and VIP, in turn, by increasing cAMP, may synergize with nicotine to enhance TH gene expression.

TH [tyrosine 3-monooxygenase:L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2], the rate-limiting enzyme in catecholamine biosynthesis (1), is subject to regulation by two mechanisms, (i) stimulus-induced transient covalent modification of the enzyme through phosphorylation, which results in an increase in its affinity for substrate and cofactors (2), and (ii) protracted stimulus-induced increases in the amount of TH mRNA and TH protein (TH induction), which only become apparent several (12–48) hours after stimulus application (1, 3–5). In the adrenal medulla, an increase in reflex sympathetic outflow to chromaffin cells results in TH induction (1, 3–6), which is believed to result in a cascade of amplification events that are triggered by a stimulus-coupled early (1–2 hr) increase in the intracellular cAMP concentration (7–9). This accumulation of cAMP is believed to be the amplification signal (5, 7, 10, 11) for a delayed increase in TH mRNA, which, in turn, precedes the observed increases in TH V_{max} and TH immunoreactivity (12, 13).

Numerous studies have been conducted in an attempt to elucidate the molecular mechanisms that operate in the transsynaptic induction of TH in the adrenal medulla (8, 14–16). Several *in vivo* studies have suggested that ACh released from the splanchnic nerve terminals that innervate chromaffin cells is the primary neurotransmitter responsible for this induction (1, 3–9, 14–16). However, the efficacy of nicotinic Ach receptor agonists at inducing TH is relatively low, compared with the induction elicited by drugs that promote a reflex increase in splanchnic nerve activity (14). Moreover, it was shown (17, 18) that the induction of TH elicited by reserpine administration to rats was blocked by prior transection of the splanchnic nerve, but the effect of nerve transection was not duplicated by the administration of the nicotinic cholinergic antagonists chlorisondamine or pempidine. Because these studies examined the effect of nicotinic receptor antagonists on adrenal medullary events that occur several hours or days after the drug application, the results did not offer an unequivocal interpretation of the events; however, they suggested (17) that the induction of adrenal TH may be triggered by the co-release, from the splanchnic nerve, of a second chemical signal in addition to ACh. Indeed, immunohistochemical studies have shown that splanchnic nerve fibers contain several neuropeptides in addition to ACh, one of which is VIP or a VIP-like substance (19, 20). VIP coexists with ACh in several areas of the central and peripheral nervous systems (21–24). A number of studies suggest that VIP, by increasing cAMP and thereby phosphorylating TH, can induce a short term activation of the enzyme (21, 23–29). Moreover, induction of TH after application of VIP to cultured PC12 cells has recently been reported (30).

The present study was designed to investigate (i) whether nicotine or VIP alone induces TH expression in cultured BAC

ABBREVIATIONS: TH, tyrosine hydroxylase; ACh, acetylcholine; VIP, vasoactive intestinal peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; BAC, bovine adrenal chromaffin.

cells, (ii) whether nicotine has a permissive action on TH induction by VIP, and (iii) whether cAMP functions as a second messenger in mediating these effects. In the present study, we demonstrated that nicotine alone produced only a small induction of TH, which was partially inhibited by a VIP antagonist, whereas the induction produced by VIP was significantly greater and was also inhibited by the VIP antagonist. More importantly, nicotine together with VIP, at concentrations that alone did not increase TH activity or TH mRNA, produced an induction of TH and increased the cAMP concentration of BAC cells. These synergistic responses thus support the concept that cAMP may function as second messenger in the induction of TH by nicotine and VIP.

Materials and Methods

Cell preparation. Bovine adrenal glands were obtained from a local slaughterhouse, and chromaffin cells were isolated from the adrenal medulla by a modified version of previously described methods (31, 32). The glands were perfused with solution A, consisting of Ca^{2+}-free Dulbecco's phosphate-buffered saline (GIBCO), 0.5% bovine serum albumin (Sigma), and 50 mM HEPES (Boehringer Mannheim), and were then incubated for 30 min at 37°. The glands were then perfused with solution A containing 0.1% collagenase (type B; Boehringer Mannheim), 13.3 mg/liter deoxyribonuclease (Sigma), 100 mg/liter soybean trypsin inhibitor (Sigma), 40 mg/liter gentamycin, and 100,000 units/liter penicillin (GIBCO) and were then incubated for 20 min at 37°. The perfusion and incubation procedure was repeated three times. The medullae were then dissected out from the glands, minced, and passed through 130-μm nylon mesh (Tetko). The cell suspension was washed twice in solution A and purified on a self-generating 40% Percoll (Pharmacia) gradient. After centrifugation at 20,000 × *g* for 20 min at room temperature in an angle-head rotor (Sorvall, SS34), the chromaffin cells were harvested by aspiration, washed twice with solution A, and then suspended in Dulbecco's modified Eagle's medium (GIBCO) containing 10% calf serum (GIBCO), 100,000 units/liter penicillin, 40 mg/liter gentamycin, 50,000 units/liter nystatin (GIBCO), and 5 μM 5-fluorodeoxyuridine (Sigma). The cells were counted and then cultured in 24-well cluster dishes (Costar no. 3524) or poly-L-lysine-coated (Sigma) 35-mm culture dishes (Nunc). The dishes were incubated at 37° in a humidified atmosphere (5% CO_2/95% O_2).

TH enzyme assay. BAC cells (2.5×10^5 cells/well) were usually maintained in culture for 5–7 days before treatment with the appropriate agents [VIP and VIP antagonist (Bachem); forskolin (Calbiochem); HL-725 (trequinsin) (Hoechst-Roussel); and carbachol, nicotine, and hexamethonium (Sigma)]. After treatment, the medium was removed and the cells were incubated in 200 μl of 50 mM Tris-acetate buffer (pH 6.0), containing 0.2% Triton X-100, for 30 min at 4°. TH activity was measured in 50 μl of cell lysate with L-[1-^{14}C]tyrosine (48.6 Ci/mmol; NEN), in a 3,4-dihydroxyphenylalanine decarboxylase enzymatic reaction; $^{14}CO_2$ production was determined. The assay was performed according to a modified version of the method described by Waymire *et al.* (33).

TH protein assay. Quantitation of TH protein was achieved by the spot immunolabeling method (34), with an affinity-purified antibody (kindly supplied by Dr. J. W. Haycock, Department of Biochemistry and Molecular Biology, Lousiana State University, New Orleans, LA). Briefly, the method consists of sonicating cells (2.5×10^6) in 25 μl of 1% SDS and heating in a boiling water bath for 5 min. Aliquots (1 μl) of the sample (containing 0.5–2.5 μg of protein) were applied in triplicate directly to a nitrocellulose sheet and were processed for immunolabeling with ^{125}I-labeled Protein A, as described (34). Autoradiograms of the nitrocellulose sheets were quantitated with an LKB 2202 Ultrascan laser densitometer and a Hewlett-Packard 3390A integrator. The arbitrary absorbance units were corrected for the protein content of the original samples.

cAMP determination. The agents to be tested were directly added to the cultured chromaffin cells (2.5×10^5 cells/well). Incubations were terminated by removing the culture medium, and cAMP was extracted by incubation with 200 μl of 0.1 M HCl for 10 min. The samples were acetylated before the cAMP content of 10–25 μl of extract was determined with a radioimmunoassay kit (Amersham).

RNA isolation and gel blot hybridization. Total BAC cell RNA was isolated from four 35-mm dishes by the guanidinium isothiocyanate-cesium chloride gradient procedure (35). RNA concentration was estimated by measuring absorbance at 260 nm (1 *A* unit = 40 μg/ml). Samples containing 15 μg of total RNA were subjected to electrophoresis through a denaturating 1% agarose gel containing 2.2 M formaldehyde, and RNA was then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by capillary blotting in 20× SSC [1× SSC contains 15 mM trisodium citrate and 0.15 M NaCl (pH 7)]. The nitrocellulose membranes were rinsed, baked at 80° *in vacuo* for 2 hr, and then incubated for 6 hr at 42° in 8× Denhardt's solution (1× Denhardt's solution contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) containing 50% (v/v) formamide, 750 mM NaCl, 50 mM NaH_2PO_4, 2.5 mM EDTA, 100 μg/ml denaturated salmon sperm DNA, and 0.2% SDS. The membranes were then hybridized with a ^{32}P-labeled TH cDNA probe (1×10^6 to 5×10^6 cpm/ml) at 42° for 24 hr, in the same buffer used for the prehybridization incubation, except containing 1× Denhardt's solution. Final washings were in 0.5× SSC with 0.1% SDS at 50°. The blots were exposed to Kodak X-OMAT AR film with a DuPont Cronex Lightning Plus intensifying screen, at −70°. The peak areas of the hybridized bands on autoradiograms were quantitated with an LKB 2202 UltraScan densitometer and a Hewlett-Packard 3390A integrator. Because the determination of the amount of TH mRNA is dependent on several experimental variables, the same blots were rehybridized with the p1B15 cDNA probe; this cDNA hybridizes to mRNA encoding cyclophilin, a stable, constitutively expressed, structural protein (36). The amount of TH mRNA was expressed in arbitrary densitometric units, defined as the ratio of the densitometric area of the TH mRNA hybridization band to that of the cyclophilin mRNA hybridization band. This double hybridization normalizes the variability generated by the experimental procedure.

Probe preparation. The hybridization probe used in our experiments for detecting TH mRNA was a rat RR1.2 TH cDNA clone, containing nucleotides +14 to +1165, and was obtained from Dr. Dona M. Chikaraishi (Department of Neurology, Tufts Medical Center, Boston, MA). Recombinant plasmid DNA was treated with *Eco*RI, and the cDNA was isolated by agarose gel electrophoresis and purified by chromatography on Sephadex G-50 spin columns (Boeheringer Mannheim). The probe was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham), by the random priming procedure of Feinberg and Vogelstein (37), to a specific activity of 1×10^6 to 5×10^8 cpm/μg of DNA. The p1B15 cDNA clone was a gift from Dr. I. Mocchetti (Department of Anatomy and Cell Biology, Georgetown University Medical School, Washington, DC) and was labeled by nick translation (38).

Statistics. Data were analyzed by either the two-tailed Student's *t* test or Duncan's multiple range test, as indicated (39).

Results

Effect of cholinergic agonists on BAC cell TH mRNA, TH protein, and TH activity. In order to test whether cholinergic agonists would increase TH mRNA, we first exposed BAC cells that had been maintained *in vitro* for 5–7 days to various concentrations of nicotine for 12 hr and we then measured the amount of TH mRNA. (This time point was chosen because preliminary studies indicated that the maximal increase in the amount of TH mRNA occurred after 8–12 hr of treatment with 100 μM nicotine.) Treatment of BAC cells with 1–10 μM nicotine failed to cause a measurable change in the amount of TH mRNA. However, 100 μM nicotine induced a

slight, but significant, increase in the amount of TH mRNA (Fig. 1).

TH activity was determined in parallel experiments. Enzyme activity was always measured at saturating substrate and cofactor concentrations; thus, any observed increases in TH activity would be expected to reflect changes in V_{max} and not changes in the affinity of the enzyme for its substrate or cofactors. Enzyme activity was not changed in a significant manner after continuous exposure of BAC cells to 1 or 10 μM nicotine for 48 hr but was increased by 30% over control after exposure of cells to 100 μM nicotine (Fig. 2). However, the increase in TH activity obtained after 48 hr of continuous exposure to 100 μM nicotine was minute, compared with that seen with 100 μM forskolin [TH activity (nmol of CO_2/hr/10^6 cells): control, 3.5 ± 0.040; forskolin, 9.9 ± 0.11 (mean ± standard error; three experiments)]. Because phosphorylation of TH can increase enzyme activity through an increase in V_{max} (40, 41), we assessed the actual change in TH protein with the spot immunolabeling assay described by Haycock (34). A 25 ± 3.5% (six experiments) increase in TH protein was observed in BAC cells incubated for 48 hr with 100 μM nicotine. This increase is similar to that measured with the TH activity assay (Fig. 2).

The time course of TH induction was investigated by exposing BAC cells to 100 μM nicotine for various times but always measuring TH activity 48 hr after the initiation of treatment. A significant (approximately 20%) increase in TH activity was not apparent until after at least 24 hr of continuous exposure to nicotine (Fig. 3). Incubation of BAC cells with forskolin for 6 hr was sufficient to produce a significant activation of TH, as measured after 48 hr [control, 3.5 ± 0.040; forskolin, 4.47 ± 0.13 nmol of CO_2/hr/10^6 cells (mean ± standard error; three experiments)]. Continuous exposure to forskolin for 48 hr was required to achieve a 3-fold increase in activity relative to the control value. Forskolin was also the most efficacious, of the agents tested, at inducing TH mRNA. After 12 hr of exposure, a 7-fold increase in TH mRNA was observed [TH mRNA (arbitrary densitometric units): control, 1; forskolin, 7.2 ± 0.94 (mean ± standard error; three experiments)].

Because BAC cells possess both nicotinic and muscarinic ACh receptors, the efficacy of carbachol at inducing TH was tested. Only at a concentration as high as 1 mM did carbachol increase TH by 25% [control, 3.5 ± 0.040; carbachol, 4.39 ± 0.11 nmol of CO_2/hr/10^6 cells (mean ± standard error; three experiments)]. Thus, the dose of carbachol required to induce TH was at least 1 order of magnitude higher than that of nicotine. Oxotremorine, a muscarinic receptor agonist, at a concentration of 1 mM was completely devoid of effect [control, 3.5 ± 0.040; oxotremorine, 3.6 ± 0.21 nmol of CO_2/hr/10^6 cells (mean ± standard error; three experiments)].

Effect of VIP on BAC cell TH mRNA, TH protein, and TH activity. VIP was tested for its capacity to induce TH by exposing BAC cells to 10 μM VIP for various (3–48 hr) periods of time. The maximum increase in TH mRNA (3-fold) was reached within 12 hr of treatment (Fig. 4); thereafter, the amount of mRNA steadily declined and reached control values after 48 hr [TH mRNA (arbitrary densitometric units): control, 1; VIP (12 hr), 2.8 ± 0.17; VIP (48 hr), 1.1 ± 0.067 (mean ± standard error; three experiments)]. Lower concentrations of VIP were devoid of significant effect on TH mRNA, as measured after 12 hr of exposure to the peptide (Fig. 4). Thus, VIP was more efficacious than nicotine at increasing the amount of TH mRNA in BAC cells.

VIP also increased TH activity and TH protein significantly at concentrations of 5 and 10 μM (Table 1). TH was measured after 48 hr of continuous treatment with VIP, even though a significant effect was apparent after only 12 hr of exposure (the increase in enzyme still being measured 48 hr after the initiation of treatment).

Effect of VIP applied together with cholinergic agonists on TH induction. Because the extent of TH induction achieved by high concentrations of either nicotine or VIP was much smaller than the maximal induction elicited by forskolin, we investigated whether nicotine and VIP, in doses that alone were devoid of effect, could be synergistic and induce TH when applied together. BAC cells were incubated for 12 hr with both nicotine and VIP at concentrations of 1 μM, at which either agent alone is not able to induce TH. Under these conditions,

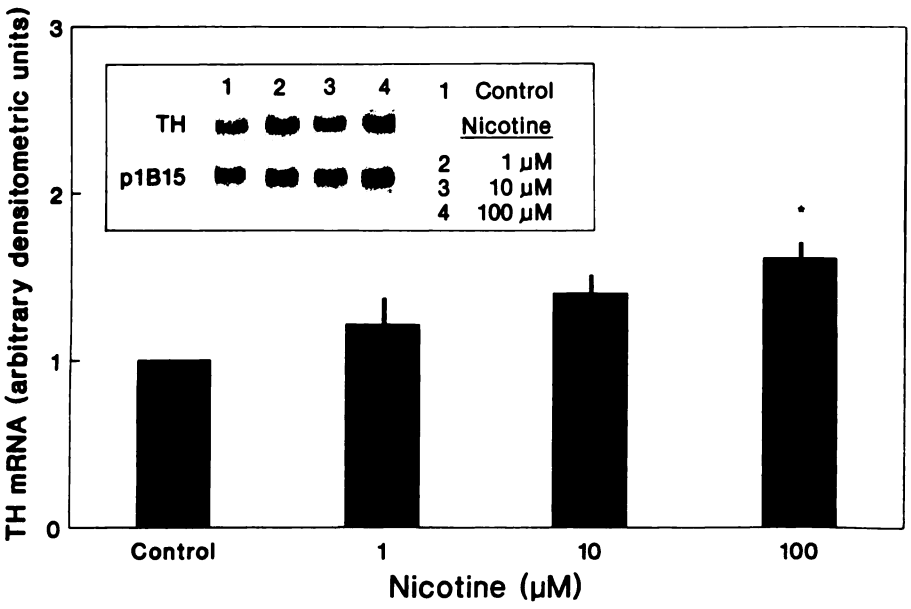

Fig. 1. Effect of nicotine on TH mRNA in BAC cells. BAC cells were cultured for 5–7 days and then exposed to various concentrations of nicotine. After 12 hr of exposure, the cells were scraped and total RNA was isolated. The amount of TH mRNA was determined by Northern analysis and is expressed in arbitrary densitometric units relative to the amount of cyclophilin (p1B15) mRNA. Values represent the mean ± standard error (three experiments). *, Significantly different ($p < 0.05$) from control, by Duncan's multiple range test. *Inset*, representative Northern blot.

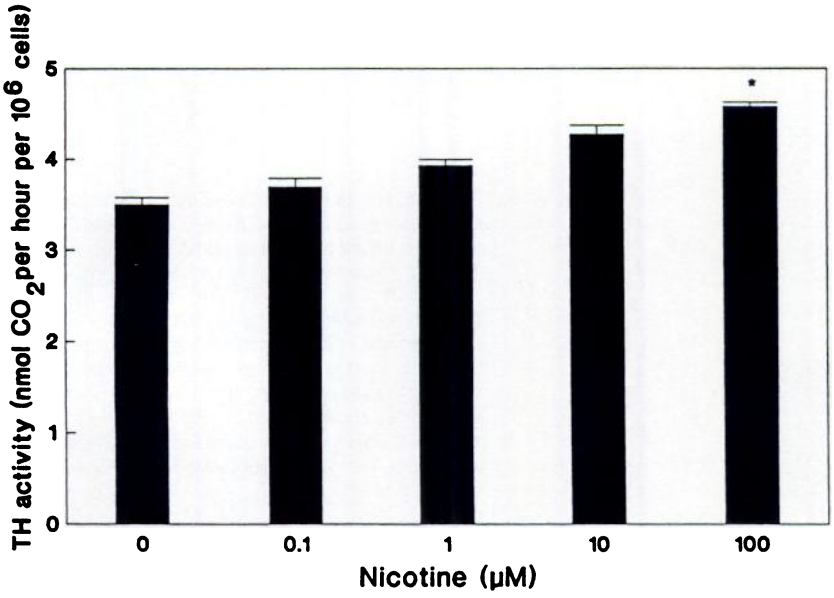

Fig. 2. Effect of nicotine on TH activity in BAC cells. BAC cells were cultured for 5–7 days and then exposed to various concentrations of nicotine. After 48 hr of continuous exposure, TH activity was measured at saturating substrate and cofactor concentrations. Values represent the mean ± standard error (three experiments). *, Significantly different ($p < 0.01$) from control, by Student's *t* test.

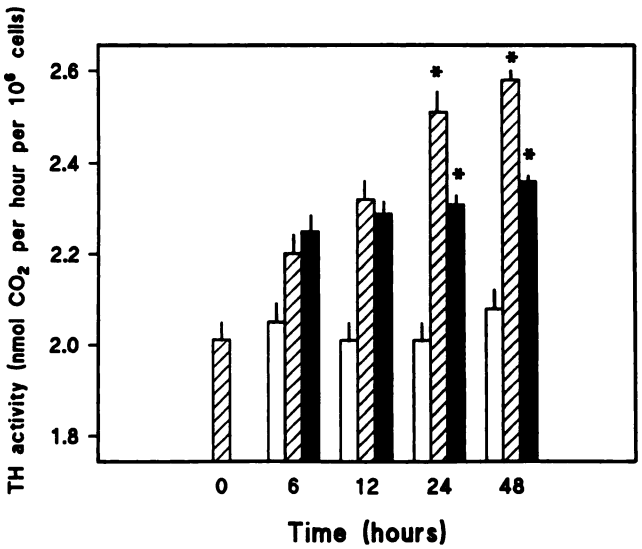

Fig. 3. Effect of [*p*-chloro-D-Phe^6,Leu^{17}]-VIP on nicotine-induced TH activity. BAC cells were cultured for 5–7 days and exposed to 10 μM VIP antagonist (□), 100 μM nicotine (▨), or these two agents together (■) for various times. TH activity was measured 48 hr after initiation of treatment, at saturating substrate and cofactor concentrations. Values represent the mean ± standard error (three experiments). *(▨), Significantly different ($p < 0.05$) from VIP antagonist alone or (■) from nicotine alone, Student's *t* test.

a 2-fold increase in TH mRNA was observed (Fig. 5). The effect of VIP (1 μM) on TH was also tested at various concentrations of nicotine. After 48 hr of treatment, in the presence of 1 μM VIP, nicotine at concentrations of 0.1 and 1 μM increased TH activity (Fig. 6A). Similar results were obtained when 1 μM nicotine was applied together with various concentrations of VIP (Fig. 6B) or when 10 μM carbachol was applied together with various concentrations of VIP [TH activity (nmol of CO_2/hr/10^6 cells): control, 3.5 ± 0.040; carbachol, 3.7 ± 0.099; VIP (1 μM), 3.6 ± 0.079; carbachol plus VIP, 4.2 ± 0.17 (mean ± standard error; three experiments; carbachol plus VIP significantly different, $p < 0.05$)]. Thus, a cholinergic agonist together with VIP, both at concentrations that *per se* are devoid of effect, can induce TH.

Effect of HL-725 on TH induction mediated by VIP and by nicotine. It is known that cAMP accumulation in BAC cells is correlated with the induction of TH (10, 11, 15). Because activation of the VIP recognition site stimulates adenylate cyclase (42) and results in an accumulation of cAMP in BAC cells, we tested the effect of VIP on TH induction in the presence of a phosphodiesterase inhibitor. The isoquinoline derivative HL-725, a potent inhibitor of type II phosphodiesterase (cGMP sensitive), was used in this study (43). At a concentration of 10 μM, HL-725 induced TH; moreover, the increase in TH induced by VIP was also potentiated by HL-725 (Table 1). The increase in TH elicited by 10 μM VIP alone after 12 hr of treatment was approximately 20% and, as shown in Table 1, after 48 hr of exposure, approximately 50%; however, 10 μM VIP together with 10 μM HL-725 increased TH in a time-dependent fashion by 70%, 129%, 180%, and 200% after 12, 24, 36, and 48 hr of exposure, respectively (in each case, activity was measured 48 hr after initiation of treatment). Another member of the secretin-glucagon peptide family, secretin, was tested for its effect on TH. Secretin (1 μM), either alone or together with HL-725, had no effect on TH activity (Table 1). In the same manner as for VIP, HL-725 potentiated nicotine-induced TH (Table 1); in addition, the increase in TH mRNA induced by 100 μM nicotine was potentiated by HL-725 [TH mRNA (arbitrary densitometric units): control, 1; nicotine, 1.9 ± 0.08; nicotine plus HL-725, 2.6 ± 0.28 (mean ± standard error; three experiments)].

Effects of antagonists on nicotine-induced and VIP-induced TH. The nicotinic receptor antagonist hexamethonium completely blocked the TH induction elicited by 100 μM nicotine. In contrast, hexamethonium had no effect on TH induction elicited by 1 μM VIP in the presence of 10 μM HL-725 (Table 2). The VIP antagonist [*p*-chloro-D-Phe^6,Leu^{17}]-VIP (44), at a concentration of 10 μM, attenuated the induction of TH elicited by 1 μM VIP in the presence of 10 μM HL-725 (Table 2). The VIP antagonist also reduced the increase in TH induced by nicotine (Fig. 3; Table 2). The increase in TH mRNA seen after 12 hr of exposure to 100 μM nicotine was also reduced in the presence of the VIP antagonist (data not shown).

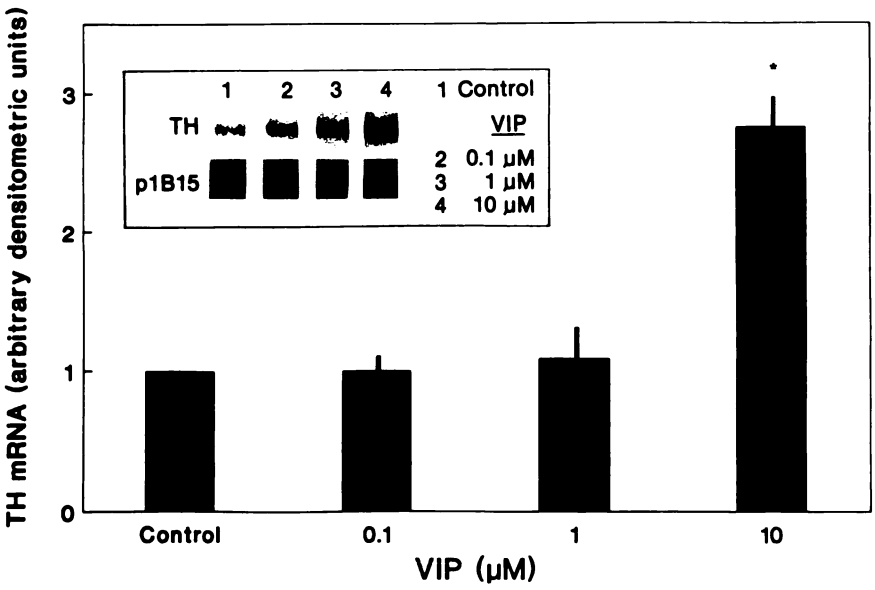

Fig. 4. Effect of VIP on TH mRNA in BAC cells. BAC cells were cultured for 5–7 days and then exposed to various concentrations of VIP. After 12 hr of exposure, the cells were scraped and total RNA was isolated. The amount of TH mRNA was determined by Northern analysis and is expressed in arbitrary densitometric units relative to the amount of cyclophilin (p1B15) mRNA. Values represent the mean ± standard error (three experiments). *, Significantly different ($p < 0.05$) from control, by Duncan's multiple range test. *Inset*, representative Northern blot.

TABLE 1

Effects of VIP, secretin, and nicotine on TH induction in the presence and absence of HL-725

BAC cells were maintained in culture for 5–7 days and then exposed to VIP, secretin, or nicotine, either alone or in the presence of 10 μM HL-725. After 48 hr of continuous exposure, TH activity was measured at saturating substrate and cofactor concentrations. TH-like immunoreactivity was determined by a spot immunolabeling assay (34). Values shown represent the mean ± standard error (three experiments).

Agent	Concentration	TH activity		TH-like immunoreactivity	
		−HL-725	+HL-725	−HL725	+HL725
	μM	nmol of CO_2/hr/10^6 cells		arbitrary units/μg of protein	
Control		3.5 ± 0.040	3.9 ± 0.087[a]	1.4 ± 0.10	1.8 ± 0.092
VIP	0.1	3.7 ± 0.079	4.4 ± 0.051		
	1	3.8 ± 0.039	4.9 ± 0.099[a]		
	5	4.3 ± 0.043[b]	5.6 ± 0.11[a]		
	10	5.5 ± 0.099[b]	10.0 ± 0.12[a]	2.3 ± 0.30[b]	5.2 ± 0.23[a]
Secretin	1	3.6 ± 0.12	4.2 ± 0.092		
Nicotine	1	3.9 ± 0.039	4.9 ± 0.091[a]		

[a] Significantly different ($p < 0.05$) from the respective values without HL-725 (Student's *t* test).
[b] Significantly different ($p < 0.05$) from control.

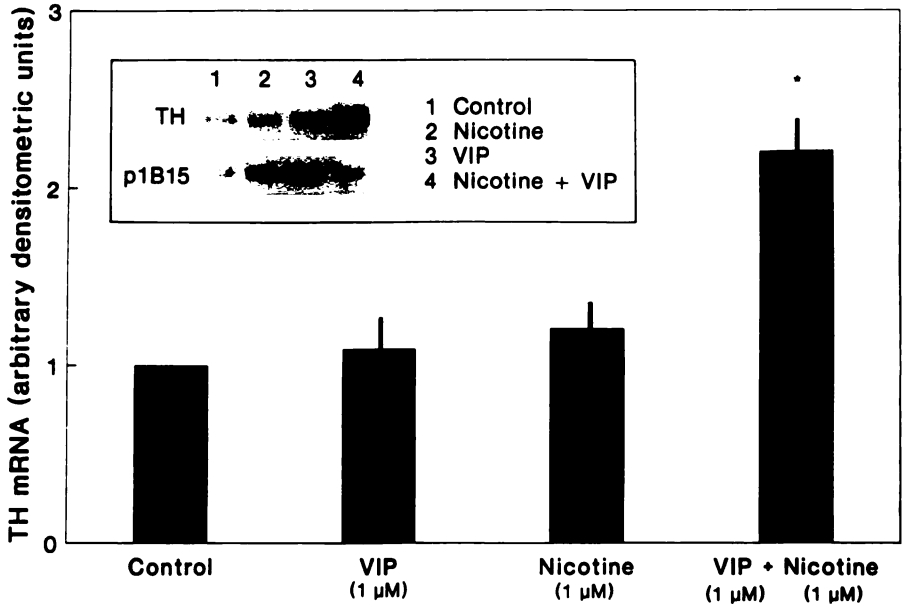

Fig. 5. Effect of VIP and nicotine together on TH mRNA in BAC cells. BAC cells were cultured for 5–7 days and then exposed to 1 μM VIP and 1 μM nicotine, separately or together. After 12 hr of exposure, the cells were harvested and the amount of TH mRNA was determined by Northern analysis. The amount of TH mRNA is expressed in arbitrary densitometric units relative to the amount of cyclophilin (p1B15) mRNA. Values represent the mean ± standard error (three experiments). *, Significantly different ($p < 0.05$) from control, by Duncan's multiple range test. *Inset*, representative Northern blot.

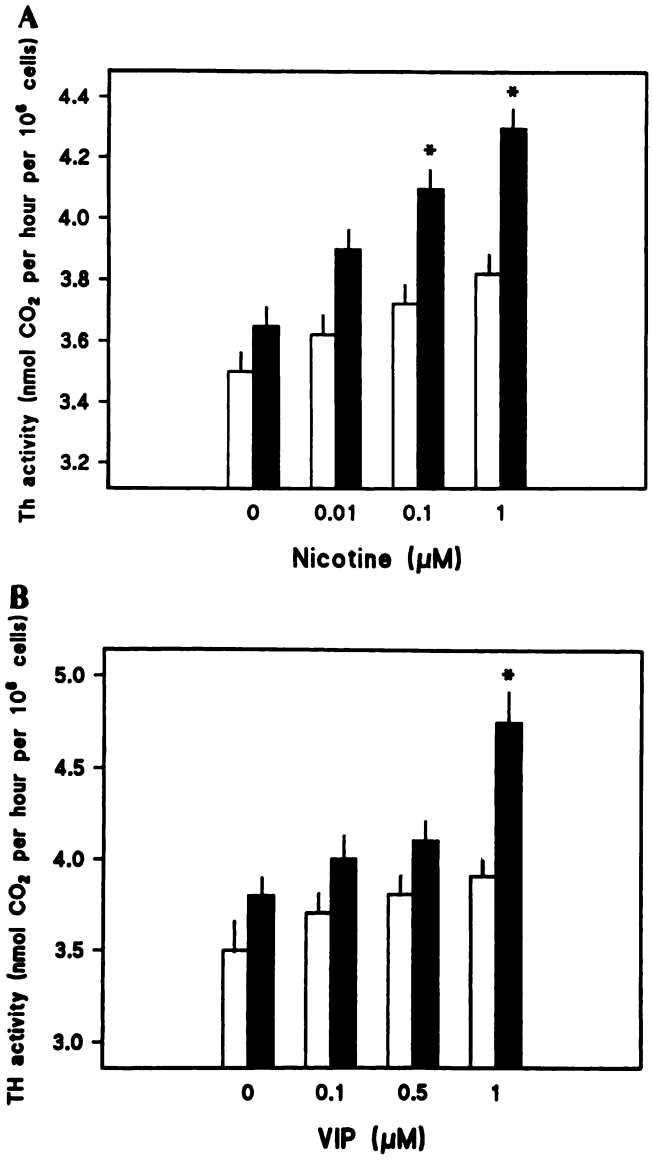

Fig. 6. Effect of VIP and nicotine together on TH activity. BAC cells were cultured for 5–7 days and then exposed for 48 hr to various concentrations of nicotine (A) or VIP (B), in the absence (□) or presence (■) of 1 μM VIP (A) or 1 μM nicotine. TH activity was measured at saturating substrate and cofactor concentrations. In each case, values represent the mean ± standard error (three experiments). *, Significantly different ($p < 0.05$) from the same concentration of nicotine without VIP (A) or VIP without nicotine (B), by Student's *t* test.

cAMP accumulation in BAC cells. The amount of cAMP in BAC cells after incubation with different agents for various periods of time was measured. Forskolin was the most potent agent, of those tested, with regard to stimulation of cAMP accumulation in BAC cells; after 5 min, a 20-fold [from 1.7 ± 0.038 to 34 ± 1.6 pmol of cAMP/10^6 cells (mean ± standard error; three experiments)] increase in cAMP was apparent, and after 1 hr a maximal, approximately 60-fold, increase was obtained. Even though the cAMP accumulation elicited by forskolin began to decline after 12 hr of exposure, a 25-fold increase over control values was still apparent after 48 hr. VIP (1 μM) alone produced a 1.5-fold increase in the cAMP concentration of BAC cells after 5 min of treatment (Fig. 7). Nicotine, at a dose of 100 μM, produced a 3.5-fold increase in cAMP accumulation over the control value after 5 min of treatment. The maximal accumulation of cAMP induced by nicotine or VIP was usually achieved between 5 and 10 min after application. VIP potentiated nicotine-induced cAMP accumulation after 5 and 60 min of exposure (Fig. 7). Thereafter, the effect declined and was not apparent after 3 hr of treatment. HL-725 (10 μM) potentiated the effects of nicotine and VIP on cAMP accumulation during a 5-min incubation [cAMP accumulation (pmol of cAMP/10^6 cells): control, 1.7 ± 0.027; HL-725, 2.1 ± 0.036; VIP (1 μM), 2.5 ± 0.038; VIP plus HL-725, 5.5 ± 0.11; nicotine (100 μM), 5.8 ± 0.21; nicotine plus HL-725, 6.8 ± 0.17 (mean ± standard error; three experiments)]. This potentiating effect of HL-725 also subsequently declined during the first 3 hr of exposure. The cAMP accumulation induced by 100 μM nicotine was completely blocked by 10 μM hexamethonium (Table 2). [*p*-chloro-D-Phe^6,Leu^{17}]-VIP, at a concentration of 10 μM, reduced not only the effect of 1 μM VIP (Table 2) but also that of 100 μM nicotine on cAMP accumulation (Table 2).

TABLE 2

Effects of hexamethonium and a VIP antagonist on nicotine-induced and VIP-induced cAMP accumulation and TH activity

BAC cells were maintained in culture for 5–7 days and then exposed to 100 μM nicotine or 1 μM VIP plus 10 μM HL-725, in the presence or absence of either 100 μM hexamethonium or 10 μM [*p*-chloro-D-Phe^6,Leu^{17}]-VIP. The cAMP content of BAC cells was measured after 5 min of exposure to the drugs, whereas the TH activity was determined after 48 hr of continuous treatment. Values (mean ± standard error, three experiments) represent the percentage of inhibition relative to values obtained in the presence of the respective agonist.

Antagonist	Inhibition of agonist-induced responses			
	Nicotine		VIP	
	cAMP accumulation	TH activity	cAMP accumulation	TH activity
	%			
VIP antagonist	47 ± 5[a]	62 ± 11[a]	45 ± 8[a]	54 ± 7[a]
Hexamethonium	100 ± 9[a]	97 ± 11[a]	2 ± 5	1 ± 8

[a] Significantly different ($p < 0.05$), by Student's *t* test, from the value in the presence of agonist.

Discussion

Nicotinic receptors play a role in the transsynaptic induction of adrenal TH (3–18). However, when we studied the effect of nicotinic receptor stimulation in primary cultures of BAC cells, with doses of nicotine or carbachol that induce significant release of catecholamines from these cells (10, 44), we found that the induction of TH mRNA and TH was only marginal (Figs. 1 and 2). This almost insignificant action of nicotine was not due to a drug-induced cellular inability to respond to other stimuli that cause a robust TH induction. In fact, forskolin and VIP, either alone or in the presence of a phosphodiesterase inhibitor, produced a marked induction of TH in the presence or absence of nicotine. Moreover, the main finding of our study is that VIP together with nicotine, at concentrations that alone were devoid of effect, increased the amount of TH mRNA and induced TH in BAC cells (Table 1; Fig. 5). The effect of VIP on TH induction was reduced by administration of the VIP antagonist [*p*-chloro-D-Phe^6,Leu^{17}]-VIP. Although, the action of nicotine was blocked by hexamethonium, it was partially inhibited also by the VIP antagonist. It is thus conceivable that, at least in part, nicotine produces its effect on TH induction by stimulating the release of VIP from cultured chromaffin cells; that is, nicotine *per se* may have a marginal direct effect on TH induction. In support of this hypothesis, Eiden *et al.*

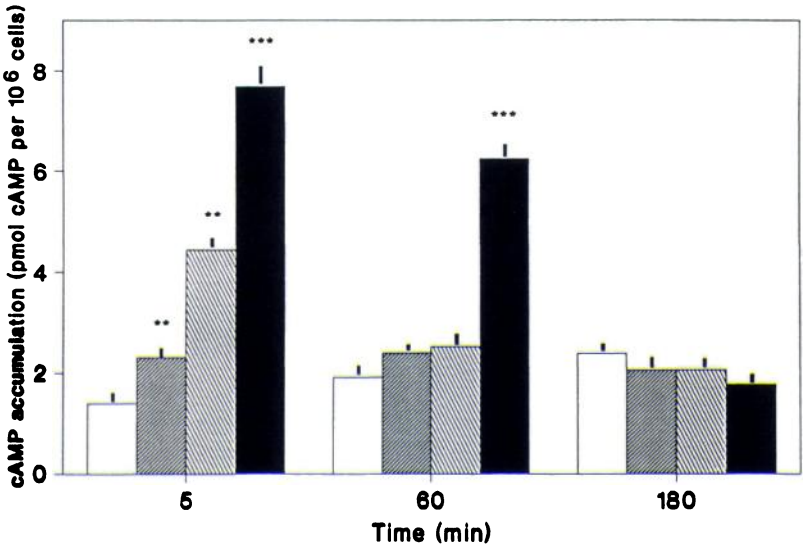

Fig. 7. Time courses of VIP- and nicotine-induced cAMP accumulation in BAC cells. BAC cells were cultured for 5–7 days and then exposed to 1 μM VIP (▨), 100 μM nicotine (▩), or these two agents together (■) for various times. The culture medium was then removed, and cAMP was extracted from the cells with 0.1 M HCl and measured by radioimmunoassay. Values shown represent the mean ± standard error (three experiments). Indicated values are significantly different (**, $p < 0.01$; ***, $p < 0.001$) from control (□), by Student's *t* test.

(45) demonstrated that VIP was detectable in BAC cells only after the cells were maintained in culture for 5 days and that nicotine, more efficiently than carbachol, elicited a release of VIP. Synergistic effects of VIP and nicotine have previously been described with regard to cAMP accumulation in BAC cells (42, 46); this finding is also confirmed by our results showing that simultaneous application of submaximal effective doses of VIP and nicotine generates a prompt, greater, and more prolonged increase in cAMP concentration than that elicited by either of the two agents alone.

Earlier studies with pharmacological and environmental stimuli showed that the induction of TH in the adrenal medulla is maximal 48 hr after stimulus application and that this induction is preceded by a relatively short-lasting activation of adenylate cyclase (4, 5). In our experiments with nicotine, an increase in TH activity was only observed after prolonged incubation with the drug. For example, it was necessary to expose BAC cells to 100 μM nicotine for at least 24 hr to obtain a small increase in TH activity measured 48 hr after stimulus application (Fig. 3). Thus, the fact that the relatively small effect of nicotine on TH mRNA and TH induction required extended exposure to large doses of the drug further suggests that the participation of an endogenous factor released from BAC cells, as a result of nicotinic receptor activation, may be required for the induction of TH in response to nicotine. Thus, it is possible that nicotine stimulates the release of VIP from these cells and that VIP, in turn, interacts with its receptors on adjacent BAC cells to trigger a chain of events that result in an increase in TH mRNA. The study of Eiden *et al.* (45), demonstrating that the content of VIP and the amount of this peptide released from BAC cells by nicotinic receptor stimulation may vary with the duration and conditions of the culture, suggests that the difference between our results and those of Stachowiak *et al.* (47) reflects the efficacy of nicotine in releasing VIP from BAC cell cultures. It is worth mentioning that, in their experiment, Stachowiak *et al.* (47) observed a 147% increase of TH mRNA after 6-hr treatment of the BAC cells with 50 μM ACh. However, the Stachowiak experiment was performed in primary culture of BAC cells maintained in serum-free chemically defined medium, whereas in our experiments 10% calf serum in Dulbecco's modified Eagle's medium was used as culture medium.

Here we have shown that 10 μM VIP increased the amount of TH mRNA in BAC cells after a relatively short period of treatment (Fig. 4). This time course is similar to that obtained by Wessels-Reicker *et al.* (30) in PC 12 cells, which showed that the peak increase in TH mRNA content (approximately 250%) was obtained after 8 hr of continuous exposure to 10 μM VIP. Moreover, the fact that, in our experiment, VIP generated almost a 3-fold increase in the amount of TH mRNA shows that VIP is able to induce TH with a greater efficacy than nicotine. Secretin, another peptide from the secretin-glucagon family, has receptors in the superior cervical ganglion but perhaps not on bovine adrenal medulla cells in culture (48) and did not induce BAC cell TH, thus emphasizing the specificity of the action of VIP in this system. Although in general there was a good correlation between the increase in TH mRNA and TH activity, the maximal percentage increase in TH activity was smaller than the maximal percentage increase in the amount of TH mRNA.

It could be argued that the small effect of high doses of nicotine on TH induction was due to receptor desensitization; long term exposure to nicotine has been shown to result in nicotinic receptor desensitization with regard to catecholamine release and VIP in cultured BAC cells (49), as well as in PC12 cells (50). However, according to our results, the induction of TH by nicotinic receptor agonists does not desensitize over time; in fact the induction of TH by nicotine does not attenuate, but rather increases, during persistent nicotine exposure. One possible explanation for our data is that large doses of nicotine are somehow toxic and produce a sustained leak of VIP from chromaffin cells, leading to an accumulation of VIP into the culture medium; thus, the time course of TH induction by nicotine may reflect the time required for VIP, released by nicotine, to accumulate in concentration sufficient to stimulate TH induction.

On the basis of *in vivo* studies into the effects of injection of nicotinic receptor agonists and antagonists on intact and denervated adrenal glands, it was concluded (7) that a prompt increase in the cAMP concentration of chromaffin cells mediates the induction of TH via activation and nuclear translo-

cation of cytosolic cAMP-dependent protein kinase (protein kinase A) (8, 9). Such importance of cAMP and of protein kinase activity as a third nuclear messenger (9, 10) in mediating TH expression is also supported by the present study. An intracellular accumulation of cAMP was shown to precede TH induction (5). In our experiments, not only did VIP increase the intracellular concentration of cAMP, but a 5-min exposure of BAC cells to 100 μM nicotine also increased the cAMP concentration of these cells, in a manner at least in part sensitive to VIP receptor inhibition. Thus, in primary BAC cells in culture, it appears that nicotinic receptor stimulation and VIP released from chromaffin cells, acting as an autocrine agent, facilitate the induction of TH via cAMP production. In support of this hypothesis, Wessel-Reicker *et al.* (30) showed that VIP induces TH via a cAMP-dependent mechanism in PC12 cells.

However, it might be unlikely that the cascade of events by which nicotinic receptor stimulation induces TH *in vivo* is similar to that observed *in vitro*. In fact, chromaffin cells *in vivo* contain only small amounts of VIP (45). Therefore, if VIP plays a role in the transsynaptic induction of TH, it is more likely to be the case that VIP released by the splanchnic nerve during paroxysmal neuronal activity facilitates the induction of TH elicited by concomitant nicotinic receptor stimulation. This does not imply that there should be a co-release from the same nerve terminal but implies a possible release from contiguous nerve terminals, one containing ACh and the other VIP.

Our data, together with earlier reports, allow us to speculate that VIP may be an important determinant of synaptic strength at cholinergic receptors of the adrenal medulla. Acting as a physiological modulator and allosterically reinforcing ACh action on nicotinic receptors, VIP may induce the accumulation of cAMP and the ensuing activation and nuclear translocation of protein kinase A, presumably resulting in the phosphorylation of the cAMP-responsive element-binding protein and activation of TH gene transcription. This action of VIP may be a permissive element in the mediation of TH induction triggered by splanchnic nerve activation.

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