Secretin and vasoactive intestinal peptide acutely increase tyrosine 3-monooxygenase in the rat superior cervical ganglion

(neuropeptide/catecholamine/sympathetic nervous system/gastrointestinal hormone)

NANCY Y. IP, CALVIN K. HO, AND RICHARD E. ZIGMOND*

Department of Pharmacology, 250 Longwood Avenue, Harvard Medical School, Boston, Massachusetts 02115

Communicated by Torsten N. Wiesel, August 26, 1982

ABSTRACT We have previously shown that stimulation of the preganglionic cervical sympathetic trunk leads to an acute increase in tyrosine hydroxylase (TyrOHase) activity in the rat superior cervical ganglion. This increase appears to be mediated in part by acetylcholine and in part by a second neurotransmitter. As a first step in an attempt to determine the identity of this noncholinergic transmitter, we have examined the ability of a number of neuropeptides to increase ganglionic TyrOHase activity *in vitro*. Secretin and vasoactive intestinal peptide (VIP) both stimulated TyrOHase activity, whereas angiotensin II, bombesin, bradykinin, cholecystokinin octapeptide, glucagon, insulin, luteinizing hormone-releasing hormone, [D-Ala², Met⁵]enkephalinamide, motilin, neurotensin, somatostatin, and substance P produced no effects. Secretin produced a significant increase in TyrOHase activity at 1 nM and a maximal elevation at 0.1 μ M. VIP produced a significant increase at 0.1 μ M and a near maximal effect at 10 μ M. Although secretin was about 2 orders of magnitude more potent than VIP, it produced a significantly smaller maximal increase in enzyme activity. Incubation of ganglia with both secretin (10 μ M) and VIP (10 μ M) produced an increase in TyrOHase activity that was not significantly different from that produced by VIP alone. The stimulatory effects of secretin and VIP were reversible within minutes after removal of the peptides. Neither incubation of intact ganglia with the cholinergic antagonists hexamethonium and atropine nor prior decentralization of ganglia altered the response to the peptides. Thus, the data demonstrate that secretin and VIP acutely increase TyrOHase activity in the superior cervical ganglion and suggest that they produce this effect by acting directly on ganglionic neurons. It remains to be determined whether secretin or VIP or a related peptide is released during preganglionic nerve firing and whether one or more of these peptides is responsible for the noncholinergic elevation of TyrOHase activity produced by preganglionic nerve stimulation.

Although acetylcholine generally has been thought to be the sole neurotransmitter in sympathetic ganglia, several lines of evidence now indicate that other substances also may act as transmitters in these ganglia. The strongest evidence comes from pharmacological studies showing that certain postsynaptic electrophysiological consequences of preganglionic nerve stimulation cannot be blocked by cholinergic antagonists. This observation was first made in the bullfrog paravertebral ganglion (1), and recently a similar phenomenon was reported in the rabbit superior cervical ganglion (SCG) (2). Interest in the possibility that certain neuropeptides may function as transmitters in some sympathetic ganglia arose after their localization in neural elements in these ganglia (3, 4). Studies on the bullfrog paravertebral ganglion have shown that a peptide that resembles luteinizing hormone-releasing hormone not only is present in the ganglion but also is released by preganglionic nerve stim-

ulation and mediates a noncholinergic slow excitatory postsynaptic potential (5, 6).

In the course of investigating the trans-synaptic regulation of tyrosine 3-monooxygenase (tyrosine hydroxylase; TyrOHase; EC 1.14.16.2), we recently found evidence for the existence of a noncholinergic transmitter in the rat superior cervical ganglion (SCG) (7, 8). Stimulation of the preganglionic nerve to this ganglion produces an acute increase in TyrOHase activity; however, only part of this increase can be blocked by cholinergic antagonists. Because immunoreactivity for four neuropeptides—substance P (9), enkephalin (10), vasoactive intestinal peptide (VIP) (11), and somatostatin (12)—has been demonstrated in the rat SCG, we have investigated the ability of these and a number of other neuropeptides to stimulate ganglionic TyrOHase activity. Of the 14 peptides examined, only secretin and VIP elevated TyrOHase activity.

MATERIALS AND METHODS

Male Sprague-Dawley rats (160–200 g) were housed in individual cages under controlled lighting (12 hr light:12 hr dark) with ad lib access to food and water for 1 wk prior to each experiment. Animals were killed by cervical dislocation, and both SCG were removed and desheathed. Individual ganglia were incubated at 37°C in a control medium consisting of Earle's balanced salt solution (GIBCO) supplemented with 0.1 mM tyrosine/0.1 mM EDTA and equilibrated with 95% O₂/5% CO₂. TyrOHase activity was assayed by measuring the rate of dopa formation. Dopa normally is not detectable in these ganglia due to its rapid decarboxylation to dopamine (13). Thus, to measure the rate of dopa synthesis, brocresine (150 μ M), an inhibitor of dopa decarboxylase (aromatic-L-amino-acid decarboxylase; EC 4.1.1.28), was added to the medium.

The standard procedure used to screen various peptides was to preincubate ganglia for 30 min in control medium and then to incubate for 30 min in medium containing brocresine and a high concentration of the peptide (10 μ M). Because lower concentrations of secretin and VIP were used in subsequent experiments, this procedure was modified to allow adequate time for the peptides to diffuse throughout the ganglion. Ganglia were preincubated for 60 min in medium containing the peptide, after which brocresine was added and the incubation was continued for a further 15 min. Bovine serum albumin (1 mg/ml, crystallized form; Miles) was added to the incubation medium in all experiments except those reported in Table 1 and Fig. 4.

At the end of the incubation period, ganglia were homogenized in 65 μ l of 1.65 M trichloroacetic acid/1 mM EDTA. The

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: TyrOHase, tyrosine hydroxylase; SCG, superior cervical ganglion; VIP, vasoactive intestinal peptide.

* To whom reprint requests should be addressed.

incubation media were added to the respective homogenates, and the combined samples were chilled on ice and then were centrifuged for 2 min in a Beckman Microfuge. The supernatants were stored at -20°C , and their dopa content was measured subsequently by HPLC and electrochemical detection (14).

To examine whether the action of secretin or VIP depended on the presence of preganglionic nerve terminals, animals were anesthetized with chloral hydrate (640 mg/kg, subcutaneous injection) 4 days prior to the experiment and a short section of each cervical sympathetic trunk was removed 5–7 mm proximal to the SCG. Sham-operated animals were used as controls in this experiment. To determine whether in intact ganglia the effect of either secretin or VIP was dependent on cholinergic stimulation, ganglia were incubated with each of the peptides in the presence and absence of hexamethonium (3 mM) and atropine (6 μM).

The data are expressed as the mean rate ($\pm$ SEM) of dopa synthesis per ganglion per 15 min or as a mean percentage ($\pm$ SEM) of the control rate. The significance of differences between groups was determined by using Student's two-tailed *t* test (for two means), except where noted.

All of the peptides used were purchased from Peninsula Laboratories (San Carlos, CA), except cholecystokinin octapeptide (Beckman Instruments), glucagon and insulin (Sigma), and VIP (from both Peninsula and Sigma). All peptides were synthetic products, except for glucagon and insulin, which were purified from pancreas. Atropine sulfate, bacitracin, chloral hydrate, hexamethonium bromide, and Trasylol were purchased from Sigma. Brocresine was purchased from Smith and Nephew.

Table 1. Effects of various peptides on dopa synthesis in the rat SCG

Peptide	Dopa synthesis, % control
Angiotensin II	94 $\pm$ 10
Bombesin	97 $\pm$ 6
Bradykinin	108 $\pm$ 9
Cholecystokinin octapeptide	91 $\pm$ 3
Glucagon	100 $\pm$ 4
Insulin	102 $\pm$ 5
Luteinizing hormone-releasing hormone	98 $\pm$ 3
[D-Ala ² , Met ⁵]Enkephalinamide	109 $\pm$ 3
Motilin	98 $\pm$ 8
Neurotensin	100 $\pm$ 4
Secretin	298 $\pm$ 16*
Somatostatin	95 $\pm$ 8
Substance P	104 $\pm$ 3
VIP	390 $\pm$ 36*

Each peptide was tested at a concentration of 10 μM . Ganglia were preincubated for 30 min in control medium and then were incubated with the peptide and brocresine for 30 min. The amount of dopa accumulated during the incubation period was determined. Three or four ganglia were studied for each peptide, except secretin and VIP ($n = 5$) and somatostatin and glucagon ($n = 7$). Rates of dopa synthesis are expressed as a percentage of the rate found in ganglia incubated for 30 min in peptide-free, brocresine-containing medium (mean $\pm$ SEM). The control rates averaged 32 ± 2 pmol of dopa formed per ganglion per 30 min. Substance P and somatostatin also were tested at 1 μM ; angiotensin II, at 0.1 and 1 μM ; bradykinin, luteinizing hormone-releasing hormone, and [D-Ala², Met⁵]enkephalinamide, at 0.01, 0.1, and 1 μM . No significant effects were found with any of these peptides at these concentrations.

* The rate of dopa synthesis was significantly greater in the presence of the peptide than in its absence; $P < 0.001$.

FIG. 1. Dose-response curves for the stimulation of TyroHase activity by secretin and VIP. Ganglia were preincubated with various concentrations of secretin (10^{-9} to 10^{-5} M) and VIP (10^{-8} to 3×10^{-5} M) or with control medium for 60 min prior to addition of brocresine. Dopa accumulation was measured during the subsequent 15 min. Each point is the mean $\pm$ SEM of four ganglia for VIP and of eight ganglia for secretin.

RESULTS

Incubation of ganglia with secretin (10 μM) for 30 min produced a 3-fold increase in the rate of dopa synthesis (Table 1). The chemically related peptide VIP (10 μM) also increased the rate of dopa synthesis (3.9-fold). On the other hand, angiotensin II, bombesin, bradykinin, cholecystokinin octapeptide, glucagon, insulin, luteinizing hormone-releasing hormone, (D-Ala², Met⁵)enkephalinamide, motilin, neurotensin, somatostatin, and substance P produced no effects when tested at the same concentration.

Dose-response curves were determined for the effects of both secretin and VIP on dopa synthesis (Fig. 1). Secretin significantly elevated dopa synthesis at 1 nM ($P < 0.025$) and caused a maximal effect at 0.1 μM . VIP significantly elevated dopa synthesis at doses above 0.1 μM and appeared to cause a near maximal stimulation at 10 μM . When data from two experiments were pooled, the effect of VIP at 0.1 μM also was significant ($P < 0.05$). We examined the ability of Trasylol and bacitracin to enhance the effects of secretin and VIP because these peptidase inhibitors have been shown to inhibit the degradation of a number of neuropeptides, including secretin (e.g., see refs. 15 and 16). However, Trasylol (500 kallikrein inhibitor units/ml) and bacitracin (20 μM) did not alter the response to submaximal concentrations of either secretin (3 nM) or VIP (0.3 μM) (Table 2).

Table 2. Lack of effect of Trasylol and bacitracin on the response to secretin and VIP

Condition	Dopa synthesis, pmol/ganglion/15 min	
	Control	Trasylol and bacitracin
No additions	25 $\pm$ 1	25 $\pm$ 1
Secretin	39 $\pm$ 3	38 $\pm$ 2
VIP	58 $\pm$ 5	53 $\pm$ 7

Ganglia were preincubated with the peptide in the presence or absence of two peptidase inhibitors (Trasylol at 500 kallikrein inhibitor units/ml and 20 μM bacitracin) for 60 min. Dopa synthesis was measured during the subsequent 15 min. The data are expressed as the mean $\pm$ SEM of four ganglia. The magnitude of the increase in dopa synthesis produced by secretin (3 nM) or VIP (0.3 μM) was not affected by the peptidase inhibitors.

Table 3. Lack of additivity of the effects of secretin and VIP

Condition	Dopa synthesis, pmol/ganglion/15 min
Control	24 ± 2
Secretin	60 ± 5*
VIP	95 ± 4*
Secretin and VIP	89 ± 10†

Ganglia were preincubated with either secretin (10 μ M), VIP (10 μ M), or secretin and VIP for 60 min prior to addition of brocresine. Dopa synthesis was measured during the subsequent 15 min. The data are expressed as the mean $\pm$ SEM of three or four ganglia.

* Comparison of secretin with VIP; $P < 0.005$.

† Comparison of secretin with secretin and VIP; $P < 0.05$.

Although VIP was less potent than secretin, it caused a significantly larger maximal stimulation of dopa synthesis ($P < 0.005$, Fig. 1 and Table 3). When ganglia were incubated simultaneously with high concentrations of both peptides (10 μ M), the increase in dopa synthesis was significantly higher than that produced by secretin alone but was not significantly different than that produced by VIP alone (Table 3).

To examine whether the effect of secretin or VIP was dependent on the presence of preganglionic nerve terminals, the cervical sympathetic trunks were cut in certain animals 4 days prior to the experiment. The preganglionic nerve terminals in the rat SCG have been shown to degenerate within this time interval (17). The increase in dopa synthesis produced by secretin in decentralized ganglia was indistinguishable from that seen in ganglia taken from sham-operated animals (Fig. 2). Incubation of intact ganglia with hexamethonium (3 mM) and atropine (6 μ M) also produced no change in the magnitude of the secretin effect (Fig. 2). Similar results were obtained for VIP (Fig. 3).

Exposure of ganglia to either VIP (5 μ M) or secretin (0.1 μ M) for 15 min in the presence of brocresine was adequate to elevate the rate of dopa synthesis by 2.8- and 1.9-fold, respectively. To determine whether these effects were reversible, ganglia were incubated with VIP without brocresine for 15 min and then were washed for various periods of time in VIP-free medium. Brocresine then was added to initiate the measurement of the rate of dopa synthesis. After 16 min of washing, the VIP-induced

FIG. 2. Effect of decentralization and cholinergic antagonists on the increase in dopa synthesis produced by secretin. Ganglia were removed from animals that had their cervical sympathetic trunks cut bilaterally (CSTX) or had been sham-operated (Sham) 4 days previously. The ganglia were preincubated with secretin (stippled bars) (1 μ M) or control (open bars) medium for 60 min prior to addition of brocresine. Dopa synthesis was measured during the subsequent 15 min. Other ganglia from sham-operated animals were preincubated with hexamethonium (Hex) (3 mM) and atropine (6 μ M) with or without secretin (1 μ M) for 60 min prior to addition of brocresine. Each value is the mean $\pm$ SEM of three or four ganglia.

FIG. 3. Effect of decentralization and cholinergic antagonists on the increase in dopa synthesis produced by VIP. The procedure followed and the abbreviations used are the same as those described in the legend to Fig. 2. VIP (hatched bars) concentration was 5 μ M. Each value is the mean $\pm$ SEM of three or four ganglia. Control, open bars.

elevation in dopa synthesis was decreased by 40% (Fig. 4). After 31 or 46 min of washing, the rate of dopa synthesis was at control values. The reversibility of the effect of secretin (0.1 μ M) was only examined after 46 min of washing, at which time it was at control values.

DISCUSSION

Although considerable information is available concerning the localization of different peptides in the nervous system, less is known about their effects on nervous tissue. In the present study, we examined the ability of 14 neuropeptides to alter the activity of TyrOHase in the rat SCG. This enzyme is of particular interest because it catalyzes the rate-limiting step in the biosynthesis of the neurotransmitter norepinephrine (18). Of the peptides studied, only secretin and VIP increased TyrOHase activity. These two peptides have a number of similarities. They are about the same molecular weight (consisting of 27 and 28 amino acids, respectively), have 9 identical amino acids in identical positions, and contain a number of similar amino acids (19). Both are secreted by cells in the small intestine and function as hormones in the gastrointestinal tract, in addition to being present in certain parts of the nervous system

FIG. 4. Reversibility of the effect of VIP on dopa synthesis. Ganglia were incubated in the absence (control) or presence of VIP (5 μ M) for 15 min, during which time their rate of dopa synthesis was measured. Other ganglia were preincubated for 15 min with VIP and then were washed for 1, 16, 31, or 46 min in VIP-free medium. Dopa synthesis was measured during the subsequent 15 min. Thus, in the group labeled 1-16 min, dopa synthesis was measured during the period from 1 min to 16 min after exposure to VIP. The effect of VIP on the rate of dopa synthesis was significantly decreased after a 16-min washing ($P < 0.05$, one-tailed t test) and was abolished after a 31-min washing. Each value is the mean $\pm$ SEM of three ganglia.

(3, 4, 20, 21). In pancreatic acinar cells, where their mechanism of action has been explored in some detail, the peptides both bind to the same two classes of receptors. However, one class has a higher affinity for secretin and the other has a higher affinity for VIP (22). Glucagon also has a number of chemical similarities to both secretin and VIP. Glucagon has 29 amino acids and shares 14 amino acid identities with secretin and 6 with VIP. However, in spite of these similarities, glucagon does not interact with either "secretin-preferring" or "VIP-preferring" receptors in the pancreas (23) nor does it increase TyrOHase activity in the SCG (Table 1).

Although both secretin and VIP stimulate TyrOHase activity, secretin is 2 orders of magnitude more potent than VIP (Fig. 1). On the other hand, VIP produces a significantly larger maximal effect. Incubation of ganglia with high concentrations (10 μ M) of secretin and VIP simultaneously showed that the effects of the two peptides were not additive. Because we know, on the basis of our previous studies with other agents, that the effect produced by the two peptides does not represent a maximal increase in TyrOHase activity (8, 13), this lack of additivity indicates that secretin and VIP do not act via independent mechanisms. Although we have no direct evidence as to whether secretin and VIP act via the same or different receptors, it is interesting to note that the effect produced by the combination of both peptides was equal to that produced by VIP alone. This result occurred in spite of the fact that the concentration of secretin used in this experiment was 100 times higher than that necessary to produce a maximal secretin effect. If both peptides were acting via a single class of receptor, the receptors under these conditions would be expected to be occupied primarily by secretin. Because the resulting increase in TyrOHase activity is similar to that produced by VIP rather than secretin, the simplest interpretation of these data is that secretin and VIP increase TyrOHase activity in the SCG via different receptors.

Previous studies have demonstrated that the activity of TyrOHase in a variety of tissues can be increased by two different biochemical mechanisms. One mechanism involves a long-term increase in the number of enzyme molecules, whereas the other involves an acute increase in the activity of preexisting molecules (for reviews, see refs. 24 and 25). The time course of the onset of the secretin and VIP effects and their relatively rapid reversibility are compatible with the peptides acting via the latter mechanism.

Because cholinergic agonists can increase TyrOHase activity acutely in the SCG (13, 26), a peptide could produce the same effect by promoting the release of acetylcholine from preganglionic nerve terminals. However, secretin and VIP did not require the presence of preganglionic nerve terminals to produce their effects. Furthermore, in intact ganglia the action of the peptides was not affected by cholinergic receptor blockers. These results suggest that both secretin and VIP act directly on ganglionic neurons to stimulate TyrOHase activity.

Although, to date, no studies have reported secretin in any sympathetic ganglion, VIP-like immunoreactivity has been found in neural processes in a number of these ganglia, including the rat SCG (11). The present study shows effects of both secretin and VIP in this ganglion. VIP has been shown previously to act on a number of autonomic effector tissues. For example, VIP produces vasodilation in the cat submandibular gland and considerable evidence indicates that VIP is the neurotransmitter that mediates the atropine-resistant vasodilation produced in this tissue by parasympathetic nerve stimulation (27). We have recently discovered a hexamethonium and atro-

pine-resistant component in the increase in TyrOHase activity produced by preganglionic nerve stimulation of the rat SCG (7, 8). Our results indicate that a second transmitter, in addition to acetylcholine, is released during this stimulation. Whether secretin or VIP or a related peptide is a neurotransmitter in the rat SCG and whether one or more of these peptides mediates the noncholinergic stimulation of TyrOHase activity remains to be determined.

We thank Dr. Margery Beinfeld, Dr. Armen Tashjian, and Dr. Stephen Sager for supplying us with some of the peptides studied. This research was supported by Grant NS 12651 from the National Institutes of Health and by a Research Career Scientist Award (MH 00162) from the National Institute of Mental Health.

1. Nishi, S. & Koketsu, K. (1968) *J. Neurophysiol.* **31**, 109–121.
2. Ashe, J. H. & Libet, B. (1981) *J. Physiol. (London)* **320**, 333–346.
3. Burnstock, G., Hökfelt, T., Gershon, M. D., Iversen, L. L., Kosterlitz, H. W. & Szurszewski, J. H. (1979) *Neurosci. Res. Program Bull.* **17**, 424–442.
4. Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J. M. & Schultzberg, M. (1980) *Nature (London)* **284**, 515–521.
5. Jan, Y. N., Jan, L. Y. & Kuffler, S. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1501–1505.
6. Jan, Y. N., Jan, L. Y. & Kuffler, S. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5008–5012.
7. Ip, N. Y., Perlman, R. L. & Zigmond, R. E. (1982) *Trans. Am. Soc. Neurochem.* **13**, 106 (abstr.).
8. Ip, N. Y., Perlman, R. L. & Zigmond, R. E. (1982) *Proc. Natl. Acad. Sci. USA*, in press.
9. Hökfelt, T., Elfvin, L. G., Schultzberg, M., Goldstein, M. & Nilsson, G. (1977) *Brain Res.* **132**, 29–41.
10. Schultzberg, M., Hökfelt, T., Terenius, L., Elfvin, L.-G., Lundberg, J. M., Brandt, J., Elde, R. P. & Goldstein, M. (1979) *Neuroscience* **4**, 249–270.
11. Hökfelt, T., Elfvin, L.-G., Schultzberg, M., Fuxe, K., Said, S. I., Mutt, V. & Goldstein, M. (1977) *Neuroscience* **2**, 885–896.
12. Hökfelt, T., Elfvin, L.-G., Schultzberg, M., Elde, R., Goldstein, M. & Luft, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3587–3591.
13. Ip, N. Y., Perlman, R. L. & Zigmond, R. E. (1982) *J. Pharmacol. Exp. Ther.*, in press.
14. Erny, R. E., Berezo, M. W. & Perlman, R. L. (1981) *J. Biol. Chem.* **256**, 1335–1339.
15. Boehm, M., Lee, Y. & Chey, W. Y. (1973) in *Endocrinology of the Gut*, eds. Chey, W. Y. & Brooks, F. P. (Slack, Thorofare, NJ), pp. 310–319.
16. Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120.
17. Raisman, G., Field, P. M., Ostberg, A. J. C., Iversen, L. L. & Zigmond, R. E. (1974) *Brain Res.* **71**, 1–16.
18. Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) *J. Pharmacol. Exp. Ther.* **148**, 1–8.
19. Mutt, V. (1982) in *Vasoactive Intestinal Peptide*, ed. Said, S. I. (Raven, New York), pp. 1–10.
20. Mutt, V., Carlquist, M. & Tatemoto, K. (1979) *Life Sci.* **25**, 1703–1707.
21. O'Donohue, T. L., Charlton, C. G., Miller, R. L., Boden, G. & Jacobowitz, D. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5221–5224.
22. Christophe, J. P., Conlon, T. P. & Gardner, J. D. (1976) *J. Biol. Chem.* **251**, 4629–4634.
23. Gardner, J. D., Conlon, T. P. & Adams, T. D. (1976) *Gastroenterology* **70**, 2935.
24. Zigmond, R. E. & Bowers, C. W. (1981) *Annu. Rev. Physiol.* **43**, 673–687.
25. Weiner, N., Lee, F.-L., Barnes, E. & Dreyer, E. (1977) in *Structure and Function of Monoamine Enzymes*, eds. Usdin, E., Weiner, N. & Youdim, M. B. H. (Dekker, New York), pp. 109–148.
26. Ikeno, T., Dickens, G., Lloyd, T. & Guroff, G. (1981) *J. Neurochem.* **36**, 1632–1640.
27. Lundberg, J. M. (1981) *Acta Physiol. Scand. Suppl.* **496**, 1–57.