Vasoactive Intestinal Polypeptide

A Neurotransmitter for Lower Esophageal Sphincter Relaxation

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Abstract. The effect of rabbit vasoactive intestinal polypeptide (VIP) antiserum on in vitro relaxation of the lower esophageal sphincter (LES) was studied in 10 cats. The stomach and esophagus were opened along the lesser curvature of the stomach and stripped of mucosa. Consecutive strips were cut and mounted in a 2.5-ml muscle chamber. They were perfused with Tyrode's solution and oxygenated continuously. After equilibration for 1 h, perfusion was stopped and one strip from the lower esophageal sphincter region was incubated in solution that contained 12–25 parts of VIP antiserum per 1,000 of Tyrode's solution, while a second strip was incubated in a solution of normal rabbit serum at the same concentration. A third strip was maintained in Tyrode's solution for the duration of the experiment. After a 1-h incubation, the strips were stimulated with 6-s square wave trains of 0.1-, 0.2-, 0.4-, and 0.8-ms pulses at 1, 2, and 5 Hz. These stimulation parameters produced LES relaxation that was completely blocked by tetrodotoxin but not by atropine or phentolamine. The strips incubated in Tyrode's solution or in normal serum relaxed reliably and consistently at all levels of stimulation. In the antiserum-treated strips, LES relaxation in response to all stimuli was significantly inhibited. Strips treated with normal serum were relaxed in a dose-dependent fashion by 10^{-7} and 10^{-6} M VIP, whereas the antiserum inhibited the relaxation induced by 10^{-7} M, but not by 10^{-6} M, VIP. Stimulation with two successive 15-min trains of electrical pulses (2 ms, 5 Hz) separated by 30 min of rest released increasing amounts of VIP into the bathing solution. VIP released during the second train of electrical stimulation was significantly ($P < 0.05$) greater than in control conditions. In the cat LES, VIP antiserum inhibits the relaxation induced by exogenous VIP or by electric stimulation of nonadrenergic, noncholinergic inhibitory nerves at a level that causes the release of VIP. These findings are consistent with the hypothesis that VIP may be an inhibitory neurotransmitter responsible for LES relaxation.

Introduction

The existence of an intrinsic neural inhibitory system in the gut wall has been recognized since the turn of the century (1–3). The inhibitory neurotransmitter is neither an adrenergic nor a cholinergic substance, but the precise nature of the neurotransmitter or neurotransmitters is not yet known. Both ATP (4, 5) and vasoactive intestinal polypeptide (VIP)[1] (6–8) have been proposed as possible neurotransmitters of the inhibitory nerves of the gut. In the lower esophageal sphincter (LES), however, VIP seems a more likely candidate than ATP (8, 9). The purpose of this study was to assess the possible role of VIP as an inhibitory neurotransmitter in the cat LES in vitro.

Methods

This study was performed on adult cats of both sexes who weighed between 2.5 and 4.5 kg. The animals were anesthetized with intramuscular ketamine hydrochloride (30 mg/kg). The stomach, LES, and esophagus were exposed and a 5-cm long segment of esophagus was measured and marked. The stomach, LES, and esophagus were removed together and opened with an incision along the lesser curvature of the stomach. The mucosal lining was rapidly rinsed with tap water to remove any gastric contents adhered to it, and the open stomach and esophagus were pinned mucosal side up on a wax block with the esophageal segment stretched to 5 cm, its in vivo length. The location of the squamo-columnar junction was marked on the specimen with a silk suture running through the thickness of the muscle as well as the mucosa.

A thickening of the muscle layer was evident when the thickness of the esophageal wall along the longitudinal cut was examined. We have shown previously (10) that the thickening of the wall and the location

1. *Abbreviations used in this paper:* LES, lower esophageal sphincter; VIP, vasoactive intestinal polypeptide.

of the squamo-columnar junction correspond to the in vivo location of the high pressure zone and that circular muscle taken from this region exhibits mechanical properties distinct from those of the rest of the esophagus.

The tissue was covered with oxygenated Tyrode's solution at room temperature and the mucosal lining was removed under the microscope with sharp dissection at the level of the submucosa. Care was taken not to remove from the muscle layer the stitch that marked the location of the squamo-columnar junction. The stitch was usually immediately proximal to the sling fibers of the stomach, which were clearly visible when the mucosa was removed. Consecutive strips were cut 2 mm apart and perpendicular to the esophageal lumen with razor blades held in a block. The strip that contained the suture, three strips proximal to it, and two strips distal to it were identified and cut in half. The resultant 12 specimens were mounted in separate muscle chambers. The design of a muscle chamber is shown in Fig. 1. Temperature was maintained by a flow of perfusate through a constant-temperature glass coil heat exchanger. Muscles were mounted on an electrode holder insert outside the bath, and the whole assembly was then lowered into the bath. The electrode-holder insert occupied all but 2.5 ml of the volume of the chamber. When the bath was used in the continuous perfusion mode, suction was applied to an overflow port in the top of the bath to maintain a constant level of solution in the chamber. Suction was sometimes also applied to an opening in the bottom of the bath to completely drain the chamber. Stretch was applied through vertical movement of the

force transducer by a microscope stage with a vernier accurate to within 0.1 mm. Vibrations produced by bubbling oxygen in the chambers were abolished by oxygenating the solution in a separate channel cut in the back of the electrode holder insert, communicating with the main chamber where the tissues were mounted. The specimens were stimulated by platinum electrodes placed longitudinally on either side of the specimen. When substances used, such as antiserum, were available in limited quantities, perfusion was stopped and a fixed amount was kept in the chamber as each chamber was individually oxygenated. Stopping the perfusion caused no temperature change and did not affect the viability of tissues over periods of several hours. Thus, it was possible to add test substances directly to individual 2.5-ml chambers rather than to large volumes of perfusate solution.

Experimental protocol. At the beginning of the experiment, the chamber, electrode inserts, electrodes, syringes, and vials were carefully rinsed with 2.5% bovine serum albumin solution to avoid binding of VIP to plastic or metal surfaces.

In each animal, four to six strips from the LES region that exhibited essentially identical high resting tension and electrically induced relaxation were used for the experiments. After the strips were mounted in the bath, they were quickly stretched to a resting tension of 2.5–3.0 g and equilibrated for 1–2 h while being perfused continuously with oxygenated Tyrode's solution. The Tyrode's perfusate contained 137 mM NaCl; 12 mM $NaHCO_3$; 1.8 mM NaH_2PO_4; 2.7 mM KCl; 2.7 mM $CaCl_2$; 5.5 mM glucose; and 1.0 mM MgCl. The perfusate was equilibrated with
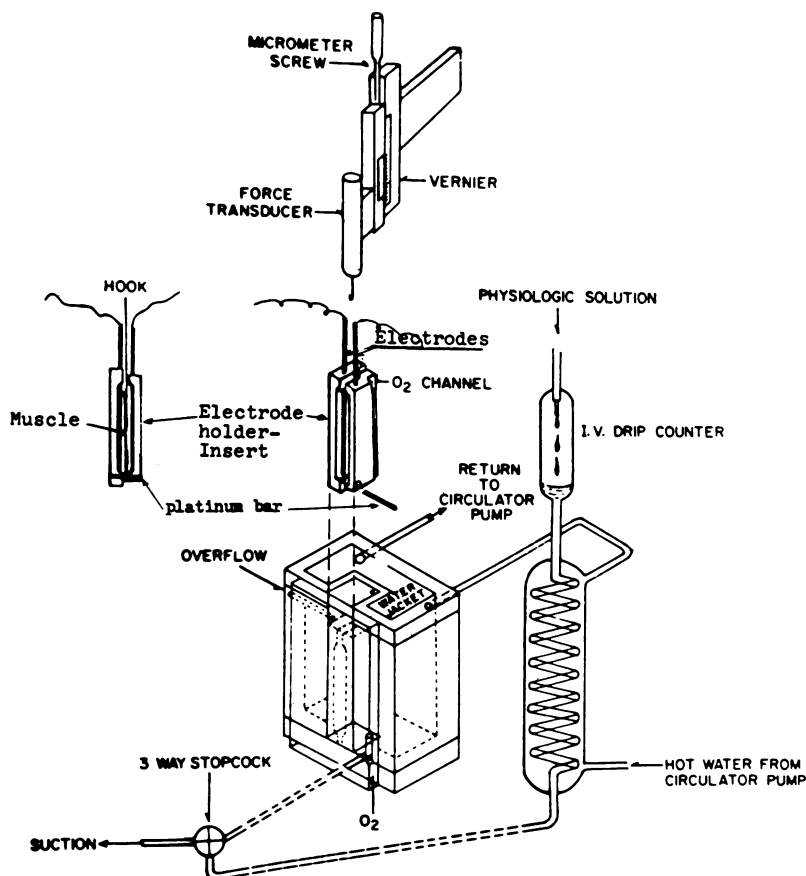
Figure 1. Muscle chamber: specimens are mounted in the electrode holder insert and secured to the platinum bar at the top of the insert. The insert is lowered into the chamber and secured in place with a screw (not shown). Physiologic solution is perfused through a drip counter into a heat exchanger and then into the bottom of the chamber. It is aspirated through an overflow hole at the top of the chamber. The chamber can be emptied rapidly by applying suction through a three-way stopcock in the bottom of the chamber. The upper end of the specimen is attached to a hook connected to a force transducer. The position of the transducer can be regulated through a micrometer screw with a vernier, which permits monitoring of deformation to within 0.1 mm. With the electrode holder in place, 2.5 ml of solution fills the chamber to overflowing.

a gas mixture that contained 95% O_2 and 5% CO_2, pH 7.2, at 37°C. During the equilibration, the tension developed in the LES strips increased, while it decreased in the gastric and esophageal strips. At the end of the experiment, when the LES strips were relaxed in 5 mM EDTA to determine their passive tension, all strips had ~1 g of passive tension. According to previously published data (10), 1 g passive tension is near the length of maximum active force development for strips of this type. After equilibration, the strips were stimulated with electrical field stimulation by 6-s trains of square waves, and response curves were obtained for frequencies varying between 1 and 10 Hz and pulse durations varying between 0.1 and 0.8 ms at supramaximal voltage (100 V). Strips from the LES area exhibited frequency-dependent and pulse-dependent relaxations for all stimuli tested.

At this point, three strips that had exhibited nearly identical frequency response curves were selected. The flow of perfusate through the muscle chambers was stopped and the strips were incubated for 2 h without a change of the solution in the chamber. One strip was incubated in Tyrode's solution that contained 12 parts of VIP antiserum per thousand (11). The VIP antiserum (7913) was specific for the carboxyl terminal 18–28 region of VIP and had negligible reactivity with glucagon, secretin, and gastric inhibitory polypeptide. The titer of this antibody for radioimmunoassay was 1:800,000 and the concentration of VIP that caused a 50% displacement of labeled VIP was 15 pM. When antibody 7913 at a dilution of 1:200 was used for immunofluorescent staining of VIP nerves, all staining was prevented by preincubation with 5×10^{-6} M VIP. A second strip was kept in the same concentration of normal serum from which the complement had been inactivated by heating at 56°C for 30 min. The third strip was incubated in Tyrode's solution alone. The three strips were stimulated at 15-min intervals with 0.1 ms, 2 Hz, 6-s trains of electrical stimuli; this stimulation produced approximately half maximal relaxation in LES strips. If after 1 h of incubation the strips incubated in antiserum showed no reduction in relaxation, the concentration of the antiserum and of the control serum was increased to 25 parts per 1,000 of Tyrode's solution. After 2 h of incubation, frequency response curves were again obtained. Cumulative dose response curves to VIP were then determined. At the end of the experiments, the muscles were exposed to high concentrations of EDTA to determine the passive tension. Relaxation was expressed as the ratio of the decrease in tension during electrical or pharmacological stimulation to the difference between the basal tension and the passive tension (10).

Tests were done in four additional animals to determine if VIP was released during electrical stimulation. After the LES strips were equilibrated for 2 h in physiologic solution, the solution was replaced. Then the chamber perfusion was stopped and, without a change of Tyrode's solution, the strips were stimulated continuously (0.2 ms, 5 Hz) for 15 min. At the end of the 15-min interval, the bath perfusate was collected, the chambers were rinsed for 30 min with Tyrode's solution, and the procedure was repeated. The bath perfusate was collected in vials and kept in ice. The vials were stored at −80°C until radioimmunoassay was performed to determine VIP content.

Data are expressed as mean±SE. Student's t tests were used to determine the statistical significance of differences between the means.

Results

Electrical stimulation of LES strips produced decreased tension that was related to frequency and duration of the impulse. Fig. 2 shows frequency response curves (1–5 Hz) of strips from the high-pressure point with the chamber continuously perfused
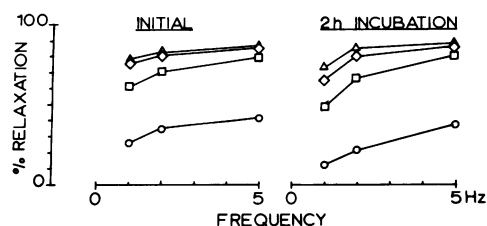

Figure 2. Frequency response curves of lower esophageal sphincter strips with the chamber continuously perfused (initial) and after 2 h of incubation in physiologic solution in an unperfused mode. The frequency response curves were obtained for 0.1, 0.2, 0.4, and 0.8 ms stimulus durations. A slight reduction in LES relaxation occurred after the 2-h incubation for only the 0.1 ms stimulus duration. The reduction, however, was not statistically significant. △, 0.8 ms; ◇, 0.4 ms; □, 0.2 ms; ○, 0.1 ms.

and after 2 h of incubation in physiologic solution in an unperfused mode. We obtained the frequency response curves for 0.1-, 0.2-, 0.4-, and 0.8-ms stimulus durations. The 2 h of incubation in an unperfused mode affected neither basal LES tension (5.1±0.63 mean±SEM g before 2 h of incubation, 5.3±0.73 g after 2-h incubation) nor the percentage relaxation induced by electrical stimulation. The relaxation induced by electrical stimulation was not blocked by 10^{-6} M concentrations of atropine, hexamethonium, phentolamine, or propranolol; this indicates that the inhibitory neural fibers that mediate the relaxation induced under these conditions are neither adrenergic nor cholinergic. These data are consistent with previously reported in vivo and in vitro studies (12, 13). 2 h of incubation without perfusion did not affect the action of inhibitory neural fibers.

Fig. 3 shows dose response curves to exogenous VIP after 2 h of incubation in 12–25 parts per 1,000 of VIP antiserum or control serum. 12 or 25 parts of VIP antiserum per 1,000 of Tyrode's solution blocked the relaxation induced by 10^{-7} M exogenous VIP but did not block the relaxation induced by
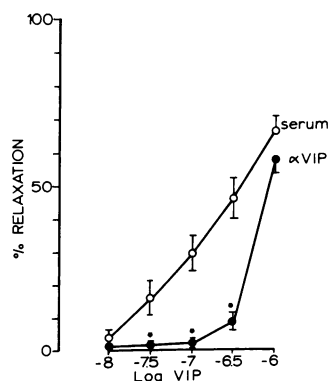

Figure 3. Dose-response curves to exogenous VIP after 2 h of incubation in 12–25 parts per 1,000 of VIP antiserum (α-VIP) or in the same concentration of normal rabbit serum.

10^{-6} M VIP. LES strips incubated in Tyrode's solution and in normal serum responded to exogenous VIP in an identical dose-dependent manner. These data are consistent with competitive inhibition of the effect of exogenous VIP by the VIP antiserum.

Fig. 4 shows frequency response curves for stimulus durations of 0.1, 0.2, 0.4, and 0.8 ms of LES strips previously incubated for 2 h in 12 or 25 parts of VIP antiserum per 1,000 of Tyrode's solution or the same concentration of normal serum in Tyrode's solution. Under all of our test conditions, incubation in normal serum did not significantly reduce relaxation with respect to the strips incubated in Tyrode's solution. VIP antiserum, on the other hand, significantly reduced relaxation under all stimulus conditions tested. The maximum percentage inhibition of relaxation occurred at 0.1- and 0.2-ms durations, as shown in Table I. Inhibition of relaxation decreased with increasing strength of stimuli. Relaxation induced by 0.8 ms, 5 Hz stimuli was reduced only 24% by the antiserum, whereas the effect of 1 ms, 10 Hz stimuli was not inhibited.

Fig. 5 shows the amount of VIP contained in the bath fluid in control conditions and after stimulation with two 15-min trains of 0.2-ms pulses at 5 Hz. The volume of tissue in the chamber was ~3% of the total volume of the chamber. After the first 15-min train, the increase in the amount of VIP contained in the bath was not significant, but after the muscle strips were stimulated with a second 15-min train, the amount of VIP present in the bath fluid was significantly greater than in the control.

Discussion

VIP causes LES relaxation by direct action on the LES muscle because the neural blocker, tetrodotoxin, does not block this effect (14, 15). VIP may be an inhibitory neurotransmitter. In our studies, highly specific VIP antiserum in doses sufficient to abolish LES relaxation induced by 10^{-7} M exogenous VIP sig-

Table I. Percentage Inhibition of LES Relaxation by VIP Antiserum (Mean±SEM)

	0.1 ms	0.2 ms	0.4 ms	0.8 ms
1 Hz	88±8	69±9	42±12	44±12
2 Hz	88±10	58±9	35±12	30±4
5 Hz	80±12	50±11	27±12	24±4

nificantly reduced relaxation induced by electrical stimulation with parameters that are known to stimulate nonadrenergic, noncholinergic inhibitory neural fibers. The inhibition of LES relaxation caused by the VIP antiserum was greatest for low duration pulses (0.1 and 0.2 ms) and decreased as pulse duration increased to 0.8 ms. A further increase in the strength of the stimulus completely overcame the effect of the antiserum. This is consistent with the finding that VIP is released in higher concentrations when stimuli of longer duration and higher frequency are used (6, 7); such stimuli may release enough VIP to surmount the effect of the antiserum. These data are consistent with a previous in vivo study (8) in which relaxation induced by vagal stimulation as well as relaxation induced by intravenous VIP were partially blocked by intraarterial infusion of VIP antiserum. In that study (8), the antiserum caused progressively less reduction in the relaxation induced by vagal stimulation as the frequency of stimulation increased. At 0.5 Hz the antiserum reduced the relaxation by ~50%, and at 10 Hz by ~20%. Similar reductions were achieved in the relaxation induced with exogenous VIP antiserum, with a 75% reduction in relaxation occurring at low doses (0.5 µg/kg) and a 55% reduction at high doses (4 µg/kg). To explain these results, the authors suggest that the antibody molecule may be too large to achieve a high enough concentration in the extracellular space at the site of the neuromuscular junction; and, furthermore, that with the antiserum concentrations at the neuromuscular junction, the antiserum and the released VIP may bind too slowly and with an affinity too low to produce a complete block. Alternatively, VIP may be one of two or more neurotransmitters released during neural stimulation. Our in vitro data agree with these in vivo observations, and they suggest a competitive mechanism of inhibition by the antiserum. Thus, as the amount of VIP released by stronger stimuli increases, the percentage inhibition
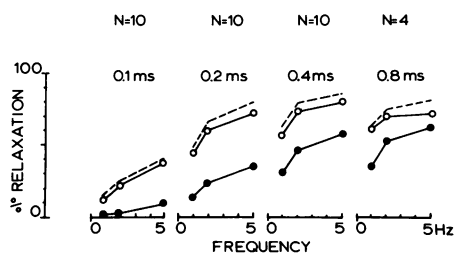

Figure 4. Frequency response curves of LES strips for stimulus durations of 0.1, 0.2, 0.4, and 0.8 ms after a 2-h incubation in normal physiologic solution (Tyrode), physiologic solution containing 25 parts per 1,000 of rabbit VIP antiserum (α-VIP) or the same concentration of normal rabbit serum (serum). VIP antiserum caused significant reductions in relaxation with all parameters tested ($P < 0.05$) with respect to both normal serum and Tyrode's solution. The slight reduction caused by incubation in serum was not statistically significant with respect to incubation with Tyrode's solution. - - -, Tyrode's solution; o, serum; •, α-VIP.
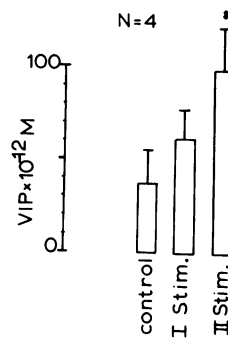

Figure 5. VIP content in the fluid chamber in control conditions, after stimulation with 15-min trains of 0.2 msec pulses at 5 Hz (first stimulus) and after a second 15-min stimulus train that followed 30 min of rest from the first stimulus. X indicates a statistically significant increase over control conditions.

produced by the antiserum decreases. The antiserum can block the effect of 10^{-7} M exogenous VIP but has no effect on 10^{-6} M exogenous VIP. It is possible that a VIP concentration $>10^{-7}$ M may be released locally at the receptor site. On the other hand, the mode of action of the VIP antiserum against exogenous VIP may be different from that of the endogenous VIP released during neural stimulation. When exogenous VIP is added to a chamber containing VIP antiserum in physiologic solution, the antiserum has full and free access to the exogenously added VIP. When VIP is released during neural stimulation, the antiserum may not, because the anti-VIP immunoglobulin is large, diffuse readily through the tissue to inhibit endogenous VIP released at the receptor site. The concentration of endogenous VIP released at the receptor site and blocked by the VIP antiserum may therefore be much lower than 10^{-7} M. No data are available on the diffusion of immunoglobulins through LES or other smooth muscle.

If the VIP antiserum diffuses readily through the LES muscle strip, fairly large amounts of VIP must be released locally to interact with the receptors. Our data on VIP release indicate that large amounts of VIP are released during electrical stimulation. Since the volume of the tissues is ~3% of the total fluid volume present in the chamber, the data indicate that during 15 min of continuous electrical stimulation, 3 pmol of VIP are released per cubic centimeter of tissue. In opossum LES, VIP content has been measured to be ~30 pmol per gram of tissue (S. Rattan, R. K. Goyal, J. H. Walsh, personal communications). The cat has more VIP nerves than do other species, and the esophagogastric junction exhibits a remarkably high VIP content, on the order of 500–600 pmol/g (16). Ultrastructural analysis of cat esophagus shows nerve profiles characterized by the presence of numerous, fairly large, dense core vesicles (16, 17). Thus, the concentration of VIP that we measured in the bath effluent is consistent with available data. It is interesting that after a first 15-min train of electrical stimuli, the amount of VIP that we collected from the effluent, although greater than in control conditions, was not significantly greater. The amount of VIP that we collected increased after a second 15-min train, and this increase over the control was statistically significant. This suggests that the tissue may trap some VIP and that, only when the tissue is saturated, VIP may overflow into the effluent.

In conclusion, our data strongly support the hypothesis that VIP may be an inhibitory neurotransmitter for the LES since it fulfills most of the criteria for a neurotransmitter: (a) VIP relaxes the LES by direct action and mimics the action of neural stimulation; (b) VIP is released by neural stimulation; (c) immunopharmacological antagonism of VIP by VIP antiserum antagonizes the effect of neural stimulation; and (d) the presence of VIP in nerve terminals in the LES region has been demonstrated previously (16).

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