Involvement of pituitary adenylate cyclase-activating peptide in opossum internal anal sphincter relaxation

SUSHANTA CHAKDER AND SATISH RATTAN
Division of Gastroenterology and Hepatology, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Chakder, Sushanta, and Satish Rattan. Involvement of pituitary adenylate cyclase-activating peptide in opossum internal anal sphincter relaxation. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G769–G777, 1998.—Despite its widespread distribution and significance in the gut, the role of pituitary adenylate cyclase-activating peptide (PACAP) in internal anal sphincter (IAS) relaxation has not been examined. This study examined the role of PACAP in nonadrenergic noncholinergic (NANC) nerve-mediated relaxation of IAS smooth muscle. Circular smooth muscle strips from the opossum IAS were prepared for measurement of isometric tension. The influence of PACAP and vasoactive intestinal peptide (VIP) antagonists and tachyphylaxis on the neurally mediated IAS relaxation was examined either separately or in combination. The release of these neuropeptides in response to NANC nerve stimulation before and after the nitric oxide synthase inhibitor N-nitro-L-arginine and NO was also investigated. Both PACAP and VIP antagonists caused significant attenuation of IAS relaxation by NANC nerve stimulation. The combination of the antagonists, however, did not have an additive effect on IAS relaxation. VIP tachyphylaxis caused significant suppression of IAS relaxation by NANC nerve stimulation. PACAP and VIP were found to be released by NANC nerve stimulation and exogenous NO. The data suggest the involvement of PACAP in IAS relaxation primarily by the activation of PACAP/VIP receptor and lack of its independent role in the relaxation. Furthermore, NO may regulate the presynaptic release of PACAP and VIP.

vasoactive intestinal polypeptide; nonadrenergic noncholinergic; nitric oxide synthase; inhibitory neurotransmitter; smooth muscle

STUDIES from different laboratories have shown that vasoactive intestinal peptide (VIP) plays a significant role in the nonadrenergic noncholinergic (NANC) nerve-mediated relaxation of the internal anal sphincter (IAS) (1, 17). On the other hand, part of the NANC nerve-mediated IAS relaxation is mediated via the nitric oxide synthase (NOS) pathway in different species including humans (3, 6, 13, 18, 21, 24, 27).

Recent studies have shown the presence of another neuropeptide, pituitary adenylate cyclase-activating peptide (PACAP) in different regions of the gastrointestinal tract (20, 25, 26, 28). Two forms of PACAP, PACAP-1(1–38) and PACAP-1(1–27), both amidated at the COOH terminus, have been isolated. The PACAP molecules show a significant homology with VIP and are considered to be the members of VIP-glucagon-secretin family of peptides (14). It has been suggested that PACAP exerts its actions via the activation of at least three types of receptors that belong to a subfamily of the seven-transmembrane-spanning G protein-coupled receptors. PACAP1 receptor is selective for PACAP and has 1,000 times more affinity for PACAP than VIP. PACAP2/VIP, and PACAP3/VIP receptors have equal affinity for PACAP and VIP (8). It has also been shown that PACAP plays a significant role in the gastrointestinal smooth muscle relaxation in response to NANC nerve stimulation (7, 9, 11). Furthermore, in humans, PACAP has been associated with the normal functioning of the gut, since a reduction in the PACAP immunoreactive neurons and levels have been shown in the gastrointestinal motility disorders, such as Hirschsprung’s disease (25). However, the role of PACAP in IAS relaxation has not been examined.

The purpose of the present investigation was to determine the role of PACAP in IAS relaxation by NANC nerve stimulation. This was carried out by investigating the influence of PACAP and VIP antagonists and tachyphylaxis in response to these neuropeptides on neurally mediated IAS relaxation either separately or in combination. To determine the regulation of PACAP and VIP release, we examined their release in response to NANC nerve stimulation before and after the NOS inhibitor.

MATERIALS AND METHODS

Preparation of smooth muscle strips. Studies were performed on circular smooth muscle strips obtained from adult opossums (Didelphis virginiana) of either sex after pentobarbital sodium anesthesia (40 mg/kg ip) and subsequent exsanguination. The entire anal canal was removed and transferred to a dissecting tray containing oxygenated (95% O2-5% CO2) Krebs solution. The composition of the Krebs solution was as follows (mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.10 glucose. The anal canal was cleaned of extraneous connective tissue and blood vessels and opened flat by an incision along the longitudinal axis. The tissue was then pinned flat, and the mucosa along with the submucosa was removed by sharp dissection. Circular smooth muscle strips were obtained from the whole circumference of the anal canal and divided into two equal strips (~2 × 8 mm). The muscle strips were tied at both ends with silk sutures for the measurement of isometric tension.

Measurement of isometric tension. IAS smooth muscle strips prepared as described above were transferred to thermostatically controlled 2-ml muscle baths (37°C) containing Krebs solution bubbled with a 95% O2-5% CO2 mixture. One end of the muscle strip was fixed to the bottom of the muscle bath with a tissue holder, and the other end was attached to an isometric force transducer (model FT03, Grass Instruments, Quincy, MA) for measurement of isometric tension. The tension of the smooth muscle strips was recorded on a Dynograph recorder (model R411, Beckman Instruments, Schiller Park, IL). After an equilibration period of 1 h, with intermittent washings, the optimal length (L0) and baseline of each smooth muscle strip were determined as previously described (15). Only those smooth muscle strips that develop...
To examine the effects of tetrodotoxin (TTX) or N-nitro-l-arginine (l-NNa) on the EFS-induced release of the peptides, the smooth muscle strips were incubated with these agents for 10 min before the application of EFS.

PACAP-38, PACAP-27, and VIP were measured by RIA using commercially available kits (Peninsula Laboratories) according to the supplier’s protocol. PACAP-38 and PACAP-27 were measured using a specific antibody for these peptides (human, ovine, or rat) raised in the rabbit. The antibodies had 100% cross-reactivity for the respective PACAPs and 0% for VIP and other related peptides. The lower limit of detection of the assay was 4.0 pg/tube, and the IC50 was 30 pg/tube. The VIP antibody used for the assay was raised against VIP (human, porcine, or rat) in the rabbit, and the antibody had 100% cross-reactivity with the native peptide and 0% cross-reactivity with PACAP-38 and PACAP-27. The detection limit for the assay was 3 pg/tube, and the IC50 was 34 pg/tube. The concentrations of the peptides in the medium were expressed as femtomoles per 100 μg of tissue weight per minute.

Binding of VIP to IAS smooth muscle membranes. Binding of VIP to IAS smooth muscle membranes was carried out according to the previously described method from our laboratory (4). Briefly, after its isolation, IAS smooth muscle was cleaned of all adherent tissues and small blood vessels. The tissue was cut into small pieces and homogenized in an ice bath in Tris buffer (25 mM, pH 7.4) containing 0.32 M sucrose by using an Ultraturrax tissue homogenizer (Teckman, Cincinnati, OH). The supernatant was separated and centrifuged at 50,000 g for 30 min at 4°C. The pellet was resuspended in Tris buffer (25 mM, pH 7.4) containing 2 mM EDTA and centrifuged at 50,000 g for 30 min. The pellet was washed twice with the same buffer by centrifugation in the same way. The final pellet was suspended in Tris buffer, and aliquots were stored at −80°C until used for VIP binding experiments. The protein contents of the membranes were determined by the method of Lowry et al. (12) using BSA as the standard.

Binding experiments were done in duplicate in Tris buffer (25 mM, pH 7.4) containing BSA (protease free, 1.5%), bacitracin (1 mg/ml), MgCl2 (2 mM), and EDTA (2 mM) in a final volume of 0.2 ml. The protein concentration of the membranes was 50 μg/ml, and the incubation with the radiolabeled VIP (45 pM) was continued for 10 min at 30°C with or without unlabeled VIP, PACAP-38, or PACAP-27. The incubation was stopped by adding 1 ml of ice-cold Tris buffer, and the membrane-bound radioactivity was separated from the unbound radioactivity by centrifugation at 16,000 g for 5 min. The pellets were washed again with the buffer by centrifugation, and the radioactivity in the pellets was counted in a gamma counter (Genesis, Laboratory Technology, Schaumburg, IL). The specific binding was determined by subtracting the radioactivity remaining in the presence of 10-6 M unlabeled VIP from the total radioactivity. The nonspecific binding was −25% of the total binding.

Drugs and chemicals. VIP (porcine), VIP-(10–28) (porcine), PACAP-(1–38) amide (PACAP-38 or PACAP), PACAP-27 amide, and PACAP-(6–38) amide (human, ovine, or rat) were from Bachem (Torrance, CA). l-NNa, N-nitro-l-arginine methyl ester, l-arginine hydrochloride, l-arginine, TTX, forskolin, and atropine methyl bromide were from Sigma Chemical (St. Louis, MO). Guanethidine monosulfate was from Ciba Pharmaceuticals (Summit, NJ). EDTA tetrasodium salt was from Fisher Scientific (Fair Lawn, NJ). 3-[125I]iodotyroisyl10-labeled VIP (2,000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). All other chemicals were of the highest purity available. Solutions of all the chemicals except forskolin were prepared in Krebs solution fresh on the day of the experiment.

G770 PACAP AND IAS RELAXATION

NACHNER NERVE STIMULATION WITH EFS. EFS was delivered from a Grass stimulator (model 588) connected in series to a Med-Lab Stimu-Splitter II (Loveland, CO). The Stimu-Splitter was used to amplify and measure the stimulus intensity using the optimal stimulus parameters for the neural stimulation (12 V, 0.5-ms pulse duration, 200–400 mA, 4-s train) at varying frequencies of 0.5–20 Hz. The electrodes used for the EFS consisted of a pair of platinum wires fixed at both sides of the smooth muscle strip. Neuromediated relaxation of IAS smooth muscle strips was quantified in response to different frequencies of EFS. The above-mentioned parameters of EFS are known to cause relaxation of IAS smooth muscle via the selective activation of NANC myenteric neurons. All the experiments were done in the presence of atropine (10−6 M) and guanethidine (3 × 10−6 M).

To investigate the role of PACAP and VIP in the neurally mediated relaxation of IAS smooth muscle, we examined the influence of PACAP antagonists, PACAP-(6–38) and PACAP-(6–27), and VIP antagonist, VIP-(10–28), on IAS relaxation in response to EFS. In another protocol, to examine the combined role of PACAP and VIP, the influence of PACAP-(6–38) plus VIP-(10–28) on the EFS-induced IAS relaxation was evaluated. The influence of VIP and PACAP tachyphylaxis on the neurally mediated IAS relaxation was also examined.

Drug responses. To determine the influence of PACAP or VIP antagonist or another neurohumoral blocking agent, the control concentration response to a given peptide or an agonist was tested first, followed by its repetition in the presence of the antagonists in the muscle bath. All the antagonists were allowed to maintain contact with the tissues for 10 min before testing the effects of the agonists.

Tachyphylaxis with PACAP or VIP was achieved by repeated administration of single doses of PACAP or VIP (10−6 M). Immediately after recovery of the response to a given dose of the peptides, the tissue was challenged repeatedly with the same dose until the response was almost abolished. This usually required four or five repeated administrations of the peptides to achieve complete tachyphylaxis.

At the end of each experiment, the smooth muscle strips were treated with 5 mM EDTA to establish the maximal relaxation (1). In all of these experiments, each smooth muscle strip served as its own control.

Measurement of PACAP and VIP release during EFS. For the measurement of the release of VIP and PACAP from the smooth muscle strips during the EFS, the muscle strips were incubated in 2-ml muscle bath filled with oxygenated Krebs solution containing 0.1% BSA, 20 mM bacitracin, and 1,000 µM aprotonin. After a 1-h equilibration period at 37°C, the muscle strips were incubated in 2-ml muscle bath filled with oxygenated Krebs solution containing 0.1% BSA, 20 mM bacitracin, and 1,000 µM aprotonin. After the final wash, the muscle strips were incubated for 10 min in the Krebs solution, and the perfusate was collected for measurement of basal release of the peptides. To examine the release of the peptides in response to EFS, the muscle bath perfusates were collected immediately after the termination of EFS (1-min train at 1 and 10 Hz). The smooth muscle strips were allowed to stand for 45 min with intermittent washings before another 10-min sample was collected for the measurement of basal peptide release, which was followed by a stimulation period of 1 min. All the samples were collected in siliconized tubes and immediately stored at −80°C until used for the assay of the peptides.
were prepared as described previously (21). Forskolin was dissolved in ethanol and diluted in Krebs solution.

Data analysis. The responses to EFS and other relaxants were expressed as the percentage of maximal relaxation caused by 5 mM EDTA. The results are expressed as means ± SE. Statistical significance between different groups was determined using the t-test, and P < 0.05 was considered significant.

RESULTS

Effects of PACAP antagonist PACAP-(6—38) on neurally mediated relaxation of IAS smooth muscle strips caused by EFS. EFS caused a frequency-dependent fall in the basal tension of IAS smooth muscle that was significantly attenuated by PACAP-(6—38). In the control experiments, 1, 2, and 5 Hz of EFS caused 48.8 ± 4.6, 59.2 ± 3.1, and 67.8 ± 3.1% falls in the basal tension of the smooth muscle strips. After pretreatment with 3 × 10⁻⁵ M PACAP-(6—38), these relaxation responses were significantly reduced to 19.5 ± 1.0, 36.2 ± 2.9 and 43.7 ± 6.9%, respectively (n = 9; P < 0.05; Fig. 1A).

Influence of VIP antagonist VIP-(10—28) on EFS-induced fall in basal tension of IAS smooth muscle strips by EFS. Similar to the PACAP antagonist, the VIP antagonist VIP-(10—28) also caused a significant inhibition of IAS relaxation caused by different frequen-
cies of EFS. In the controls, 0.5, 1, 2, and 5 Hz of EFS caused 34.8 ± 3.3, 48.8 ± 4.6, 59.2 ± 3.1, and 67.8 ± 3.1% falls in the basal tension of IAS. After pretreatment of the smooth muscle strips with 3 × 10⁻⁵ M VIP-(10–28), the fall in IAS tension with the same frequencies of EFS was significantly attenuated to 5.4 ± 1.5, 21.6 ± 7.5, 43.4 ± 6.0, and 54.5 ± 6.0%, respectively (n = 9; P < 0.05; Fig. 1B).

Both PACAP-(6–38) and VIP-(10–28) had similar effects on the basal IAS tone: an initial rise that lasted for 6–8 min followed by the recovery of the tone toward the pretreatment levels.

Effect of VIP-(10–28) plus PACAP-(6–38) on EFS-induced fall in basal tension of IAS smooth muscle. To determine the independent role of VIP and PACAP in IAS relaxation and the nature of VIP and PACAP receptors in IAS, we examined the effects of VIP-(10–28) and PACAP-(6–38) individually and in combination on IAS relaxation by different frequencies of EFS. In the control experiments, 0.5, 1, 2, and 5 Hz of EFS caused 40.4 ± 2.8, 57.2 ± 4.0, 65.7 ± 3.9, and 71.5 ± 3.6% falls in the basal IAS tension. After VIP-(10–28) (3 × 10⁻⁵ M) pretreatment, the fall in the basal IAS tension with the same frequencies of EFS was significantly reduced to 5.4 ± 1.5, 21.6 ± 7.5, 43.4 ± 6.0, and 54.5 ± 6.0%, respectively (P < 0.05; Fig. 1C). These values after VIP-(10–28) plus PACAP-(6–38) (3 × 10⁻⁵ M) were 8.2 ± 0.9, 20.3 ± 3.6, 33.3 ± 6.8, and 42.7 ± 8.4%, respectively. These relaxations were not significantly different from those observed in the presence of VIP-(10–28) or PACAP-(6–38) alone (Fig. 1C). The results suggest that VIP and PACAP released endogenously in response to EFS share a common receptor in causing IAS smooth muscle relaxation.

Effect of PACAP-(6–27) on EFS-induced IAS relaxation. In a separate series of experiments, we examined the effects of PACAP-(6–27), another PACAP antagonist, on IAS relaxation caused by different frequencies of EFS. Like PACAP-(6–38), PACAP-(6–27) also caused a rightward shift of the control EFS frequency-response curve. In control experiments, 1, 2, and 5 Hz of EFS caused 52.0 ± 7.4, 61.9 ± 3.8, and 69.2 ± 1.4% falls in IAS tension. After pretreatment with 3 × 10⁻⁵ M PACAP-(6–27), IAS smooth muscle relaxation was significantly attenuated to 19.8 ± 6.1, 40.8 ± 8.6, and 57.4 ± 6.1%, respectively (n = 4; P < 0.05; Fig. 2).

Effect of VIP and VIP plus PACAP tachyphylaxis on IAS relaxation by EFS. To further determine the role of PACAP and VIP on neurally mediated IAS relaxation, we examined the influence of their respective tachyphylaxes on IAS relaxation by different frequencies of EFS. First of all, to determine the specificity of their actions, we investigated the effects of PACAP and VIP before and after the tachyphylaxis. The fall in IAS tension by PACAP was nearly abolished by PACAP tachyphylaxis selectively, since VIP tachyphylaxis had no significant effect on PACAP responses (Fig. 3A). In control experiments, the fall in IAS tension with 3 × 10⁻⁷ and 10⁻⁶ M PACAP was 49.0 ± 6.5 and 58.7 ± 6.4%, respectively. In the presence of PACAP tachyphylaxis, the fall in IAS tension with same concentrations of PACAP was 5.8 ± 1.9 and 7.5 ± 2.2%, respectively. On the other hand, these responses in the presence of VIP tachyphylaxis were 33.6 ± 6.5 and 51.6 ± 5.9%, respectively (Fig. 3A).

Interestingly, however, the fall in IAS tension by VIP was equally attenuated by both VIP and PACAP tachyphylaxes (Fig. 3B). In the control experiments, 3 × 10⁻⁷ and 10⁻⁶ M VIP caused 49.5 ± 5.2 and 64.0 ± 1.7% falls in basal IAS tension, respectively. In the presence of VIP tachyphylaxis, the same concentrations of VIP caused 3.9 ± 1.3 and 8.0 ± 0.29% falls in IAS tension, respectively. The fall in IAS tension with 3 × 10⁻⁷ and 10⁻⁶ M VIP after PACAP tachyphylaxis was significantly antagonized to 5.0 ± 0.3 and 9.2 ± 2.2%, respectively (P < 0.05; n = 4; Fig. 3B).

We next examined the influence of VIP and PACAP tachyphylaxes on the NANC nerve-mediated IAS relaxation. In the control experiments, 0.5, 1, 2, and 5 Hz of EFS caused 36.2 ± 4.4, 53.4 ± 5.3, 65.5 ± 4.8, and 76.6 ± 3.8% falls in the basal tension of IAS, respectively. After VIP tachyphylaxis, the percent fall in IAS tension with the same frequencies of EFS was 26.0 ± 3.3, 38.5 ± 3.5, 47.3 ± 4.8, and 64.6 ± 2.1%, respectively. The combination of VIP and PACAP tachyphylaxis provided interesting and surprising data. The combined tachyphylaxis, rather than producing a further inhibition of IAS relaxation, caused the reversal of the inhibition toward the normal EFS responses. In the presence of VIP plus PACAP tachyphylaxis, the fall in the basal tension of IAS in response to 0.5, 1, 2, and 5 Hz was 46.9 ± 5.3, 61.4 ± 6.1, 65.6 ± 8.4, and 77.8 ± 5.9%, respectively, and was not significantly different from controls (P > 0.05; n = 5; Fig. 3C).

Data on the fall in the basal tension of IAS in response to EFS when the protocol was reversed; i.e., PACAP tachyphylaxis followed by VIP tachyphylaxis

![Graph](image-url)
was similar. In these experiments, percent fall in basal IAS tension by different frequencies of EFS in the presence of combined tachyphylaxis was 49.9 ± 11.7, 61.7 ± 8.2, 69.0 ± 8.2, and 73.3 ± 7.2%, respectively. These values were found not to be significantly different from their counterpart control values (P > 0.05; n = 5; Fig. 3C).

Typical effects of EFS, PACAP, and VIP before and after PACAP and VIP antagonists and tachyphylaxis are shown in Fig. 4.

Effects of antagonists and tachyphylaxis of PACAP and VIP on fall in basal IAS tension by NO and forskolin. To examine the specificity of the actions of PACAP and VIP antagonists and their respective tachyphylaxes, the effects of NO and forskolin on the basal IAS tension were investigated before and after these treatments. Both NO (Fig. 5) and forskolin (Fig. 6) caused concentration-dependent relaxation of IAS smooth muscle that was not modified by either the presence of PACAP and VIP antagonists or tachyphylaxis (Figs. 5 and 6; P > 0.05; n = 6).

Release of PACAP and VIP by EFS and NO. EFS (1 and 10 Hz) caused release of both VIP and PACAP from IAS smooth muscle strips. PACAP released under the present experimental conditions was primarily of PACAP-38 form, and only a small amount of PACAP-27 was released. Interestingly, the levels of PACAP-38 released in response to 1 and 10 Hz of EFS were significantly higher than those of VIP (Fig. 7).

The release of VIP by EFS was significantly reduced by the neurotoxin TTX and the NOS inhibitor L-NNA (Fig. 8). In the control experiments, 1 and 10 Hz of EFS caused 347.4 ± 70.7 and 326.3 ± 69.9 fmol·100 mg tissue⁻¹·min⁻¹ release of VIP above the basal levels, respectively (n = 6). The basal release of PACAP-38, VIP, and PACAP-27 in these experiments was 76.7 ± 2.9, 16.1 ± 3.0, and 5.8 ± 1.1 fmol·100 mg⁻¹·min⁻¹, respectively. The values for the basal release of PACAP-38 and VIP in the presence of L-NNA were 53.3 ± 6.7 (P < 0.05) and 7.6 ± 2.5 (P < 0.05) fmol·100 mg⁻¹·min⁻¹, respectively. These values in the presence of TTX were 51.1 ± 6.1 (P < 0.05) and 11.3 ± 2.4 (P < 0.05) fmol·100 mg⁻¹·min⁻¹, respectively. The neurotoxin pretreatment reduced the EFS-stimulated release of VIP to 117.5 ± 27.9 and 91.7 ± 24.8 fmol·100 mg⁻¹·min⁻¹ in response to 1 and 10 Hz of EFS, respectively (P < 0.05; n = 6). The EFS-stimulated increase in the release of VIP by 1 and 10 Hz was also significantly reduced to 226.5 ± 56.7 and 173.7 ± 50.7 fmol·100 mg tissue⁻¹·min⁻¹, respectively, in the presence of L-NNA (P < 0.05; n = 6).

To examine the regulation of PACAP and VIP release, we investigated the effect of exogenous NO on the release of the neuropeptides. The data in Fig. 9 show that NO caused an increase in the release of both PACAP and VIP above the basal levels. NO (10⁻⁷ M)-induced increase in the release of VIP and PACAP was 45.5 ± 10.7 and 264.7 ± 52.5 fmol·100 mg⁻¹·min⁻¹ above the basal levels, respectively.

Receptor binding studies. The data showing the displacement of radiolabeled VIP by unlabeled VIP and
PACAP are given in Fig. 10. The data show displacement of radiolabeled VIP by unlabeled VIP, PACAP-38, and PACAP-27 in a concentration-dependent manner. VIP and PACAP-38 were found to be nearly equipotent in causing the displacement of $^{125}$I-VIP. PACAP-27 on the other hand was found to be approximately one-tenth as potent in causing the displacement of $^{125}$I-VIP compared with VIP and PACAP-38. The IC$_{50}$ values for VIP and PACAP-38 were $(1.3 \pm 0.2) \times 10^{-9}$ M vs. $(6.5 \pm 1.8) \times 10^{-9}$ M, respectively ($P > 0.05$), and for PACAP-27 the calculated IC$_{50}$ was $(2.3 \pm 0.6) \times 10^{-7}$ M.

DISCUSSION

This study suggests that PACAP may be involved in the NANC nerve-mediated relaxation of IAS and that endogenously released PACAP activates either a common PACAP/VIP receptor or has a considerable overlap for the actions of both the peptides. This is supported by the findings that binding of radiolabeled VIP to IAS smooth muscle membranes was equally displaced by VIP, PACAP-38, and PACAP-27.

PACAP is partly responsible for IAS relaxation in response to NANC nerve stimulation. We have previously shown that PACAP causes relaxation of IAS by its action directly at the smooth muscle cells (23). The present studies show that selective antagonists of PACAP, PACAP-(6–38), and PACAP-(6–27) cause significant attenuation of IAS relaxation caused by NANC nerve stimulation.

The studies further demonstrate the release of PACAP in response to NANC nerve stimulation. Interestingly, in IAS, PACAP was released primarily in the form of PACAP-38. Although PACAP-immunoreactive neurons in IAS were not examined, a number of studies have shown the presence of these neurons in other regions of the gut (20, 25, 26, 28).

In IAS, the receptors responsible for the PACAP-induced relaxation of IAS appear to share common
properties with those of VIP. This is supported by the observations that both VIP and PACAP displaced radioactive-labeled VIP from IAS smooth muscle membranes receptors with the similar potency. Previous studies from our laboratory (22) have shown that PACAP- or VIP-induced relaxation of IAS was blocked by their respective antagonists. Interestingly, the combination of both antagonists failed to cause an attenuation of the EFS-induced IAS relaxation more than that achieved in the presence of either of the antagonists. The data suggest that, in contrast to the independent role of VIP, the role of PACAP in the NANC nerve stimulation-induced IAS relaxation is limited to the involvement of a common VIP/PACAP receptor.

The data suggest an involvement of VIP/PACAP receptor in the neurally mediated relaxation of IAS. There was a significant suppression of the neurally mediated IAS relaxation by VIP tachyphylaxis. The influence of PACAP tachyphylaxis on the neurally mediated IAS relaxation was far more complex. In the lower frequencies of EFS, PACAP tachyphylaxis was found to cause an augmentation of IAS relaxation. Furthermore, PACAP tachyphylaxis caused the restoration of IAS relaxation suppressed by VIP tachyphylaxis.

**Fig. 5.** Effects of PACAP and VIP antagonists (A) and PACAP and VIP tachyphylaxis (B) on fall in IAS tension by different concentrations of nitric oxide (NO). Note a concentration-dependent fall in basal tension of IAS in response to NO that was not modified by either PACAP and VIP antagonists or their corresponding tachyphylaxes (P > 0.05; n = 6).

**Fig. 6.** Effects of PACAP and VIP antagonists (A) and PACAP and VIP tachyphylaxis (B) on fall in IAS tension by different concentrations of forskolin. Note a concentration-dependent fall in basal tension of IAS by forskolin that was not modified by either PACAP and VIP antagonists or their corresponding tachyphylaxes (P > 0.05; n = 6).
laxis. Although, the exact significance of these findings is not known, the actions may be explained on the basis of the complex actions of PACAP (10, 19, 23). In IAS, PACAP causes not only relaxation but also a contraction (23). IAS smooth muscle contraction in response to PACAP was found to be mediated via substance P. In other gastrointestinal smooth muscles, PACAP has recently been shown to produce contraction either via substance P and acetylcholine release (10) or by its action directly at the smooth muscle (19). Both substance P and muscarinic stimulation are well known to produce gastrointestinal smooth muscle contraction via the activation of protein kinase C (PKC) (2).

The activation of PKC is known to interfere with the NOS pathway (16), which plays a major role in the NANC nerve-mediated IAS relaxation and a part of the VIP-induced relaxation of IAS (5, 21, 24). It would be interesting to examine whether PACAP tachyphylaxis, by inhibiting PKC, unmasks the NOS system leading to the augmentation of IAS relaxation. There is also a possibility of upregulation of VIP and NOS inhibitory neurotransmission during PACAP tachyphylaxis, but it is not known why the actions of PACAP antagonists were different from those of PACAP tachyphylaxis. The experiments with specific PACAP and VIP antibodies may help further in the evaluation of the independent and relative role of PACAP and VIP in IAS relaxation.

PACAP tachyphylaxis caused significant suppression of IAS relaxation in response not only to PACAP but also to VIP. Conversely, however, VIP tachyphylaxis blocked the effect of VIP only. Although the exact reason for these observations and the mechanism of their action are not quite clear, it is possible that...
PACAP has multiple sites of actions and that one of the sites is by the activation of a receptor that is shared by VIP. At such receptors, PACAP may possess higher affinity compared with VIP. This is corroborated by our ongoing studies showing that PACAP antagonists were more potent in displacing membrane bound [125I]-VIP than the VIP antagonist VIP-10-(28).

The data also suggest that NO upregulates the release of both PACAP and VIP, since exogenous administration of NO caused an increase in the release of both these peptides. Furthermore, similar regulation was found in response to the endogenous release of NO as the inhibition of NO biosynthesis by the NOS inhibitor caused an opposite effect, i.e., decrease in the release of VIP that was increased by the stimulation of NANC nerves. We conclude that the role of PACAP in the NANC nerve-mediated relaxation of IAS is limited to the activation of a common VIP/PACAP receptor.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-35385 and an institutional grant from Thomas Jefferson University.

Address for reprint requests: S. Rattan, 901 College, Thomas Jefferson University, 1025 Walnut St., Philadelphia, PA 19107.

Received 1 December 1997; accepted in final form 1 June 1998.

REFERENCES


