Excitatory and Inhibitory Actions of Pituitary Adenylate Cyclase-Activating Peptide (PACAP) in the Internal Anal Sphincter Smooth Muscle: Sites of Actions

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ABSTRACT

Unlike its effects on the rest of the GI tract, the effects of pituitary adenylate cyclase-activating peptide (PACAP) on the internal anal sphincter (IAS) are not known. We examined the actions of PACAP-38 (here PACAP) and PACAP-27 on the basal IAS tone of circular smooth muscle strips before and after the administration of different neurohumoral antagonists. PACAP caused a concentration-dependent fall in the basal tone of the IAS. Interestingly, however, at higher concentrations, PACAP caused a biphasic response: an initial contraction followed by a relaxation. Both the contractile and the relaxant responses were insensitive to atropine, guanethidine, apamin or tetrodotoxin. Both the contractile and the relaxant effects were inhibited by PACAP 6-38 (a selective antagonist of PACAP), vasoactive intestinal polypeptide 10-28 (a vasoactive intestinal polypeptide antagonist) and PACAP tachyphylaxis.

The role of VIP as an inhibitory neurotransmitter in the internal anal sphincter is well known (Biancani et al., 1985; Nurko and Rattan, 1988). Recently, another neuropeptide PACAP closely related to VIP has been identified in the central as well as the peripheral nervous systems (Miyata et al., 1990; Suda et al., 1992; Shen et al., 1992; Uddman et al., 1991; Ny et al., 1995; Portbury et al., 1995; Rawlings, 1994). PACAP is a 38-amino acid peptide with amidation at the C-terminus (PACAP-38). Endogenously, PACAP may also occur in the form of PACAP-27, a 27-amino acid form (Miyata et al., 1990). PACAP has been shown to be released from the myenteric neurons of the GI tract from the esophagus (Uddman et al., 1991; Ny et al., 1995) to the small and large intestines in animals (Portbury et al., 1995) as well as in humans (Shen et al., 1992). Furthermore, PACAP has been shown to be released from the myenteric neurons of the GI tract (Grider et al., 1994; Katsoulis et al., 1996). Like VIP, PACAP has been shown to be a potent and direct relaxant of different smooth muscles of the gut (Katsoulis et al., 1993a; Grider et al., 1994; Ny et al., 1995; Katsoulis et al., 1996). It has also been suggested that PACAP plays a significant role in the inhibitory neurotransmission of the gut (Grider et al., 1994; Jin et al., 1994; Katsoulis et al., 1996). Moreover, the inhibitory actions of PACAP and VIP may share a common receptor.

Actions of PACAP in the gut may vary from no effect (Pradhan et al., 1991) to either relaxation (Katsoulis et al., 1993a; Grider et al., 1994; Ny et al., 1995; Katsoulis et al., 1996) or contraction (Katsoulis et al., 1993b; Mizumoto et al., 1992). The sites and mechanism of action of PACAP may also vary widely in different preparations of the GI tract. The inhibitory effects may be exerted directly at the smooth muscle (Katsoulis et al., 1993a; Grider et al., 1994; Ny et al., 1995; Katsoulis et al., 1996); the excitatory effects may be ex-
plained on the bases of the release of ACh and substance P either by the direct activation of myenteric neurons or by the negative coupling of adenosine receptors to adenylate cyclase in the myenteric neurons of the gut (Christofo and Wood, 1993).

As a tonic smooth muscle, the IAS offers an important model to investigate both the excitatory and the inhibitory actions of an agent. The actions of VIP in the IAS are well known: it produces frank relaxation of the smooth muscle (Biancani et al., 1985; Nurko and Rattan, 1988; Chakder and Rattan, 1993). The actions of PACAP in the IAS, however, have not been investigated. Interestingly, PACAP, especially in the higher concentration range, caused a biphasic response in the IAS: an initial contraction followed by a relaxation. The purpose of the present investigation was to examine the sites of divergent actions of PACAP in the IAS smooth muscle.

Materials and Methods

Preparation of IAS smooth muscle strips. Studies were performed on circular smooth muscle strips obtained from adult opossums (Didelphis virginiana) of either sex. After i.p. pentobarbital anesthesia, the animals were killed by exsanguination and the anal canals were removed. The anal canal was cleaned of extraneous connective tissues, and the blood vessels were opened flat by an incision along the longitudinal axis in a dissecting tray containing oxygenated Krebs’ solution at room temperature. The composition of the Krebs’ solution was as follows (in mM): NaCl, 118.07; KCl, 4.69; CaCl₂, 2.52; MgSO₄, 1.16; NaH₂PO₄, 1.01; NaHCO₃, 25 and glucose, 11.10. The tissue was then pinned flat, and the mucosa, along with the submucosal layer, was removed by a sharp dissection. Circular smooth muscle strips were obtained from the whole circumference of the anal canal and were then divided into two equal strips (~2 mm wide and 8 mm long). The muscle strips were tied at both ends with silk sutures for measurement of isometric tension.

Measurement of isometric tension. The IAS smooth muscle strips were transferred to thermostatically controlled 2-ml muscle baths containing oxygenated Krebs’ solution that was bubbled constantly with a mixture of 95% oxygen and 5% carbon dioxide. 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 PT03, Grass Instruments Co., 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 hr, with intermittent washings, the optimal length and the base line of each smooth muscle strip were determined as described previously (Moummi and Rattan, 1988). Only those strips that developed spontaneous steady tension and relaxed in response to EFS were used in the study. The muscle baths were pretreated with 2.5% bovine serum albumin, and the pipette tips were silic-onized.

EFS. EFS was carried out using a pair of platinum electrodes placed at both sides of the smooth muscle strips. The stimulus was delivered from a Grass stimulator (model S88; Grass Instruments Co.) at various frequencies (0.5–20 Hz; 4-sec train, 0.5-ms pulse duration; 20–30 V).

Drug responses. To examine the relaxant effects of VIP, PACAP-38 and PACAP-27 on the basal tone of the IAS smooth muscle strips, we added the agonists cumulatively to the muscle bath. For the examination of the contractile effect of PACAP-38 on the IAS smooth muscle, the agonist was added in single bolus doses, and after the maximal response to an added dose was achieved, the smooth muscle strips were washed for at least 1 hr before the next dose was added. When the effects of an antagonist, a neurotoxin or an ion-channel blocking agent were examined on the response of a particular agent, the control concentration-response to that agent was first obtained. The smooth muscle strip was then washed for an hour, and the tension was allowed to return to the pretreatment level. The effect of the agonist was then repeated after pretreatment of the smooth muscle strip for 10 min with the antagonist or the blocking agent. In the case of single-dose experiments, the tissues were washed for 1 hr after one dose of agonist and antagonist, and the experiment was repeated after the addition of another dose.

The concentrations of different antagonists, neurotoxins and ion-channel blocking agents that we used have previously been documented in different systems to be either maximal or supramaximal in blocking the effects of their respective agonists or in blocking the specific ion channels in the smooth muscle cells or myenteric neurons. Tachyphylaxis with PACAP or VIP was achieved by repeated administration of single doses of 1 × 10⁻⁶ M PACAP or VIP. Immediately after recovery of the responses of the peptides, the tissue was challenged repeatedly with the same dose of the peptide until the response was abolished. This usually required 4 to 5 repeated administrations of the peptides for significant tachyphylaxis.

Data analysis. The relaxation of the smooth muscle strips in response to different stimuli was expressed as percent of maximal relaxation (100%) caused by 5 mM EDTA. The contractile responses, on the other hand, were quantified as percent of maximal contraction (100%) caused by bethanechol. All the values are expressed as mean and S.E. of different experiments. For comparison of different groups for significant difference, Student’s t test or analysis of variance (ANOVA) was used, and a P value < .05 was considered significant statistically.

Results

Effect of PACAP-38 (PACAP) and PACAP-27 on the basal tension of the IAS smooth muscle. PACAP-38 caused a concentration-dependent fall in the basal tension of the smooth muscle strips (fig. 1). However, at concentrations higher than 10⁻⁶ M, it caused a biphasic response: an initial contraction followed by a relaxation. In order to explore this phenomenon further, we examined the effects of the higher concentrations of PACAP as single doses. Interestingly, the initial rises in the basal IAS tension with PACAP became even more apparent with single doses (fig. 2). Therefore, for in-depth studies, to determine the site of excitatory action of PACAP in the IAS, we obtained the data on rises in the IAS tension with single doses. Interestingly, PACAP-27 up to 3 × 10⁻⁶ M, caused only a fall in the basal tension of the IAS smooth muscle (fig. 1). In order to examine the sites of both inhibitory and excitatory actions of PACAP, the studies were carried out only with PACAP-38. Unless otherwise stated, we have used the term PACAP as synonymous with PACAP-38.

Effect of PACAP 6-38 and VIP 10-28 on PACAP-38-induced fall and rise in the basal tension of the IAS smooth muscle. PACAP 6-38 (3 × 10⁻⁵ M), a well-known antagonist of PACAP caused significant and selective antagonism of both the inhibitory and the excitatory actions of
PACAP (n = 5–6; P < .05; fig. 3). PACAP-6-38, on the other hand, was found to have no significant effect either on the relaxant actions of isoproterenol or on the contractile actions of bethanechol.

The VIP antagonist VIP 10-28 (3 x 10^{-5} M) caused significant antagonism of the fall and the rise in the smooth muscle tension induced by different concentrations of PACAP-38 (n = 5; P < .05; fig. 4); this suggests that VIP and PACAP-38 share a common receptor.

Effect of PACAP tachyphylaxis on the fall and rise in the basal IAS tension caused by PACAP-38. The fall and rise in the basal tension of the IAS smooth muscle strips induced by PACAP-38 were significantly blocked by PACAP tachyphylaxis. In the control experiments, 1 x 10^{-6} and 3 x 10^{-6} M PACAP-38 caused a 49.0 ± 6.5% and a 58.7 ± 6.4% fall in the basal tension of the smooth muscle strips. After tachyphylaxis with PACAP, these values were 5.8 ± 1.9% and 7.5 ± 2.2%, respectively (n = 4; P < .05; fig. 5).

For the contractile actions, in the controls, 1 x 10^{-6} and 3 x 10^{-6} M PACAP-38 produced a 33.4 ± 8.4% and a 84.9 ± 6.4% fall in the basal tension of the smooth muscle strips. After tachyphylaxis with PACAP, these values were 6.3 ± 1.5% and 9.2 ± 2.8%, respectively (n = 4; P < .05; fig. 6).
8.6% rise in the basal tension of the IAS smooth muscles. After PACAP tachyphylaxis, these values were 8.7 ± 4.6% and 12.0 ± 1.1% respectively (n = 4, P < .05; fig. 5).

Effect of the neurotoxin TTX and of the NOS inhibitor L-NNA on PACAP-38-induced fall and rise in the basal tension of the IAS smooth muscle strips.

The neurotoxin TTX (1 × 10⁻⁶ M) had no significant effect on PACAP-38-induced fall or rise in the basal tension of the IAS smooth muscle strips (n = 5–7, P > .05; fig. 6).

L-NNA (3 × 10⁻⁵ M) caused a decrease in the PACAP-induced fall in the basal IAS tension, which suggests that a part of the inhibitory action of PACAP-38 is NO-dependent. The declines in IAS tension induced by 1 × 10⁻⁷, 3 × 10⁻⁷, 1 × 10⁻⁶ and 3 × 10⁻⁶ M PACAP-38 before L-NNA were 25.4 ± 5.3%, 47.0 ± 8.2%, 67.0 ± 8.2% and 73.0 ± 8.3%; after L-NNA, these values were 10.8 ± 2.7%, 22.2 ± 4.7%, 47.2 ± 8.2% and 53.4 ± 8.1%, respectively (n = 7, P < .05; fig. 7).

In contrast to its effect on relaxation, L-NNA had no significant effect on the IAS smooth muscle contractions caused by PACAP-38 (n = 4, P > .05; fig. 7). However, comparison of the effects of 1 × 10⁻⁵ M PACAP revealed a significant increase in PACAP-induced contraction by L-NNA from 79.6 ± 4.5% to 100.0 ± 0% (n = 4; P < .05).

Effect of atropine and guanethidine on PACAP-38-induced fall and rise in the basal tension of the IAS smooth muscle strips.

The muscarinic receptor antagonist atropine (1 × 10⁻⁶ M) and the adrenergic blocking agent guanethidine (3 × 10⁻⁵ M) had no significant effect on PACAP-38-induced fall and rise in the IAS smooth muscle tension. PACAP-38 at concentrations of 1 × 10⁻⁶, 3 × 10⁻⁶ and 1 × 10⁻⁵ M, caused 51.6 ± 5.1%, 58.8 ± 5.1% and 63.8 ± 5.4% fall in the basal IAS tension. After atropine, the same concentrations of PACAP caused 47.9 ± 7.5%, 62.8 ± 7.9% and 75.5 ± 7.8% fall in the smooth muscle tension, respectively (n = 6, P > .05). For the contractile actions, in the control experiments 1 × 10⁻⁶, 3 × 10⁻⁶ and 1 × 10⁻⁵ M PACAP-38 produced 30.3 ± 6.1%, 63.1 ± 8.8% and 93.5 ± 6.5% rise in the basal IAS tension respectively. After atropine these values were 37.2 ± 7.0, 76.5 ± 6.0 and 92.2 ± 4.5% respectively (n = 6, P > .05).

After guanethidine pretreatment, the same concentrations of PACAP-38 produced 37.4 ± 8.0%, 50.5 ± 7.9% and 62.2 ± 8.7% fall and 26.6 ± 6.8%, 59.9 ± 6.4% and 100.0 ± 0% rise in the IAS tension, respectively (n = 6, P < .05).

Effect of substance P antagonist spantide on PACAP-38-induced fall and rise in the basal tension of the IAS smooth muscle.

PACAP-38-induced fall in the basal IAS tension, which suggests that a part of the inhibitory action of PACAP-38 is NO-dependent.
basal tension of the IAS smooth muscle strips was not affected significantly by the substance P antagonist spantide \((3 \times 10^{-5} \text{ M}) (n = 4, P > .05; \text{fig. 8}).\)

Interestingly, in contrast to the inhibitory effects, spantide caused a significant inhibition of PACAP-38-induced contractions of the smooth muscle strips. In the control experiments, \(1 \times 10^{-6}, 3 \times 10^{-6}\) and \(1 \times 10^{-5} \text{ M}\) PACAP-38 caused 46.8 ± 7.4%, 71.0 ± 9.9% and 95.8 ± 4.2% contractions of the muscle strips, respectively. After pretreatment with spantide, the same concentrations of PACAP-38 produced 10.8 ± 6.8% and 16.9 ± 6.8% contractions, respectively (\(n = 4, P < .05\); \text{fig. 8}). Spantide also caused a shortening of the latency of onset of IAS relaxations induced by PACAP-38. The fall in IAS tension by \(3 \times 10^{-6}\) and \(1 \times 10^{-5} \text{ M}\) PACAP in control began within 74.2 ± 6.1 and 80.0 ± 4.1 sec, respectively; after spantide, these values decreased significantly to 48.3 ± 4.2 and 52.5 ± 3.2 sec, respectively (\(P < .05\)).

**Effect of the ganglionic blocker hexamethonium on the fall and rise in the basal IAS tension caused by PACAP-38.** The ganglionic blocker hexamethonium \((1 \times 10^{-4} \text{ M})\) had no effect on either the fall or the rise caused by PACAP-38 in the basal tension of the IAS smooth muscle strips.

In the control experiments, \(1 \times 10^{-6}\) and \(1 \times 10^{-5} \text{ M}\) PACAP-38 caused 34.0 ± 4.4% and 95.0 ± 5.0% rise in the smooth muscle tension, respectively, whereas after treatment with hexamethonium, these values were 20.9 ± 5.7% and 81.1 ± 9.5.2%, respectively (\(n = 7, P > .05\)).

**Effect of apamin and \(\omega\)-conotoxin GVI A on PACAP-38-induced fall and rise in the basal tension of the IAS smooth muscle.** The Ca\(^{2+}\)-activated K\(^+\) channel blocker apamin (\(1 \times 10^{-5} \text{ M}\)) had no significant effect on either the fall or the rise induced by PACAP-38 in the basal IAS tension (\(n = 5, P > .05\); \text{fig. 9}).

The N-type calcium channel blocker \(\omega\)-conotoxin GVIA \((1 \times 10^{-5} \text{ M})\) caused a significant suppression of PACAP-38-induced fall and rise in the basal IAS tension. In control experiments, \(1 \times 10^{-7}, 3 \times 10^{-7}\) and \(1 \times 10^{-6} \text{ M}\) PACAP-38 caused 30.2 ± 5.6%, 48.3 ± 6.1% and 70.0 ± 6.1% fall in the smooth muscle tension, respectively, whereas after treatment with \(\omega\)-conotoxin, these values were 16.3 ± 3.4%, 24.6 ± 3.7% and 40.3 ± 4.3%, respectively (\(n = 6, P < .05\); \text{fig. 10}).

Similarly, in the control experiments, \(1 \times 10^{-6}, 3 \times 10^{-6}\) and \(1 \times 10^{-5} \text{ M}\) PACAP caused 26.4 ± 3.7%, 66.2 ± 7.5% and 96.0 ± 2.8% rise in the smooth muscle tension, respectively. After \(\omega\)-conotoxin, the same concentrations of PACAP-38 caused 10.8 ± 3.6%, 20.6 ± 7.4% and 76.8 ± 9.8% rise in the smooth muscle tension, respectively (\(n = 6; P < .05\); \text{fig. 10}).

**Discussion**

This study shows clearly that PACAP-38 (PACAP) in the IAS smooth muscle not only produces relaxation but also causes contraction of the sphincteric smooth muscle. Both the inhibitory and the excitatory actions of PACAP may share a common receptor with VIP. The inhibitory action of PACAP in the IAS appears to be exerted primarily via its action at the smooth muscle cells. The excitatory action of PACAP appears to be exerted via the activation of distinct PACAP receptor on substance P-containing postganglionic nerve terminals.

The inhibitory effect of PACAP and the receptor responsible for this action in the GI smooth muscle are well known. Conversely, the contractile action of PACAP in the smooth muscle is relatively unknown. Before the present study, the contractile action of PACAP had been demonstrated only in the guinea pig ileum \emph{in vitro} (Katsoulis et al., 1993b) and on gall bladder motility in the conscious dog (Mizumoto et al., 1993a).

**Fig. 8.** Influence of spantide on the rise and fall in the IAS tension with different concentrations of PACAP. The concentration of spantide that was effective in blocking the effect of substance P in the IAS had no significant effect on the fall of the IAS tension caused by PACAP. However, it caused a significant and concentration-dependent antagonism of the rise in the IAS tension caused by PACAP.

**Fig. 9.** Dose-response curves showing the percent maximal fall and rise in IAS tension in response to PACAP before and after apamin. Note that apamin had no significant effect on the concentration-response curve of either the excitatory or the inhibitory effect of PACAP in the IAS.
The excitatory action of PACAP is below the level of postganglionic cell body and the axon. Furthermore, \( \omega \)-conotoxin, an agent well known to block specifically the N-type Ca\(^{2+} \) channel at the postganglionic nerve terminal, caused significant attenuation of the contractile as well as the relaxant effects of PACAP. Moreover, the specific antagonist of substance P, spantide, caused almost complete obliteration of the rise in the IAS tone induced by PACAP. The involvement of substance P in the mediation of PACAP excitatory response in the IAS is similar to that in the guinea pig ileum, where a part of the contractile action of PACAP has been shown to be sensitive to antagonism by spantide. However, unlike the actions of PACAP in the IAS, a significant portion of the contractile action of PACAP in the guinea pig ileum was exerted \textit{via} the release of ACh. Interestingly, in the guinea pig ileum, there was no such later fall in the smooth muscle tension as was observed in the IAS smooth muscle.

It is interesting that the rise in the IAS tension with PACAP was seen only at the higher concentrations; only a fall in IAS tension was observed with the lower concentrations. The exact mechanism for the masking of this rise in IAS tension with the lower concentrations of PACAP or the selective activation of distinct excitatory PACAP receptor in the IAS remains to be investigated.

The other main differences between the mechanism of action of PACAP in causing the inhibition \textit{vs.} the contraction is based on the involvement of NOS activation and the involvement of Ca\(^{2+} \)-activated potassium channels. The fall in the IAS tension induced by PACAP was significantly antagonized by the NOS inhibitor L-NNA, whereas the excitatory action of PACAP remained unaffected. It is well known that a major part of the action of NO is inhibitory in the smooth muscle. However, there is a precedent for NO-induced contraction in the opossum esophageal longitudinal smooth muscle (Saha \textit{et al.}, 1993). In the IAS circular smooth muscle, however, the only response to NO was that of relaxation (Rattan and Chakder, 1992). The data suggest the involvement of the NOS pathway in the mediation of PACAP-induced IAS relaxation, but not in that of the contraction. The involvement of NOS pathway in the PACAP-induced relaxation of the IAS smooth muscle is similar to that for VIP (Rattan and Chakder, 1992; Murthy and Makhlouf, 1994).

The present study in the IAS smooth muscle focused only on the actions of PACAP-38 and PACAP-27. PACAP-27 primarily produced relaxation with potency almost similar to that of PACAP. Interestingly, PACAP-27 in the guinea pig ileum produced frank contraction. Actions of other PACAP fragments and the mechanism of the inhibitory action of PACAP in the IAS smooth muscle remain to be investigated. On the basis of the potency and the effects of PACAP-38, PACAP-27 and VIP, two types of PACAP receptors have been described in the literature. In PACAP I receptor, PACAP-38 and PACAP-27 act preferentially with similar potency higher than that of VIP. PACAP II receptor, on the other hand, is characterized by equally high potency for PACAP-38, PACAP-27 and VIP. PACAP I receptor may be further subclassified into PACAP IA, where PACAP-38 and PACAP-27 show equal high potency, and PACAP IB, where PACAP-38 is 100 to 1000 times more potent than PACAP-27. On the basis of these features, it is speculated that the PACAP receptor for the inhibitory actions of PACAP in the IAS is of the PACAP II type and for the excitatory actions is of the PACAP I type.
One of the difficulties with this characterization of PACAP receptors in the IAS is that PACAP-27 causes either no or only a small contraction of the IAS smooth muscle. Thus it is possible that the activation of PACAP receptors for the IAS contraction may involve PACAP IB, yet another PACAP I receptor subtype or a completely distinct PACAP receptor. Additionally, the insensitivity of the inhibitory effect of PACAP to apamin (unlike that of VIP) may further suggest that the PACAP receptor responsible for the actions of PACAP in the IAS smooth muscle is distinct from VIP receptor. Moreover, it is possible that comparing the potencies of PACAP and VIP by examining their effects on the basal IAS tension may not provide an exact characterization of PACAP and VIP receptors, because PACAP produces a biphasic effect, whereas VIP causes only relaxation. This may undermine the actual potency of the inhibitory effect of PACAP in the IAS smooth muscle.

In summary, the present data show that PACAP exerts a biphasic effect (an initial contraction followed by a relaxation) in the IAS. The initial contractile effects were observed with higher concentrations of PACAP and were found to be mediated by the activation of PACAP receptor at the substance P-containing nerve terminals. The PACAP receptor(s) responsible for the inhibitory action of the neuropeptide is (or are) hypothesized to be present in the IAS smooth muscle cells and on the nerve terminals of the myenteric inhibitory neurons. The exact nature and role of PACAP and the PACAP receptors in the inhibitory neurotransmission, the relationship of PACAP receptors with substance P-containing neurons and IAS smooth muscle cells and interactions with the NOS pathway and VIP remain to be determined.

References


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