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Distribution and effects of pituitary adenylate cyclase activating peptide in cat and human lower oesophageal sphincter

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1 The localization, tissue concentrations, and effects of pituitary adenylate cyclase activating peptide (PACAP) 27 and 38 were investigated in cat and human lower oesophageal sphincter (LOS), and compared with those of vasoactive intestinal peptide (VIP) and helospectin.

2 PACAP-immunoreactive nerve structures were found in the cat and human LOS, with an abundance in the circular smooth muscle layer. PACAP 27-immunoreactivity was often co-localized with VIP-immunoreactivity.

3 In cat tissue, PACAP (PACAP 27 plus PACAP 38) concentrations were 50 fold lower than VIP concentrations; in human tissue they were 10 fold lower.

4 PACAP 27, PACAP 38, helospectin I, and VIP induced concentration-dependent relaxations in circular smooth muscle preparations from cat and human LOS. The order of potency was: VIP > helospectin I ≥ PACAP 27 > PACAP 38. N^G-nitro-L-arginine, scopolamine, or apamin, did not influence the relaxant effects of PACAP 27 or VIP.

5 In cat preparations, both cyclic AMP and cyclic GMP levels were increased after exposure to PACAP 27 and helospectin I, whereas exposure to VIP was followed by an increase in cyclic AMP levels only. In human preparations, there was an increase in cyclic AMP levels without any change in cyclic GMP levels.

6 These results suggest that in the cat and human LOS, PACAP 27 and VIP can occur within the same nerve structures. PACAP 27 has a potent relaxant action, but its functional importance has to be established.

Keywords: Cyclic nucleotides; oesophagogastric junction; helospectin; immunohistochemistry; non-adrenergic, non-cholinergic nerves; radioimmunoassay; smooth muscle relaxation; vasoactive intestinal peptide (VIP)

Introduction

Pituitary adenylate cyclase activating peptide (PACAP) is a neuropeptide with structural similarities to vasoactive intestinal peptide (VIP). PACAP was first isolated from the ovine hypothalamus and exists in at least two biologically active forms, PACAP 27 and PACAP 38 (Miyata *et al.*, 1989; 1990). A wide organ distribution has been found for PACAP-immunoreactive (IR) nerve structures (Arimura, 1992). The peptide was especially abundant in the brain and testes, but could also be demonstrated in the respiratory and gastrointestinal tracts, including the lower oesophageal sphincter (LOS) (Uddman *et al.*, 1991; Arimura, 1992; Sundler *et al.*, 1992; Ny *et al.*, 1994). PACAP has been shown to affect the smooth muscle tone of isolated preparations of the gut from rat and guinea-pig (Mungan *et al.*, 1992; Schwörer *et al.*, 1992; Katsoulis *et al.*, 1993; McConalogue *et al.*, 1995), and to be released during electrical field stimulation of guinea-pig taenia coli (Jin *et al.*, 1994). Like VIP, PACAP stimulated production of nitric oxide (NO) in gastric cells (Murthy & Makhlouf, 1994).

Helospectin is also a peptide with structural homology to VIP. It was isolated from the salivary gland venom of the gila monster lizard (Parker *et al.*, 1984), and exists in two biologically active forms, helospectin I and helospectin II. Helospectin-like immunoreactivity has been found in intestinal smooth muscle, including the LOS (Absood *et al.*, 1992; Desai *et al.*, 1992; Ny *et al.*, 1994).

Since VIP has been suggested to be one of the transmitters mediating relaxation of the LOS (Goyal & Rattan, 1980; Biancani *et al.*, 1984), we have compared the tissue distribution of PACAP (27 and 38) and VIP in cat and human LOS, and investigated the effects of PACAP (27 and 38) on isolated circular smooth muscle specimens. These effects were compared with those of helospectin I, helospectin II, and VIP. Cyclic nucleotide concentrations were measured in preparations after exposure to PACAP 27 and 38, helospectin I and VIP. A possible interaction between PACAP and the NO pathway was also investigated.

Methods

Preparations

Adult male cats, with an average weight of 4.0 kg, were anaesthetized by intravenously administered α-chloralose and killed by an intravenous injection of air. The distal two thirds of the oesophagus, and adjacent parts of the stomach, were removed and opened along the longitudinal axis of the oesophagus. The LOS was identified as a thickening of the circular smooth muscle layer at the oesophagogastric junction as described in detail previously (Ny *et al.*, 1995). Human preparations were taken from 6 patients (age 51-73 years) undergoing surgery because of oesophageal or stomach cancer. These tissues were taken only from macroscopically normal tissue, as confirmed by histochemical examination. Whole wall specimens used for histological examination were put in formaldehyde. Tissue specimens used for radioimmunoassay analysis and functional studies were immediately placed in an ice-cold Krebs solution (for composition, see below). The specimens were pinned flat with the mucosal side up and stretched to its *in vivo* length. The mucosa was removed by sharp dissection and the muscularis externa was dissected free.

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Histochemistry

Tissue specimens were fixed for 4 h in an ice-cold solution of 4% formaldehyde in phosphate-buffered saline (PBS; pH 7.4), and rinsed in 15% sucrose in PBS (at least 3 rinses during 3 days). The tissue pieces were then frozen at −40°C in isopenthane and stored at −70°C, before sectioning in a cryostat. Cryostat sections were cut at a thickness of 8–10 μm and thaw-mounted onto chrome-alum slides. For the simultaneous demonstration of two antigens (Wessendorf & Elde, 1985), sections were incubated at 4°C in the presence of two primary antisera, raised in two different species (rabbit anti-PACAP and guinea-pig anti-VIP). The sections were first incubated overnight with PACAP-antiserum, rinsed in PBS, and then incubated overnight with VIP-antiserum. After rinsing for 10 min in PBS (3 rinses), the sections were incubated for 90 min with Texas Red conjugated anti-rabbit immunoglobulins, rinsed, and incubated for 90 min with fluorescein isothiocyanate (FITC)-conjugated anti-guinea-pig immunoglobulins. Subsequently, the sections were mounted in glycerol/PBS with *p*-phenylenediamine to prevent fluorescence fading (Johnson & Araujo, 1981). The sections were examined in an Olympus 3 × 50 system fluorescence microscope equipped with epi-illumination and filter settings for Texas Red and FITC-fluorescence. The immunoreaction products were documented by microphotography. The primary antisera used were: PACAP 27 rabbit antiserum from J.J. Holst (Department of Medical Physiology C, Panum Institute, University of Copenhagen, Denmark; diluted 1 : 1280 in PBS), PACAP 27 rabbit antiserum from Peninsula Inc (Belmont, CA, U.S.A.; code no. IHC 8922; diluted 1 : 600 in PBS) and guinea-pig VIP-antiserum from Euro-Diagnostica (Malmö, Sweden; code no. B-GP-340-100; diluted 1 : 640 in PBS). Preliminary experiments were also performed with a PACAP 38 antiserum (J. Hannibal, Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Denmark). The secondary antibodies used were FITC-conjugated goat anti-guinea-pig immunoglobulins (1 : 80 in PBS; Sigma, St Louis, MO, U.S.A., code no. F-6261) and Texas Red-conjugated affinity-purified $F(ab')_2$ fragments of donkey anti-rabbit immunoglobulins (1 : 80; Jackson ImmunoResearch Inc, West Grove, PA, U.S.A., code no. 711-076-152). In control experiments, no immunoreactivity could be detected in sections incubated with primary antisera absorbed with excess of the respective antigens (100 μg ml^{-1}), or in the absence of primary antisera. The structures related are referred to as PACAP- and VIP-immunoreactive (IR) as cross-reactions with other antigens, sharing similar amino acid sequences, cannot be completely excluded.

Confocal microscopy

To evaluate whether two immunoreactivities were localized within the same nerve structure, sections were analysed in a confocal laser scanning microscope (Multiprobe 2001 CLSM; Molecular Dynamics, Inc) equipped with an Ar/Kr laser with three emission wavelengths (488 nm, 568 nm, 647 nm) and an inverted Nikon Diaphot TMD microscope. The sections were scanned with 40× (1.00) and 60× (1.40) oil immersion plan apochromat lenses (Nikon) using a 50 μm pinhole. Each optical section was stored as a 512 × 512 pixel frame, with a pixel width of 0.32 or 0.6 μm (40× lens) or 0.11, 0.21 or 0.42 μm (60× lens). From each specimen, two series of optical sections were made. For 50% of the specimens, one series was first made using the 488 nm laser band for excitation, and a 530 nm (±15 nm) band pass filter for detection of the FITC fluorescence. Then starting from the same focal plane as in the first series, another series was made using 568 nm for excitation and a 590 nm longpass filter for Texas Red fluorescence. For the other half, Texas Red fluorescence was detected before FITC fluorescence. By making consecutive series, rather than simultaneous detection, signal/noise ratio was optimized and 'leakage' of FITC fluorescence into the Texas Red channel, and vice versa, was eliminated.

Measurements of PACAP 27, PACAP 38 and VIP

Transverse segments of cat and human LOS were prepared from the muscularis externa, including the inner circular smooth muscle layer, the myenteric plexus, and the outer longitudinal smooth muscle layer. The specimens were frozen and stored in liquid nitrogen until they were boiled in water/acetic acid to extract the peptides from tissue (Hannibal *et al.*, 1995). The concentrations of PACAP 27, PACAP 38 and VIP in tissue extracts were measured radioimmunochemically in at least two different solutions and expressed as pmol g^{-1} wet weight of tissue (Fahrenkrug *et al.*, 1977; 1978; Hannibal *et al.*, 1995).

Functional studies with circular smooth muscle preparations of cat and human LOS

From the muscularis externa at the oesophagogastric junction the circular smooth muscle layer was dissected free. Preparations, 1–2 mm wide and 20 mm long, were cut in the direction of the muscle fibres. Such a preparation was then cut into 2–3 minor strips, and silk-ligatures were tied at both ends. Between the knots there was a distance of 5–7 mm. The preparations were transferred to thermostatically controlled (37°C) 5 ml tissue baths containing Na^+-Krebs solution (for composition, see below) constantly bubbled with 5% CO_2 and 95% O_2 resulting in a pH of 7.4, and mounted between two L-shaped hooks. One hook was attached to a force transducer (Grass FT03) for measurement of isometric tension, and the other was connected to a sledge, which allowed adjustment of the passive tension of the strip. The recordings were made on a Grass polygraph, 7D or E. Strips from cat specimens were stretched from resting length (L_R) until a length of about 160% of L_R was reached (Ny *et al.*, 1995). Similarly, human preparations were stretched until a length of about 200% of L_R was reached (Tøttrup *et al.*, 1990). After mounting, the strips were allowed to equilibrate for one hour. During this period the preparations developed a spontaneous, active tone, and those that did not were excluded. The tension of the muscle strips was determined by exposure to a 'Ca^{2+}-free' medium containing 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; for composition, see below). The '0'-tension level obtained was considered as a 100% relaxation.

Measurement of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations

Cyclic nucleotide concentrations were measured in LOS preparations after exposure to 1 μM helospectin I, PACAP 27, PACAP 38, and VIP. This concentration of the peptides was chosen on the basis of previously published results obtained in the cat and opossum LOS, where 1–2 μM VIP was shown to produce near maximal activation of the cyclic AMP pathway (Torphy *et al.*, 1986; Szewczak *et al.*, 1990). Furthermore, in the guinea-pig taenia coli PACAP 27, 1 μM, caused maximal stimulation of the cyclic AMP pathway (McConalogue *et al.*, 1995). The cyclic nucleotide concentrations were also measured after treatment with 0.1 μM PACAP 38. When a stable relaxation was obtained, the preparations were rapidly removed from the tissue bath, frozen in liquid nitrogen and stored at −20°C until analyzed. The tissue was homogenized in 10% trichloracetic acid (TCA) in water using a glass-glass homogenizer, and centrifuged at 1500 × *g* (4°C) for 10 min. The protein content in the pellets was determined by the method described by Bradford (1976). The supernatants were extracted 5 times with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residues stored at −20°C. Residues were dissolved in 0.05 M sodium acetate, and the amounts of cyclic GMP and cyclic AMP were quantified by using [^{125}I]-cyclic GMP and [^{125}I]-cyclic AMP RIA kits (RIA-NEN, Du Pont Company, Boston, MA, U.S.A.). [^{3}H]-cyclic

AMP was added to the TCA tissue homogenate in order to determine the recovery of cyclic GMP and cyclic AMP during the ether extraction. The mean recovery was 72%.

Solutions

The solutions used had the following compositions (in mM): Na^+-Krebs solution; NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11, Ca^{2+}-free solution NaCl 119, KCl 4.6, $NaHCO_3$ 15, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11 and EGTA 0.1.

Chemicals

The chemicals used were obtained from the following sources: α-chymotrypsin, apamin, N^G-nitro-L-arginine (L-NOARG), scopolamine, vasoactive intestinal peptide (VIP) from Sigma Chemical Company (St Louis, MO, U.S.A.). Helospectin I, helospectin II, PACAP 27, PACAP 38, PACAP 6-27, and PACAP related peptide (rat and human) were obtained from Peninsula Laboratories Inc (Belmont, CA, U.S.A.). Triton X-100 and *p*-phenylenediamine were obtained from Merck (Darmstadt, Germany).

Calculations and statistics

Statistical results are expressed as means ± s.e.mean, if not otherwise stated. A statistical difference between two means was determined by an unpaired Student's *t* test, whereas for multiple comparisons an analysis of variance, ANOVA, followed by an unpaired two-tailed Dunnet's *t* test was used. A *P* value <0.05 was regarded as significant. Outliers were checked for by Dixon's gap test. (*n*) refers to the number of strips tested, each from a different animal. The IC_{50} values were determined by linear extrapolation as a 50% relaxation response compared to the baseline (the Ca^{2+}-free medium, see above).

Results

PACAP-immunohistochemistry

A similar nerve distribution pattern was seen in both feline and human LOS. There was a moderate to rich number of PACAP 27-IR nerve structures in the inner circular layer of muscularis externa, with varicose terminals running along the smooth muscle bundles (Figure 1). In comparison, the outer longitudinal layer contained a sparse number of PACAP 27-IR nerves. In the myenteric plexus, some ganglionic cell bodies were PACAP 27-IR. In the lamina propria of the mucosa and in the submucosa, some PACAP 27-IR nerve trunks were observed, and a few PACAP 27-IR nerve fibres were observed in the muscularis mucosa running along muscle bundles. The two different polyclonal PACAP 27 antisera used exhibited a similar nerve distribution pattern. No immunoreactivity could be detected in sections incubated with primary antisera absorbed with excess of antigen (Figure 1). Preliminary experiments with the PACAP 38 antiserum were unsuccessful.

Double immunostaining and confocal microscopy

In cat LOS, coinciding profiles were often observed between PACAP 27- and VIP-IR nerve fibres and cell bodies of the myenteric ganglia. Coincidence between the PACAP 27- and

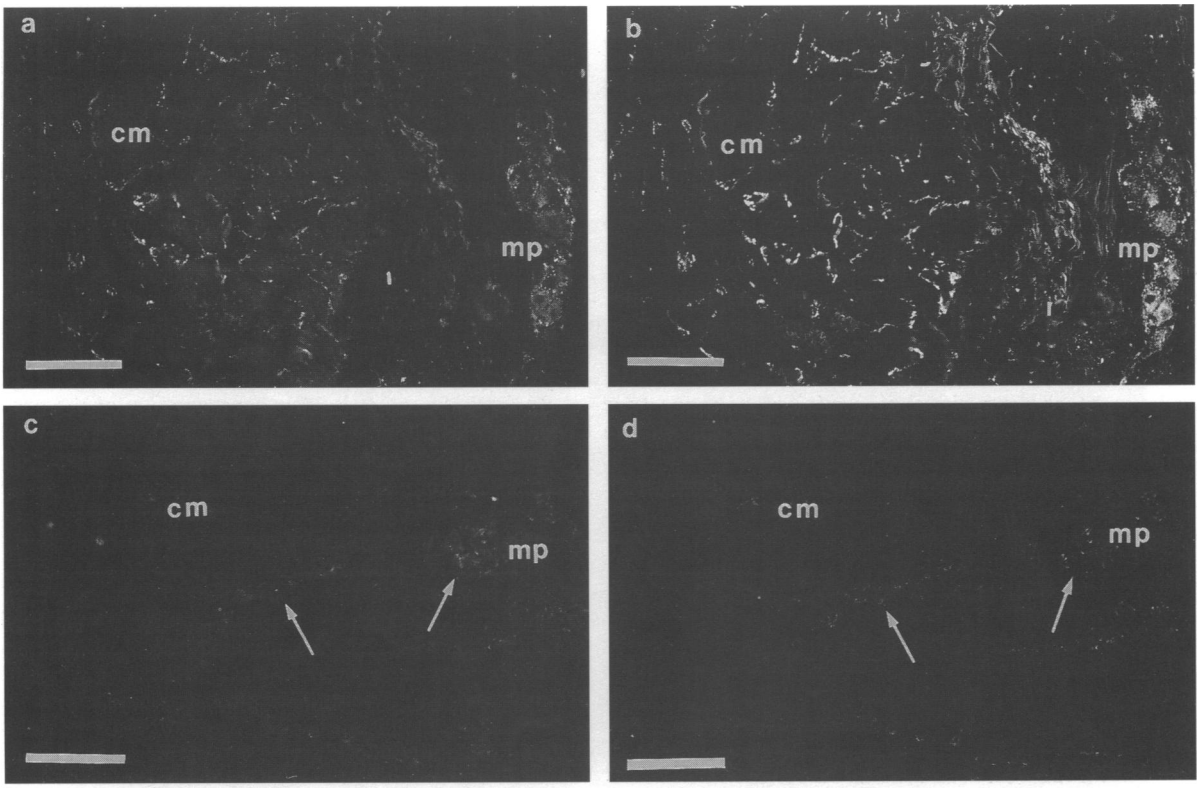

Figure 1 Conventional double label immunohistochemistry of the human lower oesophageal sphincter. (cm) Indicates circular smooth muscle layer and (mp) indicates myenteric plexus. (a) Pituitary adenylate cyclase activating peptide (PACAP-IR) varicose nerve terminals (identified by Texas Red immunofluorescence) along smooth muscle bundles. (b) Vasoactive intestinal peptide (VIP)-IR varicose nerve terminals (identified by FITC immunofluorescence) with profiles frequently coinciding with the corresponding PACAP-IR varicose nerve terminals in (a). The cell bodies in the myenteric plexus are VIP-IR, but only weakly PACAP-IR. In (c) and (d), no immunoreactivity is observed against PACAP 27 and VIP, respectively, in a section incubated with primary antisera absorbed with excess of the respective antigens. In the myenteric plexus and the circular smooth muscle layer some filaments (arrows) display unspecific autofluorescence. Bar represents 100 μm.

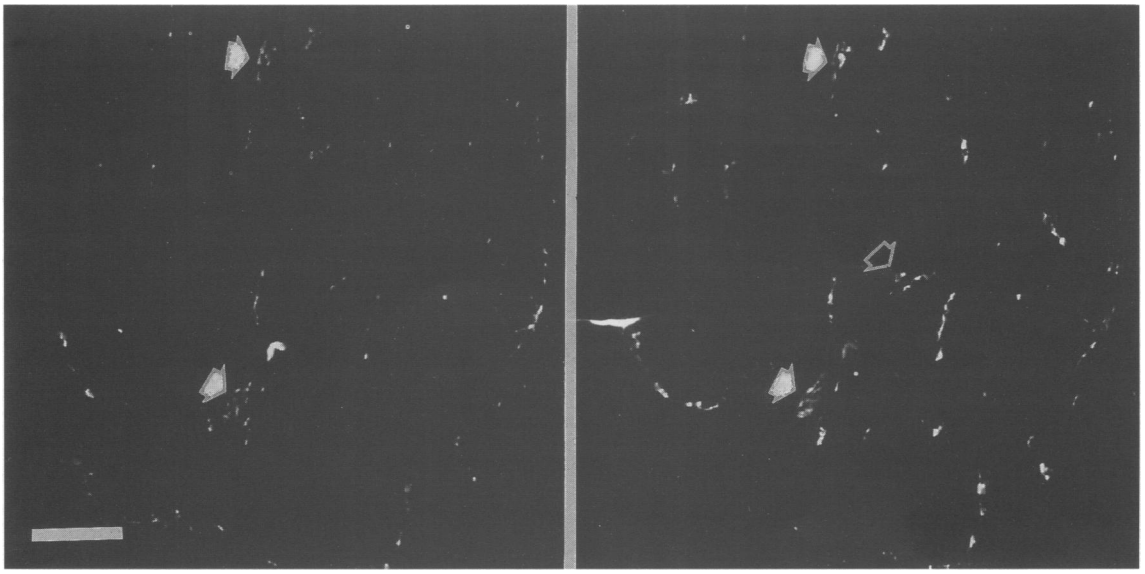

Figure 2 Confocal microscopy of the circular smooth muscle layer from the cat lower oesophageal sphincter. Shown are PACAP-IR (left panel) and VIP-IR (right panel) nerve profiles. Note that many nerve structures are both PACAP-IR and VIP-IR (solid arrows), whereas some are only VIP-IR (hollow arrows). Bar represents 50 μm. For key to abbreviations see Figure 1 legend.

VIP-IR nerve fibres was verified by confocal microscopy and varied between 65–75% (Figure 2). In human LOS double immunostaining also demonstrated coinciding PACAP 27- and VIP-IR nerve profiles. Confocal microscopy revealed that the frequency of coincidence was very high, i.e. more than 95% (Figure 3).

Measurements of PACAP 27, PACAP 38 and VIP

In cat specimens, the mean concentration of PACAP 27 was 0.12 ± 0.02 pmol g^{-1} wet weight of tissue ($n=8$; of which four preparations had concentrations below the detection limit and therefore were not included in this calculation), that of PACAP 38, 4.7 ± 0.3 pmol g^{-1} wet weight of tissue ($n=8$), and that of VIP 246.7 ± 34.4 pmol g^{-1} wet weight of tissue ($n=8$). In human specimens, the concentrations were for PACAP 27, 0.13 ± 0.03 pmol g^{-1} wet weight of tissue ($n=6$; of which two preparations had concentrations below the detection limit and therefore were not included in this calculation), for PACAP 38, 3.9 ± 0.8 pmol g^{-1} wet weight of tissue ($n=6$), and for VIP, 44.8 ± 11.6 pmol g^{-1} wet weight of tissue ($n=6$; Figure 4).

Functional studies

Cat preparations The effects of helospectin I, helospectin II, PACAP 27, PACAP 38 and VIP were tested at concentrations of 1 nM – 1 μM. All the peptides relaxed the circular smooth muscle preparations in a concentration-dependent manner. The order of potency was (as determined by their IC_{50} values): VIP (11.9 nM) > helospectin I (22,6 nM) > helospectin II (61.8 nM) > PACAP 27 (90.6 nM; Figure 5). An IC_{50} value for PACAP 38 could not be calculated since the relaxation produced by this peptide, in the highest concentration used, was 24 ± 6%. PACAP related peptide (human and rat) at con-

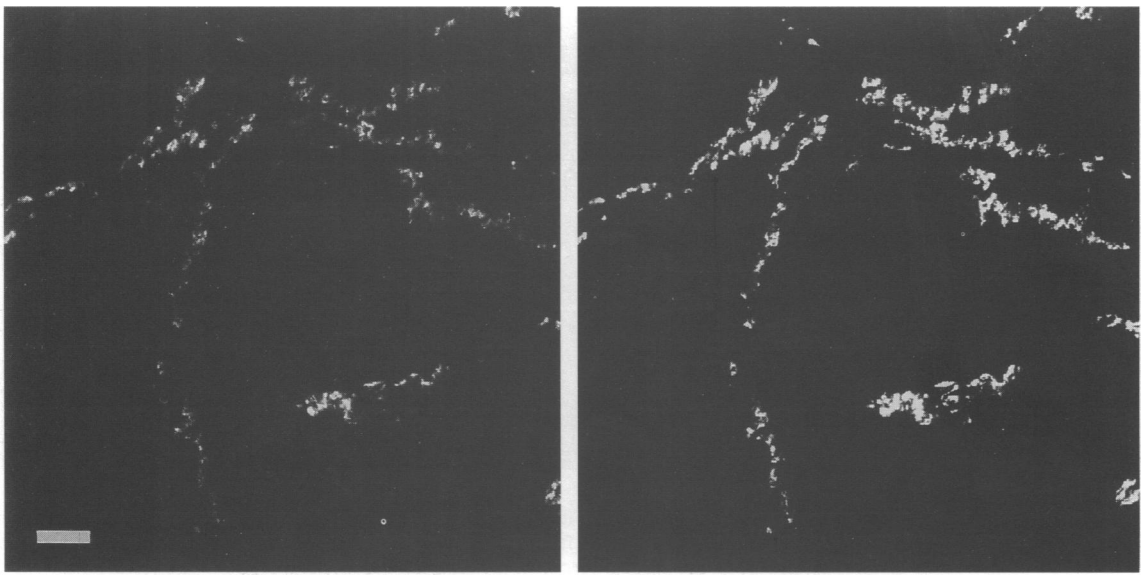

Figure 3 Confocal microscopy of the circular smooth muscle layer from the human lower oesophageal sphincter. Shown are PACAP-IR (left-panel) and VIP-IR (right panel) nerve profiles. Note that almost all nerve structures are both PACAP-IR and VIP-IR. Bar represents 10 μm. For key to abbreviations used see Figure 1 legend.

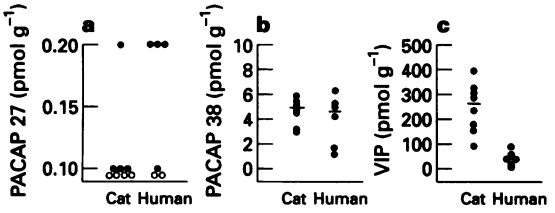

Figure 4 Concentrations (pmol g^{-1} wet weight of tissue) of (a) PACAP 27, (b) PACAP 38 and (c) VIP in the muscularis externa (including inner smooth muscle layer, myenteric plexus and outer longitudinal smooth muscle layer) of the cat ($n=8$) and human lower oesophageal sphincter ($n=6$). Line represents median value and open rings, tissue concentrations below the limit of detection of PACAP 27. For key to abbreviations used see Figure 1 legend.

centrations of 1 nM – 1 μM, had no effect on the smooth muscle tone. The effects of all peptides were abolished by pretreatment of the preparations with the peptidase α-chymotrypsin (2 units ml^{-1}) for 30 min (Figure 6 shows PACAP 27 data only). This pretreatment had no effect on the spontaneously developed tone. The NO synthesis inhibitor, N^G-nitro-L-arginine (0.1 mM; Figure 6), scopolamine (1 μM), and apamin (1 μM) did not affect relaxations induced by PACAP 27 or VIP. The PACAP fragment, PACAP 6-27, did not affect relaxations induced by PACAP 27.

Human preparations Helospectin I, PACAP 27, PACAP 38, and VIP (1 nM – 1 μM) relaxed the preparations in a concentration-dependent manner. The order of potency (as determined by their IC_{50} values) was: VIP (23.3 nM) > PACAP 27 (51.2 nM) > helospectin I (112.8 nM; Figure 7). No IC_{50} value for PACAP 38 could be calculated, since the relaxation produced by this peptide, at the highest concentration used, was 38 ± 7%.

Cyclic GMP and cyclic AMP concentrations

Cyclic nucleotide concentrations were measured in cat and human tissue exposed to helospectin I, PACAP 27, PACAP 38, and VIP at a concentration of 1 μM. In cat preparations, helospectin I, PACAP 27 and VIP, in comparison with controls, significantly ($P<0.05$) increased the levels of cyclic AMP (39–55%). Cyclic GMP levels were also significantly increased ($P<0.05$; 60–75%) for helospectin I and PACAP 27 (Figure 8). In cat LOS ($n=5$) exposed to 0.1 μM PACAP 38 no significant changes in cyclic nucleotides were found. In human preparations, the peptides evoked a significant ($P<0.05$) increase (28–49%) of the cyclic AMP levels, but there were no changes in the cyclic GMP levels (Figure 9). There were no statistically significant differences between the effects of the various peptides on the elevated levels of each respective cyclic nucleotide.

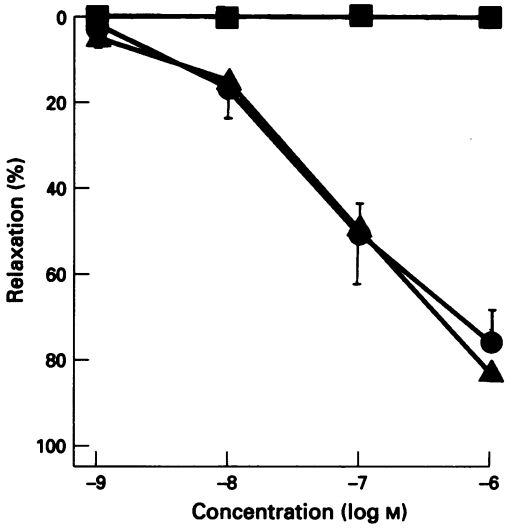

Figure 6 Effects of PACAP 27 on resting tension in preparations from the cat lower oesophageal sphincter after pretreatment with N^G-nitro-L-arginine (L-NOARG, 10^{-4} M; ●); $n=6$, pretreatment with α-chymotrypsin (2 units ml^{-1}; ■); $n=6$, and no treatment i.e. control (▲). Values are means ± s.e.mean.

Discussion

The present results show a moderate to rich occurrence of PACAP 27-IR nerve structures in the feline and human LOS. These findings are in line with previous morphological studies

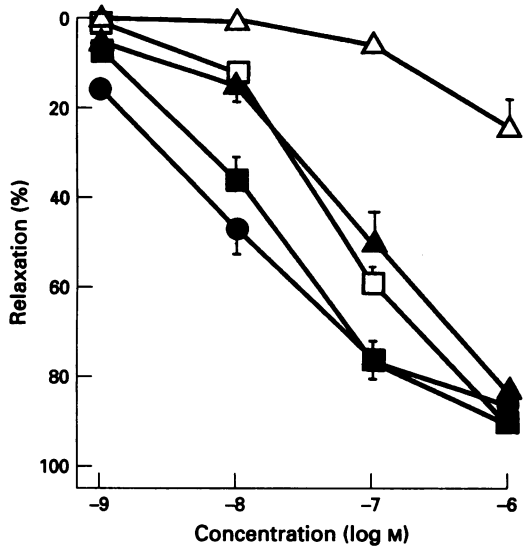

Figure 5 Effects of PACAP 27 (▲); $n=10$, PACAP 38 (△); $n=9$, helospectin I (■); $n=10$, helospectin II (□); $n=10$ and VIP (●); $n=10$, on resting tension in preparations from the cat lower oesophageal sphincter. Values are means ± s.e.mean. For key to abbreviations used see Figure 1 legend.

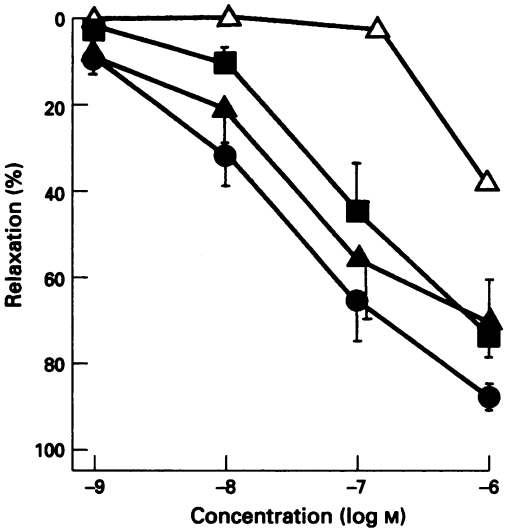

Figure 7 Effects of PACAP 27 (▲); $n=5$, PACAP 38 (△); $n=2$, helospectin I (■); $n=5$ and VIP (●); $n=5$, on resting tension in preparations of the human lower oesophageal sphincter. Values are means ± s.e.mean. For key to abbreviations used see Figure 1 legend.

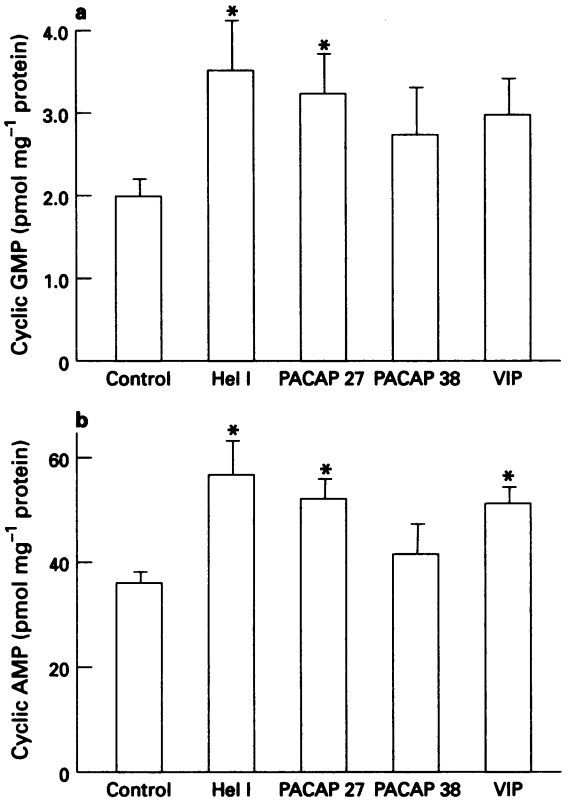

Figure 8 (a) Concentrations of cyclic GMP (pmol mg^{-1} protein) in preparations of the cat lower oesophageal sphincter, control, i.e. resting tension ($n=11$), and after treatment with 1 μM of the following peptides; helospectin I ($n=6$), PACAP 27 ($n=6$), PACAP 38 ($n=5$), and VIP ($n=6$). Values are means ± s.e.mean. *$P<0.05$. (b) Concentrations of cyclic AMP (pmol mg^{-1} protein) in preparations of the cat lower oesophageal sphincter, control, i.e. resting tension ($n=11$), and after treatment with 1 μM of the following peptides; helospectin I ($n=6$), PACAP 27 (n = 6), PACAP 38 (n = 5), and VIP (n = 6). Values are means ± s.e.mean. *$P<0.05$. For key to abbreviations used see Figure 1 legend.

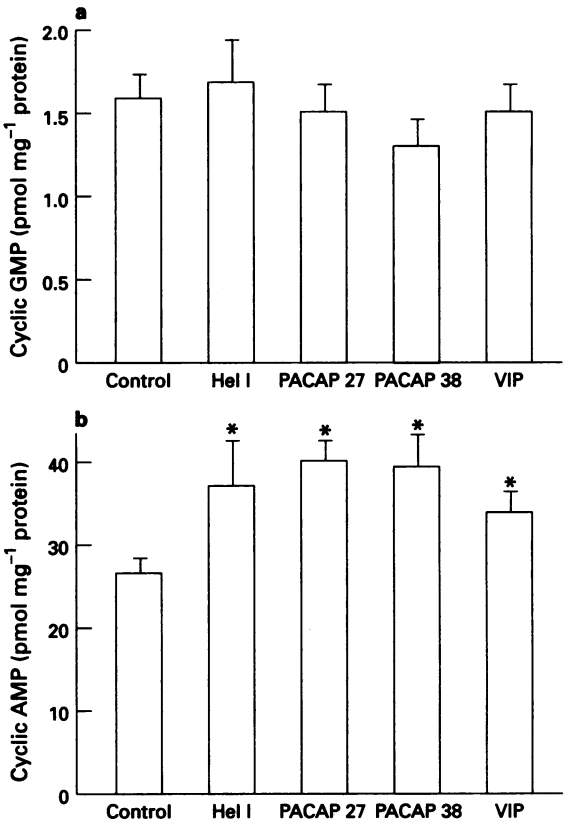

Figure 9 (a) Concentrations of cyclic GMP (pmol mg^{-1} protein) in preparations of the human lower oesophageal sphincter, control, i.e. resting tension ($n=5$), and after treatment with 1 μM of the following peptides; helospectin I ($n=5$), PACAP 27 ($n=5$), PACAP 38 ($n=2$), and VIP ($n=5$). Values are means ± s.e.mean. (b) Concentrations of cyclic AMP (pmol mg^{-1} protein) in preparations of the human lower oesophageal sphincter, control, i.e. resting tension ($n=5$), and after treatment with 1 μM of the following peptides; helospectin I ($n=5$), PACAP 27 ($n=5$), PACAP 38 ($n=2$), and VIP ($n=5$). Values are means ± s.e.mean. *$P<0.05$. For key to abbreviations used see Figure 1 legend.

(Uddman *et al.*, 1991; Ny *et al.*, 1994). A dense PACAP 27-IR nerve pattern was seen in the circular smooth muscle layer, whereas markedly fewer nerve fibres were observed in the longitudinal smooth muscle layer and in the muscularis mucosa. As judged by immunohistochemistry and confocal microscopy, PACAP 27- and VIP-IR nerve strucures were often co-localized within the same nerve fibres (cat LOS 65–75%; human LOS 95–100%). A high degree of coinciding nerve profiles for PACAP and NO synthase (NOS), the NO synthesizing enzyme, and for VIP and NOS has previously been described in the cat LOS (Ny *et al.*, 1994). Whether or not a similar co-localization can be also found in the human LOS remains to be established.

Previous studies have shown that VIP is an effective relaxant of the cat and human LOS, and it has been suggested that VIP is a transmitter in this region (Goyal & Rattan, 1980; Biancani *et al.*, 1984). In the present study, it was demonstrated that not only VIP, but also PACAP and helospectin are effective relaxants of the cat and human LOS. In the cat, the potency of helospectin I was comparable to that of VIP, but was higher than that of PACAP 27. Helospectin II had a lower potency than helospectin I, suggesting that the C-terminal serine at the helospectin molecule is important for the effect. In the human LOS, the potency of VIP was higher than that of helospectin I, but not different from that of PACAP 27, possibly reflecting differences in potency of the peptides between species. The markedly lower potency of PACAP 38 implies that if PACAP has any functional importance in the LOS, the active form is most probably PACAP 27. However, in both cat and human LOS, the concentration of PACAP 27 was extremely low. This was surprising considering the immunohistochemical results. The tissue concentrations of PACAP 38 were not different between the species, which contrasts with the concentrations of VIP, which were 5 times higher in the cat than in the human preparations. It has been demonstrated previously that the VIP concentrations in cat LOS are higher than those found in the human LOS (Alumets *et al.*, 1979; Aggestrup *et al.*, 1986; McGregor *et al.*, 1984). Low concentrations of PACAP 27 compared with the concentrations of PACAP 38 have been also found in human corpus cavernosum tissue (Hedlund *et al.*, 1995, unpublished observations). It may therefore be speculated that if PACAP has any functional importance for regulation of tone in the LOS, there is a conversion of PACAP 38 to PACAP 27. However, PACAP may have other functions within the LOS, and its primary function may not be direct regulation of sphincter tone. Previous morphological studies have suggested that PACAP-containing afferent pathways from the dorsal root ganglia to the dorsal horn of the lumbo-sacral spinal cord are involved in the spinal control of nociception in rats (Moller *et al.*, 1993). It may be speculated that PACAP is localized to afferent nerves in the LOS, and that it has a sensory function. Thus, these functional, morphological, and radioimmunological findings suggest that VIP is the major peptide transmitter in the LOS, but does not exclude that when appropriately stimulated, nerve fibres in the LOS release not only VIP, but a cascade of different peptides including PACAP.

At least two different receptors for PACAP have been identified in the gastrointestinal tract (Christophe, 1993). One is specific for PACAP and binds PACAP with high affinity, but has no affinity for VIP. A second receptor, which was recently cloned (Ishihara *et al.*, 1992), binds both VIP and PACAP with similar affinity. A method to discriminate at the functional level between the two receptors has been suggested by using the K^+ channel blocker, apamin (Schwörer *et al.*, 1992). No apamin sensitive relaxation was induced by PACAP 27 in the cat LOS, suggesting that PACAP and VIP act via a common receptor.

VIP is considered to activate the cyclic AMP second messenger pathway. This seems to be the case also for helospectin and PACAP. Previous studies have demonstrated that PACAP 27 and PACAP 38 have a 1000 fold greater ability than VIP to activate adenylate cyclase in the ovine pituitary gland (Arimura, 1992). In the human and cat LOS, this appears not to be the case, since cyclic AMP was increased to the same level after exposure to PACAP, VIP, and helospectin.

Investigations of LOS of various species have demonstrated that NO is an important mediator of relaxation of the sphincter (Tøttrup *et al.*, 1991; 1993; De Man *et al.*, 1991; Ny *et al.*, 1995). An interplay between NO and VIP has been suggested to take place in at least some parts of the gastrointestinal tract (Makhlouf & Grider, 1993), and recently an interaction of NO and PACAP was described (Murthy & Makhlouf, 1994). A co-localization of NOS and VIP, and also of NOS and helospectin, and NOS and PACAP, has been demonstrated in the cat LOS (Ny *et al.*, 1994; 1995). This raises the possibility of an interaction between NO and helospectin and/or NO and PACAP, respectively, in the gastrointestinal tract. An increase of not only cyclic AMP, but also cyclic GMP levels, was observed in cat, but not in human, tissue after exposure to helospectin I and PACAP 27. Whether this was due to release of NO by the peptides remains unresolved. However, the NO synthesis inhibitor, L-NOARG, did not influence the relaxation induced by exogenously administered PACAP, helospectin, or VIP. This indicates that even if NO is released by these peptides, it is not crucial for their relaxant action. NO and VIP may also be parallel neurotransmitters, as has been suggested to be the case in the dog colon (Keef *et al.*, 1994).

In summary, the present study has shown that in the cat and human LOS, PACAP and helospectin are potent relaxants, possibly acting via the same receptor as VIP. Since PACAP is not more potent than VIP and occurs in markedly lower concentrations, the physiological importance of PACAP in the LOS is unclear. A possible interaction between PACAP and VIP, and between PACAP and other transmitters in the LOS, remains to be elucidated.

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