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Pituitary adenylate cyclase-activating polypeptide, helospectin, and vasoactive intestinal polypeptide in human corpus cavernosum

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1 The distribution and effects of pituitary adenylate cyclase-activating polypeptide (PACAP-27 and -38), helospectin (Hel-1 and Hel-2), and vasoactive intestinal polypeptide (VIP), were investigated in isolated preparations of human corpus cavernosum (CC).

2 Immunohistochemistry revealed coinciding profiles of nerve structures that showed immunoreactivities for VIP and PACAP, and VIP and Hel. Confocal microscopy showed the co-existence of VIP- and PACAP-immunoreactivities, and VIP- and Hel-immunoreactivities in most (90%) varicose nerve structures.

3 As determined by radioimmunoassay, the amounts of VIP, PACAP-27, and PACAP-38 in the preparations were 61.7 ± 11.6, 0.1 ± 0.05, and 3.7 ± 0.5 pmol g^{-1} wet weight of tissue (pmol g^{-1} wet wt.), respectively. In tissue from patients with diabetes, the content of VIP was lower (13.7 ± 0.5 pmol g^{-1} wet wt.), whereas that of PACAP (-27 and -38) was unchanged.

4 Cyclic nucleotide levels were determined in preparations exposed to PACAP-27, PACAP-38, Hel-1, Hel-2, and VIP. All the peptides, but Hel-2, significantly increased the concentrations of cyclic AMP, whereas the levels of cyclic GMP were unchanged.

5 The peptides concentration-dependently relaxed noradrenaline-contracted preparations. The order of potency was VIP > PACAP 27 > Hel-1 > Hel-2 > PACAP-38.

6 Hel-1, VIP and PACAP-27 effectively counteracted electrically induced contractions. At 10^{-6} M, the highest peptide concentration used, the inhibitory effects obtained reached $96 \pm 3\%$, $87 \pm 6\%$, and $80 \pm 3\%$, respectively.

7 The results suggest that PACAP and Hel-1 are co-localized with VIP in nerve structures within the human cavernous tissue, and that the peptides are effective relaxants of CC preparations *in vitro*. The role of the investigated peptides for penile erection remains to be established.

Keywords: Non-adrenergic, non-cholinergic; relaxation; immunohistochemistry, confocal microscopy, radioimmunoassay; cyclic AMP

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) has been isolated from ovine hypothalamus and found to belong to the vasoactive intestinal polypeptide (VIP)-family. Two forms of PACAP have been identified, PACAP-38, and a C-terminally truncated form of 27 amino acids, PACAP-27 (Miyata *et al.*, 1989, 1990). PACAP has structural similarities to VIP, and is derived from a 176 amino acid long precursor protein (Kimura *et al.*, 1990). PACAP-like immunoreactive (IR) nerve fibres and binding sites for the peptide have been demonstrated in both the central and peripheral nervous systems (Köves *et al.*, 1990; Gottschall *et al.*, 1990; Vigh *et al.*, 1991; Shivers *et al.*, 1991; Sundler *et al.*, 1992; Christofi & Wood, 1993; Salomon *et al.*, 1993).

Helospectin (Hel), i.e. helospectin-1 (Hel-1), with 38 amino acids, and helospectin-2 (Hel-2), with 37 amino acids, have been isolated from the salivary gland venom of the lizard *Heloderma horridum* (Parker *et al.*, 1984). These peptides are also structurally related to VIP (Robberecht *et al.*, 1985), and have been identified in endocrine cells and nerve structures of the central nervous system and in the mammalian intestine (Bjartell *et al.*, 1989; Luts *et al.*, 1991; Kivipelto *et al.*, 1992; Absood *et al.*, 1992c).

PACAP and Hel exhibit smooth muscle relaxant properties similar to those of VIP, and all three peptides are believed to act via adenylate cyclase activation (Grundemar & Högestätt, 1990; Absood *et al.*, 1992a,b; Naruse *et al.*, 1993). Furthermore, PACAP and VIP, as well as Hel and VIP, have been found to co-exist in neurones and nerve cell bodies throughout the gut of several species (Sundler *et al.*, 1992; Absood *et al.*, 1992c; Desai *et al.*, 1992; Ny *et al.*, 1994a,b). Considering that VIP, PACAP, and Hel have common biological actions, we found it of interest to investigate their distribution and functional effects in human penile erectile tissue, where it has been suggested that VIP acts as a neurotransmitter (see Andersson & Wagner, 1995). Some of the results have been published previously in abstract form (Hedlund *et al.*, 1994).

Methods

Preparations

Tissue specimens of corpus cavernosum (CC) were obtained from 19 patients aged 20 to 75 years (mean 57 years). Five of them (known to have normal erectile capacity) underwent cystourethrectomy (no preoperative external radiation) because of bladder malignancy. Four patients, previously treated with oestrogen, went through a gender reassignment operation (penis amputation). Four patients with a non-diabetic erectile dysfunction underwent surgery for implantation of penile

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prostheses. Erectile tissue was also obtained from two donor patients with brain damage after an intracerebral tumour and an intracranial haemorrhage, respectively. The functional responses to K^+, noradrenaline, and electrical stimulation of nerves were similar in the preparations from the different individuals. In addition, specimens obtained from four patients with diabetic erectile dysfunction (surgery for implantation of penile prostheses) were used for radioimmunological determination of VIP and PACAP concentrations only. The specimens were immediately placed in chilled Krebs solution (for composition, see below). Tissue prepared for immunohistochemistry and radioimmunoassay, and strip preparations ($1 \times 2 \times 5$ mm) for functional studies were dissected. All preparations were used within 12 h. The procedure for obtaining erectile tissues was approved by the Ethics Committee, University of Lund.

Immunohistochemistry

Tissue specimens were immersion-fixed for 4 h in a freshly prepared, ice-cold solution of 4% formaldehyde in phosphate buffered saline (PBS; pH 7.4) and rinsed at least 3 times over 48 h in ice-cold PBS containing 15% sucrose (pH 7.4). The specimens were frozen in isopentane at −40°C and stored at −70°C. Cryostat sections were cut at a thickness of 8 μm and prepared for immunohistochemistry as previously described (Alm *et al.*, 1993). In the immunohistochemical procedure, the sections were incubated at 4°C in the presence of one, or the combination of two primary antisera (Table 1A and B), raised in different species, for the simultaneous demonstration of two antigens (Wessendorf & Elde, 1985). In some experiments, sections were first incubated over night with one of the primary antisera, rinsed in PBS, and then incubated over night with the other primary antiserum. After rinsing for 10 min in PBS (3 rinses), the sections were incubated for 90 min with a secondary antiserum, rinsed, and when appropriate, incubated for 90 min with another secondary antiserum. The secondary antiserum/antisera used was/were dependent on the characters of the primary antiserum/antisera (Tables 1A and B). After rinsing, the sections were mounted in glycerol/PBS with *p*-phenylenediamine (Merck, Darmstadt, Germany) to prevent fluorescence fading (Johnson & Araujo, 1981). No differences in immunolabelling pattern could be detected between the two methods of incubation with primary antisera.

The immunoreactive (IR) nerve structures were subjectively evaluated with respect to type (nerve trunks, varicose and non-varicose nerve fibres) and number as described previously by Lundberg *et al.* (1988). In the evaluation of the latter parameter, immunoreactivity for protein gene product 9.5 (PGP) was used as an 'internal standard' for total innervation. PGF is a cytoplasmatic protein that can be used as a pan-neuronal marker for the demonstration of all types of nerve fibres (Gulbenkian *et al.*, 1987; Lundberg *et al.*, 1988).

The sections were examined in an Olympus 3×50 System fluorescence microscope equipped with epi-illumination and filter settings for FITC and Texas Red immunofluorescence. The immunoreaction products were documented by microphotography.

The primary and secondary antisera used are listed in Table 1A. 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}). The PACAP-27 antisera used did not cross-react with Hel-1 or with VIP. As cross reactions with other antigens sharing similar amino acid sequences cannot be completely excluded, the structures related are referred to as PACAP-, Hel-, and VIP-IR.

Confocal microscopy

To evaluate whether PACAP- and VIP-, and Hel- and VIP-immunoreactivities were localized within the same nerve structure, sections were analysed in a confocal laser scanning microscope (Multiprobe 2001™ CLSM; Molecular Dynamics, Inc.), as previously described in detail (Ny *et al.*, 1994a,b).

Measurements of PACAP and VIP

Tissue specimens of human CC, each weighing approximately 200 mg, were frozen and stored in liquid nitrogen until extraction of peptides from the tissue were undertaken in boiling water/acetic acid (Hannibal *et al.*, 1995). The PACAP-27 antiserum used (91084-2) was the same as that used for immunohistochemistry. VIP-antiserum and PACAP-38 antiserum (5603-7 and 733C-5; Dept. Clin. Biochem., Bispebjerg Hospital Copenhagen, Denmark.) were used. The concentration of PACAP-27, PACAP-38, and VIP in tissue extracts were measured by radioimmunoassay in at least two different solutions and expressed as pmol g^{-1} wet weight of tissue (pmol g^{-1} wet wt.) (Hannibal *et al.*, 1995; Fahrenkrug & Schaffalitzky de Muckadell 1977; 1978).

Table 1A Primary and secondary antibodies used

Primary antibodies	*Concentration**	*Source*	*Secondary antibodies*	*Concentration*	*Source*
1 PGP* 9.5 (ra) (RA 95101)	1:2000	UltraClone Ltd, Cambridge, England	I FITC*-conjugated goat anti-guinea pig Ig (F-6261)	1:80	Sigma, St Louis, Mo, U.S.A.
2 VIP* (gp*) (B-GˆP-340-X)	1:640	Milab Ltd, Malmö, Sweden			
3 PACAP*-27 (ra) (Ab 91084-2)	1:1000	Prof. J.J. Holst, Dept. Med. Physiol. C, Univ. Copenhagen, Denmark	II FITC-conjugated swine anti-rabbit Ig (F205)	1:80	Dakopatts, Stockholm, Sweden
4 PACAP-27 (ra*) (IHC 8922)	1:600	Peninsula Ltd, St Helens, England			
5 Helospectin-1 (ra) (B63-1)	1:640	Milab Ltd, Malmö, Sweden	III Texa Red-conjugated affinity purified donkey anti-rabbit IgG (H+L) (711-076-152)	1:80	Jackson Immuno-Research Inc, West Grove, Pa, U.S.A.

B Combinations of antibodies used

Combinations of primary antibodies	*vs*	*Combinations of secondary antibodies*
1		II
2+3		I+III
2+4		I+III
2+5		I+III

*PACAP = pituitary adenylate cyclase-activating polypeptide, PGP = protein gene product, VIP = vasoactive intestinal polypeptide, ra, and gp = antibodies raised in rabbits and guinea-pigs, FITC = fluorescein isothiocyanate. Concentrations refer to dilutions in PBS.

Measurements of cyclic nucleotides

Guanosine and adenosine 3':5'-cyclic monophosphate (cyclic GMP and cyclic AMP) were analysed in (−)-noradrenaline (NA)-contracted CC preparations (controls), and in NA-contracted preparations exposed to VIP, PACAP-27, PACAP-38, Hel-1, or Hel-2 (10^{-6} M). When the effects of the peptides reached a maximal and stable level, usually within 2 min, the strips were immediately frozen in liquid nitrogen and then stored in 0.5 ml of 10% trichloracetic acid (TCA) at −70°C. The tissue was homogenized manually with a glass-glass mortar and then centrifuged at 1500 *g* (4°C) for 10 min. The pellets were dissolved and the protein content was determined by the protein assay described by Bradford (1976), with bovine serum albumin used as a standard. The supernatants were extracted 5 times with 5 ml of water-saturated diethyl ether. The aqueous phases were evaporated and the residues stored at −20°C. The 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 (RIANEN, Du Pont Company, Boston, MA, U.S.A.). A [^{3}H]-cyclic GMP recovery marker was added to the TCA tissue homogenate in order to determine the recovery of cyclic nucleotides during the ether extraction. The mean recovery was 71%.

Functional studies

Silk ligatures were applied at both ends of the strip preparations. The strips were then mounted between two L-shaped hooks in thermostatically controlled (37°C) organ baths (5 ml) containing Krebs solution (for composition, see below), aerated with a mixture of 95% oxygen and 5% carbon dioxide to maintain pH at 7.4. As a standard procedure, the fluid in the organ baths was changed every 30 min for fresh Krebs solution, kept as mentioned above. Tension was registered by means of a Grass FTO 3C force transducer connected to a Grass 7D polygraph (Grass Instrument Co., Quincy, MA., U.S.A.). Electrical field stimulation (EFS) was with a Grass S48 stimulator connected to a pair of platinum electrodes (4 mm long) placed in parallel at both sides of the CC preparations in the organ baths. Single square wave pulses were delivered at supramaximum voltage (20–40 V) with a duration of 0.8 ms and at a frequency producing approximately 80% of the maximum contractile response (20–32 Hz). A preparation was regarded as stable when the amplitudes of three consecutive electrically induced contractions did not differ by more than 10%. The polarity was changed after each pulse with a polarity-changing unit. The train duration was 5 s and the stimulation interval 120 s.

Experimental procedure

An equilibration period of approximately 1 h, during which tension was adjusted, was allowed for the preparations to attain stable tension levels of 5.8 ± 0.2 mN ($N=15$, $n=61$). Previous investigations (Hedlund & Andersson, 1985) have reported good and reproducible responses to agonists and EFS at this tension level. To verify the contractile ability of the CC preparations, an isotonic K^+ solution (for composition, see below) was added to the organ baths. The responses obtained amounted to 17.0 ± 1.0 mN. The relaxant effects of PACAP-27, PACAP-38, Hel-1, Hel-2 and VIP, added cumulatively, were investigated in CC preparations contracted with NA; 10^{-6} M. In addition, the inhibitory effects of PACAP-27, Hel-1 and VIP were compared in strips activated by EFS.

Drugs and solutions

A Krebs solution of the following composition was used (mM): NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5 MgCl 1.2, NaH_2PO_4 1.2 and glucose 11. An isotonic K^+ solution (124 mM) was prepared in which the NaCl of the normal Krebs solution was replaced by equimolar amounts of KCl.

The following drugs were used: NA (Aldrich-Chemie GmbH & Co, Steinheim, Germany), PACAP-27, PACAP-38, Hel-1, Hel-2 (Peninsula Laboratories Inc. St. Helens, U.K.), VIP (Sigma Chemical Company, St. Louis, MO, U.S.A.). Stock solutions were prepared and then stored at −70°C. Subsequent dilutions of the drugs were made with 0.9% NaCl (for NA, the NaCl was supplemented with ascorbic acid as an antioxidant).

Calculations

The effects of the relaxant drugs are expressed as percentage relaxation of the agonist-induced contraction. Student's unpaired two-tailed *t* test was used for statistical comparison of two means. Statistical significance for the cyclic AMP data was calculated by using analysis of variance with Bonferroni/Dunnes correction. A probability of $P<0.05$ was accepted as significant. When appropriate, results are given as mean ± standard error of the mean (s.e.mean). *N* denotes the number of individuals and *n* the number of strip preparations. All statistical comparisons were made using *N*. The IC_{50} values denote the concentration producing 50% relaxation of the agonist-induced contraction. They were determined by linear interpolation.

Results

Immunocytochemistry and confocal microscopy

PGP-IR nerves were the most frequently occurring category of nerves. Gracile varicose terminals in rich amounts accompanied bundles of smooth muscle cells. Only a few nerves were found in the adventitia of the cavernous artery and smaller arteries, along which single coarse PGP-immunoreactive nerve trunks were running. Gracile PACAP-, Hel-, and VIP-IR varicose terminals were found in moderate numbers along bundles of smooth muscle cells, and dispersed in the adventitia, and along the borders of the adventitia and media in arteries of various sizes. No coarse PACAP-, Hel-, and VIP-IR nerve trunks were observed. No overt differences in number of PACAP-IR nerve structures could be seen between the two PACAP antisera used.

Double immunostainings revealed that there were coinciding profiles for nerve terminals that were PACAP- and VIP-, and Hel- and VIP-IR (Figures 1 and 2). Confocal microscopy revealed that there was a 90% coexistence between PACAP- and VIP-, and Hel- and VIP-IR nerves (Figures 3 and 4).

Measurements of PACAP and VIP

In the CC specimens ($N=8$, $n=8$), the mean concentration of VIP was 61.7 ± 11.6 pmol g^{-1} wet wt. that of PACAP-27 was 0.1 ± 0.05 pmol g^{-1} wet wt. ($N=7$; $n=7$; concentration below the level of measurement in 1 specimen), and that of PACAP-38 was 3.7 ± 0.5 pmol g^{-1} wet wt. In tissue from 4 patients with diabetes mellitus, the VIP concentration was 13.7 ± 1.2 pmol g^{-1} wet wt., that of PACAP-38 was 3.7 ± 0.8 pmol g^{-1} wet wt. and that of PACAP-27 was 0.1 ± 0.05 pmol g^{-1} wet wt.

Measurements of cyclic GMP/cyclic AMP

Cyclic nucleotide levels were measured in tissue exposed to Hel-1, Hel-2, PACAP-27, PACAP-38, and VIP in a concentration of 10^{-6} M. All peptides, but Hel-2, significantly increased the concentrations of cyclic AMP in comparison with controls (Figure 5). PACAP-38 ($N=5$, $n=5$; $P<0.001$) was the most effective of the peptides and produced a 132% increase in cyclic AMP-levels, followed by PACAP-27 ($N=6$, $n=6$; $P<0.001$), Hel-1 ($N=5$, $n=5$; $P<0.05$), VIP ($N=5$, $n=5$; $P<0.01$), and Hel-2 ($N=5$, $n=5$) with a 106%, 58%,

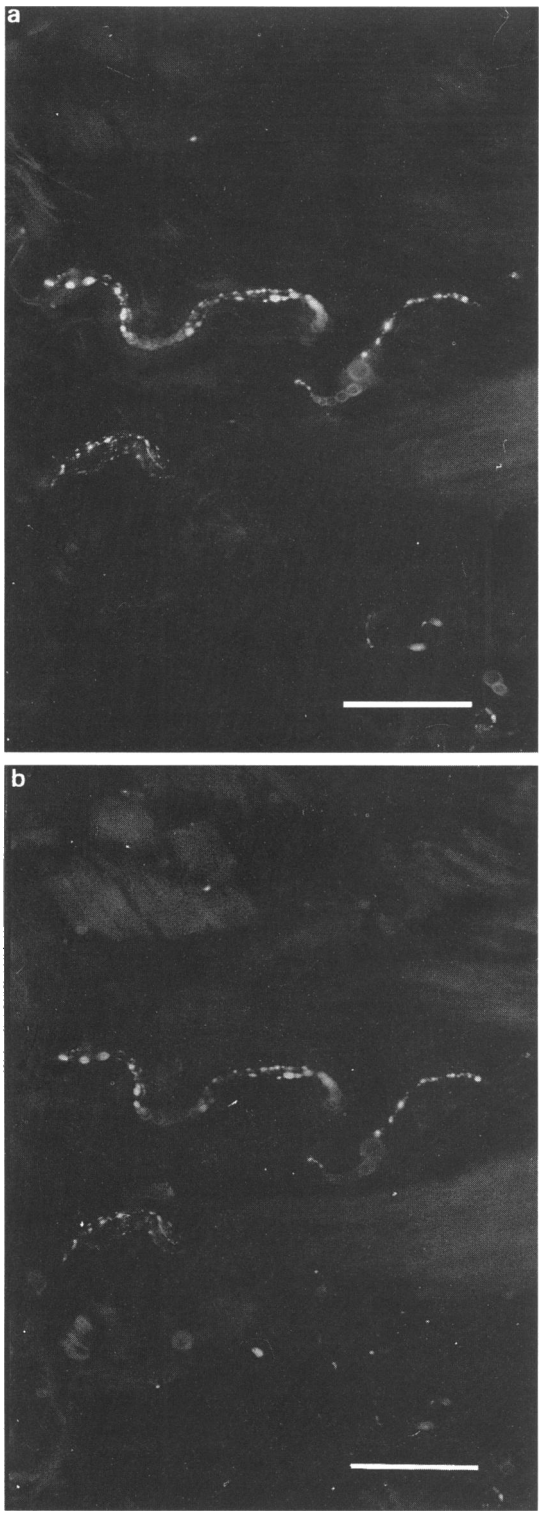

Figure 1 (**a**) VIP-IR varicose terminals: FITC fluorescence; (**b**) PACAP-IR varicose terminals: Texas Red fluorescence. Same section as in (**a**). Bar = 50 μm.

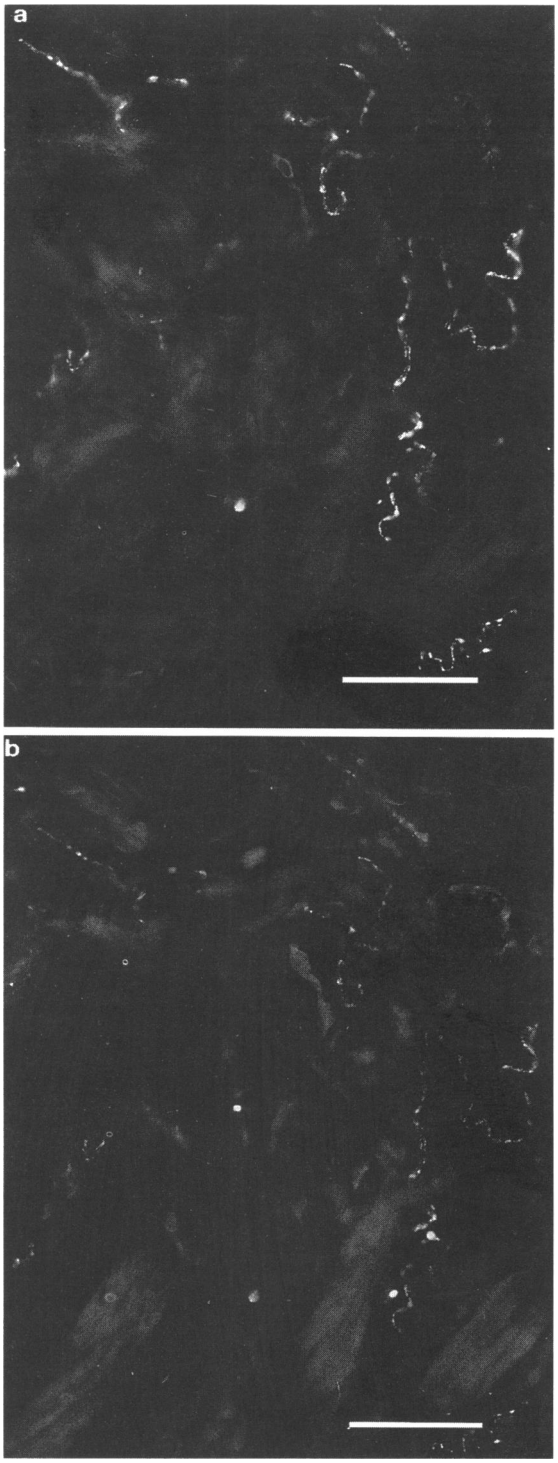

Figure 2 (**a**) VIP-IR varicose terminals: FITC fluorescence; (**b**) Hel-1-IR varicose terminals: Texas Red fluorescence. Same section as in (**a**). Bar = 50 μm.

57% and 27% increase, respectively. The control cyclic GMP value (3.3 ± 0.6 pmol mg^{-1} protein) was not significantly different from those obtained after exposure to VIP, PACAP-27, PACAP-38, Hel-1 or Hel-2.

Functional studies

Transient, spontaneous contractions were found in 11 out of 54 preparations. NA 10^{-6} M produced stable contractions amounting to 11.0 ± 0.7 mN ($N = 15$, $n = 54$). PACAP-27, Hel-1, and VIP concentration-dependently relaxed precontracted preparations (Figures 6 and 8). The $-\log IC_{50}$ values for VIP, PACAP-27 and Hel-1, were 7.00 ± 0.12 ($N = 12$, $n = 13$), 6.87 ± 0.20 ($N = 7$, $n = 12$), and 6.49 ± 0.12 ($N = 6$, $n = 8$), respectively. At 10^{-6} M, the highest peptide-concentration used, the relaxant effects obtained in NA-contracted strips were 79 ± 4%, 77 ± 6%, and 69 ± 5%, respectively. PACAP-38 and Hel-2 also produced relaxations in a concentration-dependent manner ($N = 5$, $n = 5$), and the effects at 10^{-6} M were 28 ± 9% ($-\log IC_{50}$ value noncalculable) and 65 ± 6% ($-\log IC_{50}$ value = 6.25 ± 0.11), respectively.

While relaxing the preparations, the peptides induced oscillations in tension in 13 out of 35 strips. This phenomenon

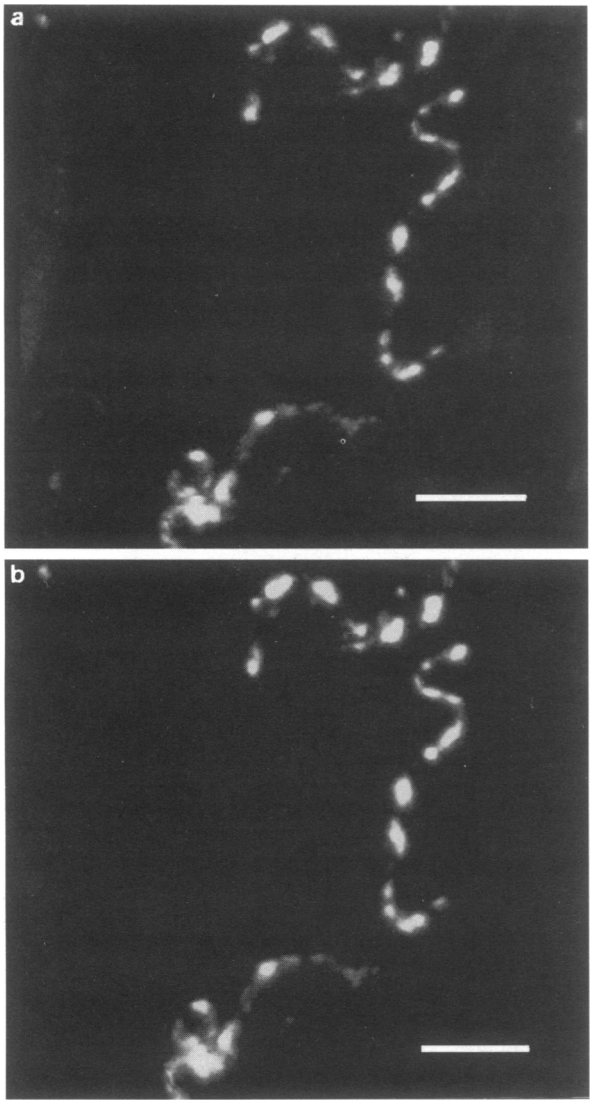

Figure 3 Confocal microscopy. Varicose nerve terminal showing co-localization of (**a**) VIP-immunoreactivity (FITC immunofluoresence) and (**b**) PACAP-immunoreactivity (Texas Red immunofluoresence). Each image is a maximum intensity projection of a 3D-median filtered (3 × 3 × 3) section series, consisting of 60 confocal sections (0.29 μm between adjacent sections). Bar = 10 μm.

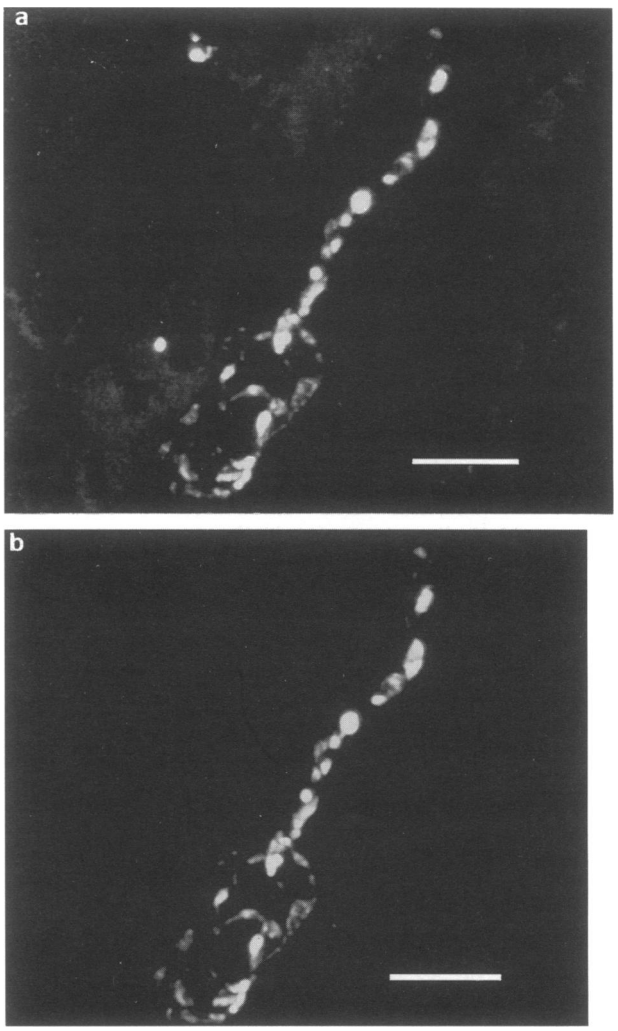

Figure 4 Confocal microscopy. Varicose nerve terminal showing co-localization of (**a**) VIP-immunoreactivity (FITC immunofluoresence) and (b) Hel-immunoreactivity (Texas Red immunofluoresence) . Each image is a maximum intensity projection of a 3D-median filtered (3 × 3 × 3) section series, consisting of 60 confocal sections (0.29 μm between adjacent sections). Bar = 10 μm.

was most frequently seen with PACAP-27, where oscillations were observed in half of the preparations, and with PACAP-38, which induced oscillations in 80% of the stips.

For NA-contracted preparations used later than within 12 h, the relaxant effects of PACAP-27 and VIP were reduced to 15 ± 7% ($N = 5$, $n = 6$), and 30 ± 16% ($N = 4$, $n = 4$), respectively. Data obtained from these experiments are not included in the presented results.

In electrically contracted preparations, Hel-1, VIP, and PACAP-27 effectively counteracted the contractile responses (Figure 7 and 8). The $-\log IC_{50}$ values were 7.70 ± 0.11 ($N = 6$, $n = 6$), 7.46 ± 0.26 ($N = 6$, $n = 6$), and 7.04 ± 0.8 ($N = 6$, $n = 6$), respectively. Inhibitory effects of 96 ± 3%, 87 ± 6% and 80 ± 3% were achieved at a peptide concentration of 10^{-6} M.

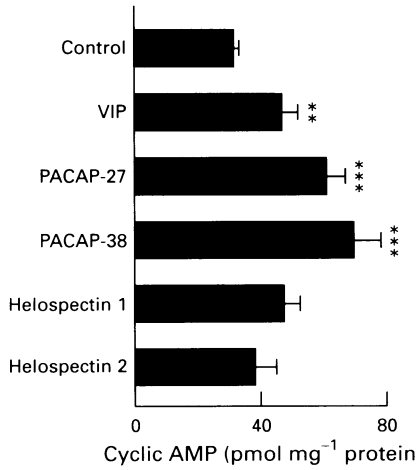

Figure 5 Tissue concentration of cyclic AMP 2 min after peptide administration to precontracted (NA 10^{-6} M) preparations. Control ($N = 11$, $n = 11$), VIP ($N = 9$, $n = 9$), PACAP-27 ($N = 6$, $n = 6$), PACAP-38 ($N = 5$, $n = 5$), helospectin-1 ($N = 5$, $n = 5$), and helospectin-2 ($N = 5$, $n = 5$).

Discussion

Non-adrenergic, non-cholinergic (NANC) relaxation of CC smooth muscle is considered to be an essential step in the induction and maintenance of penile erection. Nitric oxide (NO) or a NO-like substance is probably the most important in-

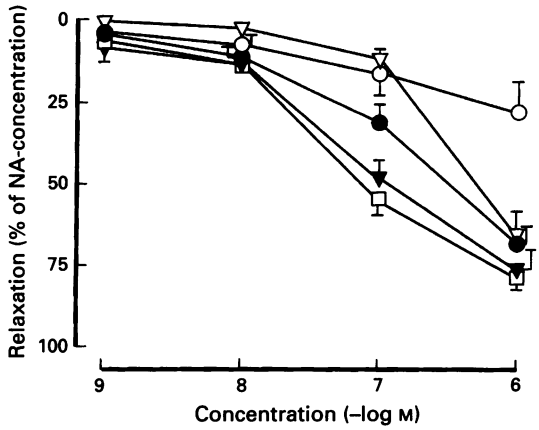

Figure 6 The relaxant effect of PACAP-27 (●; $N=12$, $n=13$), PACAP-38 (○; $N=5$, $n=5$) helospectin-1 (▼; $N=6$, $n=8$), helospectin-2 (▽; $N=5$, $n=5$) and VIP (□; $N=9$, $n=9$) on cavernous preparations contracted by (−)-noradrenaline 10^{-6} M. Values are given as mean ± s.e.mean.

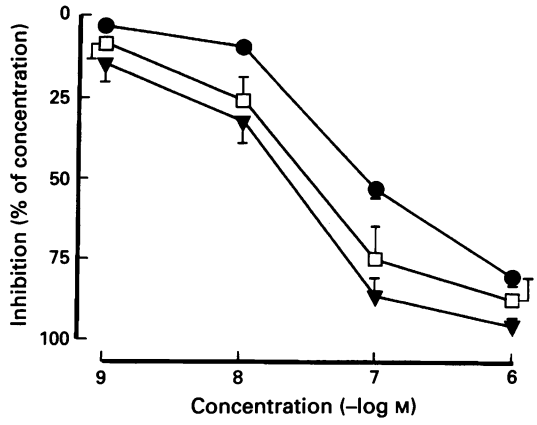

Figure 7 Relaxant effect of PACAP-27 (●; $N=6$, $n=6$), Hel-1 (▼; $N=6$, $n=6$) and VIP (□; $N=6$, $n=6$) on contractions induced by electrical field stimulation (supramaximum voltage, pulse duration 0.8 ms, frequency 20–32 Hz, train duration 5 s, stimulation interval 120 s). Values are given as mean ± s.e.mean.

hibitory NANC transmitter (Andersson, 1993; Klinge & Sjöstrand, 1994; Andersson & Wagner, 1995). By immunohistochemistry, NOS has been localized to the pelvic plexus and the cavernous nerves, and their terminal endings within corporeal tissue (Burnett *et al.*, 1992; 1993; Alm *et al.*, 1993), and NO has been shown to mediate both endothelium- and neuronally mediated relaxation in isolated CC tissue from various species (Andersson, 1993; Klinge & Sjöstrand, 1994; Andersson & Wagner, 1995). However, several peptides have been suggested to contribute to the NANC-mediated relaxation of penile erectile tissues, including VIP, peptide histidine methionine and calcitonin gene-related peptide (Andersson, 1993).

Many immunohistochemical studies have demonstrated VIP in autonomic nerves in the smooth muscle of the human corpus cavernosum and corpus spongiosum, and in penile vessels, mainly arteries and arterioles (Polak *et al.*, 1981; see Andersson 1993), and the density of VIP-containing nerves has been described as exceeding that of adrenergic nerves (Gu *et al.*, 1983). Human penile erectile tissue has been found to contain high concentrations of VIP (Polak *et al.*, 1981; Shirai *et al.*, 1990). In isolated cavernous preparations, VIP has been shown to produce relaxation (Larsen *et al.*, 1981; see Andersson, 1993), being more effective in counteracting contractions elicited by spontaneous myogenic activity and electrical

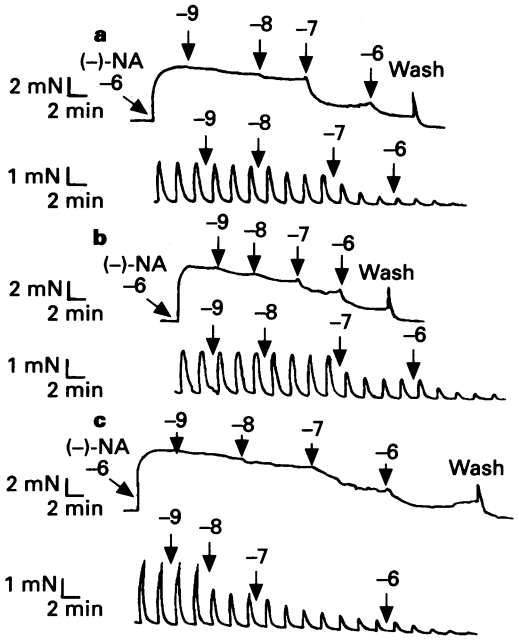

Figure 8 Tracings showing the effects of VIP (**a**), PACAP-27 (**b**), and Hel-1 (**c**) on preparations contracted by (−)-noradrenaline ((−)-NA; upper panels) and electrical field stimulation (lower panels). Stimulation parameters as in Figure 7.

stimulation than relaxing NA-contracted preparations (Andersson, 1993). Intracorporeal injection of VIP in anaesthetized dogs induced erectile responses (Jünemann *et al.*, 1987; Takahashi *et al.*, 1992), but failed to do so in impotent men (Adaikan *et al.*, 1986; Kiely *et al.*, 1989; Roy *et al.*, 1990), and in healthy volunteers (Wagner & Gerstenberg, 1987). These findings have suggested that VIP may be important for penile erection, but a definite role has not been established.

The present results confirmed previous findings showing that the majority of VIP-immunoreactive nerves accompanied cavernous smooth muscle bundles and occurred in nerve endings and in arteries. However, they also revealed the occurrence of PACAP- and Hel-immunoreactivity in nerve terminals following smooth muscle cells. Moreover, double immunostainings showed coinciding profiles for the distribution of nerve endings that were VIP- and PACAP-IR, and VIP- and Hel-IR. With the antiserum available to us, we were unable to demonstrate the occurrence of PACAP-38.

Throughout the gut of several species, PACAP and VIP as well as Hel and VIP have been found to be co-localized within nerve structures and suggested to be involved in local motor-, secretory- and vasoregulation (Sundler *et al.*, 1992; Absood *et al.*, 1992c; Desai *et al.*, 1992). The present confocal microscopic findings further support the co-localization of PACAP and VIP, and Hel and VIP in terminal neurones within the cavernous tissue, suggesting a close relation between the peptides in local modulatory action of penile smooth muscle tone. Like VIP and PACAP-27, Hel-1 produced concentration-related relaxations of NA-contracted CC preparations. VIP was more potent than Hel-1 and PACAP-27, but the differences in IC_{50} values were small. The finding of diminished effects of VIP and PACAP-27 (Hel was not tested) in experiments performed later than 12 h after the surgical intervention was unexpected, particularly since the contractile ability of the preparations was preserved, and the relaxation in response to EFS and to carbachol was unimpaired. The reasons for this are unclear.

The relaxation induced by PACAP-27, PACAP-38, Hel-1, and Hel-2 was associated with an increase in the concentrations of cyclic AMP, suggesting that these peptides, like VIP, act through the cyclic AMP system. One of the receptors for PACAP (type-I; e.g., Christophe., 1993), has a high affinity for

PACAP and no affinity for VIP. Another receptor (type-II; Ishihara *et al.*, 1992), has a similar affinity for both VIP and PACAP. Interestingly, PACAP-38 produced the most pronounced increase in cyclic AMP, but was the least effective in causing relaxation. The possibility cannot be excluded that PACAP-27, PACAP-38, and VIP act via a common receptor to produce a cyclic AMP increase and relaxation, PACAP-27 being more effective than PACAP-38. However, PACAP-38 may, in addition, via the specific PACAP-receptor, more effectively than PACAP-27 (c.f. Morrow *et al.*, 1993), stimulate an increase of a cyclic AMP pool not associated with relaxation. This may explain the discrepancy between the increases in cyclic AMP and the capacity to relax the preparations.

The receptor for Hel-1 is not known, but possibly also Hel-1 can activate the common VIP/PACAP-receptor.

In electrically stimulated preparations, PACAP-27, VIP, and Hel-1 effectively inhibited the contractile responses. As previously seen with VIP (Andersson, 1993), the peptides had less effect in NA-contracted preparations than in those exposed to EFS. The reason for this may be that the peptides have both prejunctional and postjunctional effects, the former leading to a diminished release of NA from the adrenergic nerve terminals.

In agreement with previous investigations, the present results showed that CC contained high concentrations of VIP. The levels of PACAP, i.e. the levels of PACAP-38 and PACAP-27 taken together, were 1/10 of the VIP content.

Interestingly, the total tissue concentration of PACAP was almost exclusively accounted for by PACAP-38. Considering that PACAP-38 was less potent than PACAP-27, our findings could reflect that PACAP-38 in the event of neuronal activation has to be converted to the more active PACAP-27 to exert an effect. Even if this is the case, the fact that the potency of PACAP-27 was not higher than that of VIP, and that the total amount of PACAP (−27 plus −38) was considerably lower than that of VIP, make it reasonable to question the physiological role of PACAP in penile erection. Previous investigators have shown that the concentration of VIP in CC decreases in patients with diabetic erectile dysfunction (Lincoln *et al.*, 1987). Although preliminary, our results confirmed this, but also showed that the concentrations of PACAP were not different from those found in the controls. Provided that there is a causal relation between the CC concentration of VIP and erectile dysfunction, this finding further supports the doubts about the role of PACAP in erectile function. In the rat, PACAP has been found in afferent pathways from the dorsal root ganglia to the dorsal horn in the spinal cord of the lumbo sacral region, and it has been suggested that it is involved in the spinal regulation of nociception (Moller *et al.*, 1993). PACAP may be localized to afferent nerves also in the CC, and instead having a modulatory action of smooth muscle tone, PACAP may be involved in penile sensory functions.

As mentioned above, neuronally derived NO seems to be essential for NANC-mediated relaxation of CC tissue. An interplay between NO and VIP has been suggested to occur in at least some parts of the gastrointestinal tract (Mahklouf & Grider, 1993), and recently an interaction of NO and PACAP was described (Jin *et al.*, 1994). The finding that NOS and VIP were found within the same nerve structures in human CC (Jünemann *et al.*, 1993), raises the question whether there is an interplay not only between NO and VIP, but also between NO and Hel-1 and/or NO and PACAP in the CC. The physiological consequences of such interactions are not known.

Thus, the present results suggest that VIP, PACAP, and Hel-1 are localized within the same neuronal structures in human erectile tissue. The three peptides are structurally related and relax smooth muscle in different organs, including the CC. However, the roles of VIP and related peptides as neurotransmitters and/or neuromodulators in the nervous control of penile erection still have to be established. The present results do not indicate that the VIP-related peptides have a prominent role in this context. An interaction between NO and VIP/VIP-related peptides seems to be a possible mechanism for relaxation, but has to be demonstrated.

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