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The induction of nitric oxide-mediated relaxation of human isolated pulmonary arteries by PACAP

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1 The effects of pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP) were analysed in human isolated circular segments of pulmonary arteries. Guinea-pig pulmonary arteries were used for comparison. The responses obtained were analysed in relation to the vascular endothelium and the nitric oxide (NO) synthase inhibitor N^G-monomethyl L-arginine (L-NMMA).

2 PACAP and VIP induced concentration-dependent relaxations of precontracted pulmonary arteries. The maximal dilator response (I_{max},%) and the potency (pEC_{50} value) were the same for both peptides, and there were no differences in the effects obtained on human and guinea-pig segments. PACAP and VIP were both more potent that acetylcholine (ACh).

3 Removal of the vascular endothelium abolished the PACAP induced dilator response in pulmonary arteries from both species. The VIP induced dilatation was unaffected, whereas the response to ACh was abolished. L-NMMA given before PACAP inhibited the dilatation. Furthermore, L-NMMA also reversed the dilatation already induced by PACAP and excess concentrations of L-arginine restored the dilator response of the L-NMMA treated arteries.

4 PACAP is a potent dilator of human pulmonary arteries. Although the dilator effect seems to be similar in amplitude to the one induced by VIP, the present results suggest differences in the underlying mechanisms of action (endothelium-dependency) between the two peptides.

Keywords: Pituitary adenylate cyclase-activating peptide (PACAP); vasoactive intestinal peptide (VIP); nitric oxide (NO); N^G-monomethyl L-arginine (L-NMMA); pulmonary artery; neuropeptide

Introduction

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide belonging to the secretin-glucagon-vasoactive intestinal peptide (VIP) family of peptides. It was originally isolated from ovine hypothalamus on the basis of its ability to stimulate adenylate cyclase in rat cultured pituitary cells (Miyata *et al*., 1989). It occurs in two forms: PACAP 27 and PACAP 38, where PACAP 27 constitutes the N-terminal, 'VIP-like', portion of PACAP 38 (Miyata *et al*., 1990). PACAP-like immunoreactive nerve fibres are distributed in the lungs of guinea-pig and man and *in vitro* PACAP 38 induces a concentration-dependent relaxation of guinea-pig isolated pulmonary arteries (Cardell *et al*., 1991; Luts *et al*., 1993). Preliminary studies indicated that PACAP-induced relaxation may rely on a functioning endothelium. However, data from studies in other vascular beds have indicated that the PACAP-induced relaxation may be endothelium-independent (Warren *et al*., 1991; Minkes *et al*., 1992; Kästner *et al*., 1995). In the present study, we have tried to evaluate the vasodilator responses induced by PACAP and VIP in human and guinea-pig pulmonary arteries in relation to the vascular endothelium and the nitric oxide (NO) synthase inhibitor N^G-monomethyl L-arginine (L-NMMA).

Methods

Human lung tissue was obtained at lobectomy performed for carcinoma of the lung. A macroscopically normal part of the lung was excised and immersed in a cold (+4°C) buffer solution (for composition, see over). Young male guinea-pigs (200–300 g) were killed by a blow to the neck and the lungs were removed and immersed in the same type of buffer solution. Small pulmonary arteries were dissected (the third to fourth branches in guinea-pigs and the fifth to seventh branches in man). Each vessel segment was divided in matching cylindrical segments. Care was taken to avoid excess manipulation of the tissue in order to minimize damage to the walls. The specimens were used in experiments within a couple of hours.

The vasomotor reactivity was analysed in tissue-baths (Högestätt *et al*., 1983). The segments were mounted on two L-shaped metal prongs (100 μm in diameter). One prong was connected to a force-displacement transducer (FT03C, Grass Instr., U.S.A.) attached to a computer (486 LOOP, Phoenix Technologies Ltd., U.S.A.) for recording of isometric tension. The other prong was connected to a displacement device, allowing fine adjustments (with an accuracy of 2.5 μm) of the distance between the two parallel prongs. The suspended segments were immersed in small (2.5 ml volume), water-mantled, temperature controlled (37°C) tissue baths containing a Na^+-Krebs solution. The solution was continuously equilibrated with 5% CO_2 in O_2 resulting in a pH of 7.4.

The segments to be tested were given an initial passive load through adjustment of the distance between the two metal prongs. A tension of 0.5–2.0 mN was applied to the segments. The tension was chosen with regard to variations in outer diameter and length between the individual segments. The specimens were subsequently allowed to stabilize at the selected level of tension for 90 min. The contractile capacity of each tissue segment was then examined through exposure to a potassium rich (60 mM) buffer solution. Only segments showing strong and reproducible contractions (<10% variation between the two tests) were included in the studies. In order to study relaxation capacity, the segments were submaximally precontracted by histamine 5-hydroxytryptamine (5-HT) or endothelin-1 (ET-1).

In most experiments the concentration-response relationship for agonists was determined by the cumulative application

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of increased drug concentrations. There were no differences in the response to PACAP when concentration-response curves obtained by cumulative application were compared to those obtained by a single dose procedure (Cardell *et al*., 1990). The integrity of the vascular endothelium was assessed by obtaining a dilator response to ACh (Furchgott, 1984). In separate experiments the endothelium was removed by rubbing the intimal surface with a small wooden stick inserted via one cut end. The absence of the endothelium was verified through the use of ACh (see above) and staining with 5% silver nitrate followed by light microscopy (Abrol *et al*., 1984).

I_{max}(%) represents the maximal dilator response induced by an agonist, expressed as a percentage of the precontraction induced by a precontracting agent. The pEC_{50} value represents the negative logarithm of the agonist concentration eliciting half the maximal response, EC_{50}. To obtain this value the log concentration-response relationship was approximated by linear regression analysis of the data within the 5–95% interval. Since the linear regression in some experiments only were reliant on 3 to 4 points, these data were also fitted to a logistic hypobolic equation with tension as a function of concentration (Aceves *et al*., 1985; Randall *et al*., 1989). The pEC_{50} values obtained through linear regression analysis and by the logistic hypobolic equation, did not differ significantly.

Solutions and drugs

Buffer solutions (a) Standard buffer solution (mM): NaCl 119, KCl 4.6, $CaC1_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and glucose 11. (b) 60 mM K^+ buffer solution: as above, with equimolar amounts of NaCl substituted with KC1. Analytical-grade chemicals and double-distilled water were used for preparing all solutions.

Drugs Pituitary adenylate cyclase activating peptide 38 (Peninsula, U.S.A.), acetylcholine hydrochloride (Sigma, U.S.A.), endothelin-1 (Auspep, Australia), histamine dihydrochloride (Sigma, U.S.A.), 5-hydroxytryptamine creatinine sulphate (Sigma, U.S.A.), L-arginine (Sigma U.S.A.), Nω-nitro-L-arginine (Sigma, U.S.A.), vasoactive intestinal peptide (Peninsula, U.S.A.). PACAP and VIP were dissolved and further diluted in saline containing bovine serum albumin (1%) and used in the experiments within 30 min. The concentrations of the agents are expressed as the final molar concentration in the bath.

Statistics

Results are expressed as mean ± s.d. Statistical comparisons were made by Student's *t* test and *P* values less than 0.05 were accepted as being statistically significant.

Results

PACAP induced a concentration-dependent relaxation of human and guinea-pig pulmonary arteries, precontracted with histamine or 5-HT. The maximal relaxation obtained, did not differ significantly between humans and guinea-pigs (I_{max}(%): 35 ± 21, $n=10$ and 26 ± 10, $n=6$, respectively), nor could any differences in potency be seen (pEC_{50} values; 8.21 ± 0.48 and 8.24 ± 0.53, respectively. VIP induced a dilatation response, nearly identical to the one induced by PACAP, whereas ACh induced a less potent relaxation ($P<0.05$). Maximal vasodilatation was achieved with PACAP 10^{-7} M, VIP 10^{-7} M, ACh 10^{-5} M (guinea-pig) and ACh 3×10^{-5} M (human), respectively. Larger doses of PACAP, VIP and ACh, did not elicit any further dilatation. Human data are summarized in Table 1 and concentration-response curves obtained in both human and guinea-pig isolated tissues are shown in Figure 1.

Only pulmonary arterial segments with intact endothelial cells could be relaxed by exposure to ACh. In endothelium denuded segments PACAP induced no (or occasionally, a very small, <7%) dilatation. These segments could further be dilated by VIP in a concentration-dependent manner (Figure 2). The same results were obtained when VIP was given to endothelium-denuded segments, which had not been pretreated with PACAP (Figure 3). Endothelium denuded segments from humans and guinea-pigs responded in the same principal way. Pretreatment with L-NMMA (100 μM) markedly reduced the

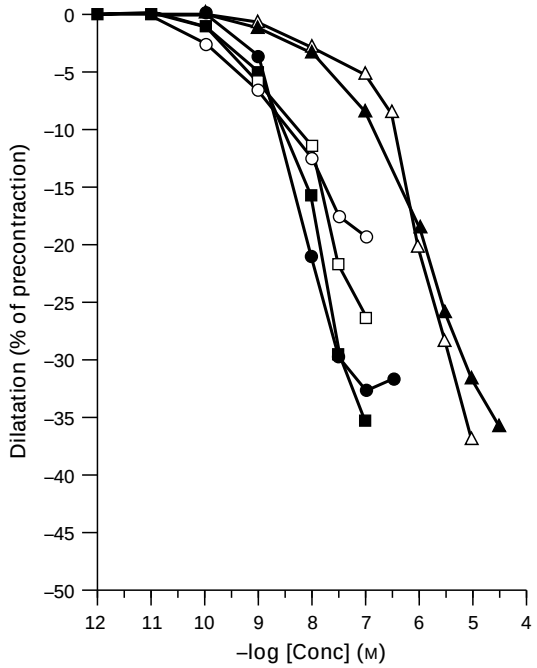

Figure 1 Concentration-dependent relaxations induced by PACAP (■, □), VIP (●, ○) and ACh (▲, △) in human (solid symbols) and guinea-pig (open symbols) pulmonary arteries. Responses are expressed as a percentage of precontraction and each point is the mean of 5–10 experiments.

Table 1 Relaxation of human pulmonary artery

		n	*Precontraction* (mN)	I_{max} (%)	pEC_{50}
PACAP	E+	10	1.11 ± 0.60	35 ± 21	8.21 ± 0.48
	E–	5	1.18 ± 0.93	3 ± 5	
VIP	E+	5	1.18 ± 0.64	33 ± 21	8.30 ± 0.14
	E–	5	1.13 ± 0.56	24 ± 13	8.49 ± 0.18
ACh	E+	10	0.72 ± 0.46	36 ± 11	6.15 ± 0.64
	E–	10	1.00 ± 0.65	0	

PACAP, VIP and acetylcholine (ACh) induced dilatations of histamine precontracted guinea-pig pulmonary arteries with (E+) and without (E–) an intact endothelium. Maximal relaxation expressed as percentage of precontraction (I_{max}) and sensitivity expressed as the negative logarithm of the contraction eliciting half the maximal relaxant response value (pEC_{50}). The values represent mean ± s.d.

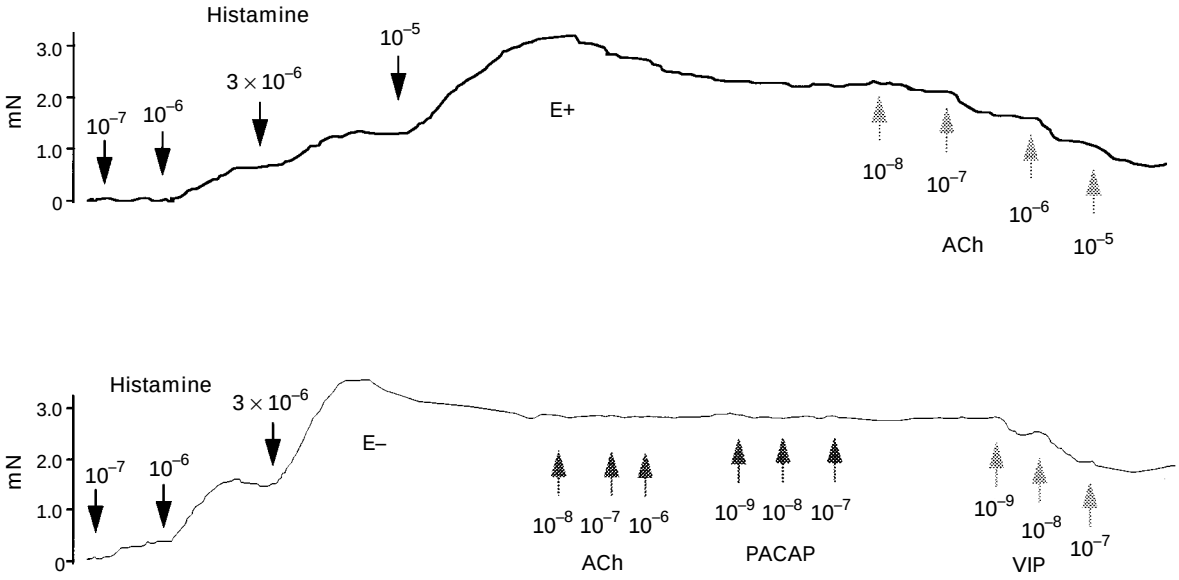

Figure 2 Typical examples of acetylcholine (ACh), PACAP and VIP-induced dilatations of histamine precontracted guinea-pig pulmonary arteries with (E+) and without (E−) an intact endothelium. The upper trace shows that acetylcholine induced a concentration-dependent dilatation, indicating a functioning endothelium. The lower trace shows a matched arterial segment where the endothelium has been removed. In the latter, acetylcholine as well as PACAP failed to induce any relaxation, whereas application of VIP resulted in a concentration-dependent dilatation, indicating a difference in endothelium-dependence between the two peptides. All concentrations are molar.

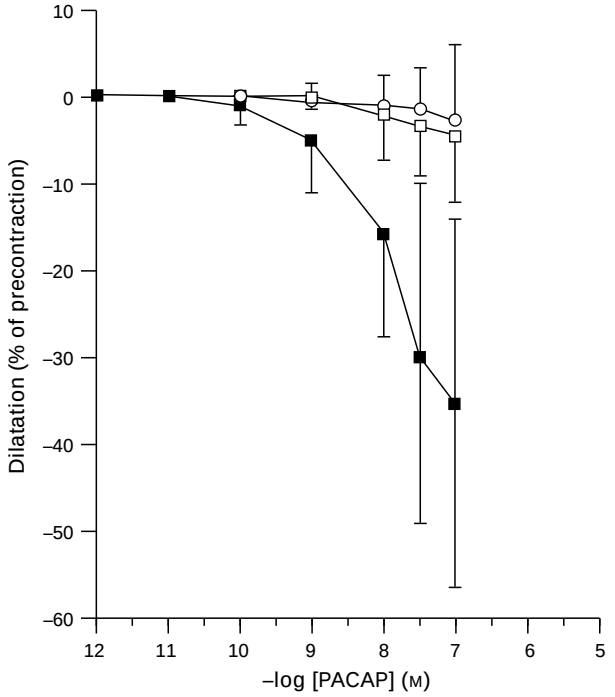

Figure 3 Dilator response to PACAP in human precontracted pulmonary arteries in the absence and presence of L-NMMA (100 μM), and after endothelium removal. Control pulmonary arteries with intact endothelium in the absence (■) and presence (□) of L-NMMA and pulmonary arteries denuded of endothelium (○). Responses are expressed as a percentage of precontraction and each point is the mean with s.d. shown as vertical lines ($n=5-10$).

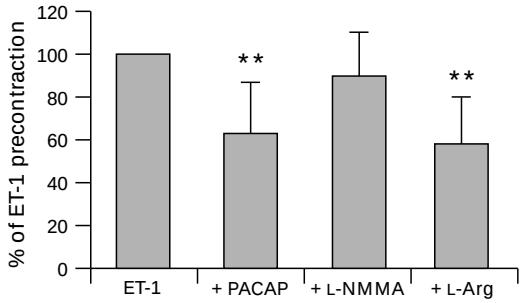

Figure 4 Maximal changes in tension levels of human pulmonary arteries, precontracted with endothelin-1 (ET-1) (first column). These segments were dilated by application of a single dose of PACAP (3×10^{-8} M) (second column), then the dilatation was suppressed by the addition of L-NMMA (100 μM) (third column). The dilator response could be restored by the addition of L-arginine (L-Arg) in an excessive concentration (100 μM) (fourth column). The values represent mean ± s.d of 6 experiments. $^*P<0.05$.

PACAP-induced relaxation (humans, I_{max}(%): 3 ± 5, $n=5$, $P<0.05$) (Figure 3) and abolished the ACh-induced dilatation ($n=5$).

The precontraction levels of both histamine and 5-HT precontracted segments ($10^{-6}-10^{-5}$ M) were changed by application of L-NMMA (100 μM) to the tissue-baths. Therefore, when the effects of L-NMMA on PACAP-relaxed segments were analysed, endothelin-1 (ET-1) (10^{-7} M) was used for precontraction. In the concentration used, the ET-1-induced response was not affected by application of L-NMMA (100 μM) or by removal of the endothelium. Human and guinea-pig pulmonary arterial segments precontracted by ET-1 were relaxed on the application of a single dose of PACAP (3×10^{-8}M) human, E_{max}(%); 34 ± 27, $n=6$). The inhibition obtained could be reversed by addition of L-NMMA (100 μM) and restored again by application of excess concentrations of L-arginine (100 μM) (Figure 4).

Discussion

PACAP induced a concentration-dependent dilatation of human isolated pulmonary arteries. The dilatation amplitude obtained was identical to the one induced by VIP in matched segments. However, the PACAP-induced dilatation seems to rely on a functioning endothelium, whereas the dilatation to VIP appears to be endothelium-independent.

The PACAP gene has been cloned in several species, and its transcript has been found in several tissues, including lung (Kimura *et al*., 1990; Hosoya *et al*., 1992; Shioda *et al*., 1994). In the respiratory tract of guinea-pig and rat small numbers of PACAP-immunoreactive fibres can be seen (Cardell *et al*., 1991). By contrast, the respiratory tract of man receives a comparatively rich supply of PACAP-containing fibres. The majority of these fibres also store VIP (Luts *et al*., 1993). In general, PACAP causes a systemic vasodilatation *in vivo* and a vasorelaxation *in vitro* (Cardell *et al*., 1991; Warren *et al*., 1991). The mechanisms involved in the vascular response to PACAP are to a large extent unknown (Nanha *et al*., 1991; Minkes *et al*., 1992). In the present study, the dilatation amplitudes induced by PACAP and VIP in human pulmonary artery were identical. This is in agreement with previous findings on guinea-pig isolated pulmonary arteries (Cardell *et al*., 1991). In human coronary arteries and rabbit aorta rings, PACAP 38 appears to be 5–10 and 100 times more potent than VIP, respectively (Warren *et al*., 1991; Kästner *et al*., 1995), whereas PACAP-38 has been shown to be less potent than VIP on porcine coronary arteries (Huang *et al*., 1992).

The human respiratory tract contains a comparatively rich number of nonadrenergic, noncholinergic (NANC) nerve fibres (Burnstock, 1972; Richardson & Béland, 1976). The NANC system seems to mediate airway smooth muscle relaxation, but the neurotransmitters involved in NANC dilatation have not been unequivocally identified. Although certain neuropeptides, like VIP, have been candidates for some time, the interest has recently focused on small molecules such as NO and carbon monoxide (Bult *et al*., 1990; Verma *et al*., 1993). Probably several substances act together as co-transmitters in order to induce a dilator response and it has been suggested that VIP may stimulate the synthesis of NO and *vice versa* (Shimosegawa & Toyota, 1994; Foda & Said, 1995). Our finding that the VIP-induced dilatation of human pulmonary artery seems to be endothelium-independent is in line with previous findings on this vessel (Greenberg *et al*., 1987). The present data also indicate that PACAP relies on the endothelium to induce pulmonary vasodilatation and that this dependency may involve the release of NO from the endothelium. This assumption is based on the finding the PACAP-induced dilatation is inhibited by L-NMMA, a well established NO synthase inhibitor. Furthermore, in arteries precontracted by ET-1, L-NMMA reversed the vasodilatation previously induced by PACAP. Excess concentrations of L-arginine, restored the dilator response of the L-NMMA treated arteries, further indicating the involvement of NO. The precontracting agent ET-1, was chosen since it, in the concentration used, is independent of the endothelium (Cardell *et al*., 1990). Other studies have indicated that PACAP induced dilatation of porcine coronary arteries and rabbit aorta, may be independent of a functioning endothelium (Warren *et al*., 1991; Kästner *et al*., 1995) and *in vivo* analysis of the systemic and pulmonary vascular responses to PACAP and VIP in cat have shown that L-NAME has no effect on the pulmonary responses to either peptide (Minkes *et al*., 1992). However, it is well known that the endothelium-dependence as well as the involvement of NO for VIP varies with the vessel type and species investigated (Bodelsson & Stjernquist 1992; Hattori *et al*., 1992; Luu *et al*., 1993; Morris 1993; Jansen-Olesen *et al*., 1994; Gyoda *et al*., 1995) and a similar phenomenon may be true for PACAP. In the present study the integrity of the vascular endothelium was assessed by obtaining a dilator response to ACh and vascular segments failing to respond were excluded. However, it is possible that some vessels with a reduced endothelium function were among the included segments. Thus, the PACAP-induced dilator response investigated seems to be dependent on the endothelium, the variability seen, especially at higher concentrations, may at least partly, be due to pathology-associated endothelial dysfunction in some of the vessels employed.

Like VIP, PACAP exerts its biological effects by binding to high-affinity receptors (Shivers *et al*., 1991). Three different G-protein coupled receptors that bind PACAP with high affinity have been cloned (Harmer & Lutz, 1994; Rawlings, 1994). One receptor (the PACAP type I receptor) recognizes VIP poorly and the chemically related peptide secretin, a second receptor (the PACAP type II or VIP_1 receptor) recognises both VIP and secretin, while a third receptor (the PACAP type III or VIP_2 receptor recognizes VIP but not secretin (Usdin *et al*., 1994). Several splice variants of the PACAP type I receptor, have been identified and found to be expressed in rat (Spengler *et al*., 1993; Journot *et al*., 1994). In the present investigation, PACAP and VIP were found to be equipotent. Conceivably, PACAP induces its dilator effects on human pulmonary arteries through a PACAP type II or type III receptor. Different sub types of the PACAP receptor may account for the finding that PACAP relaxes precontracted human pulmonary arteries by a mechanism which is dependent upon an intact endothelium as opposed to an endothelium-independent mechanism observed in other species (Warren *et al*., 1991; Kästner *et al*., 1995).

In conclusion, PACAP seems to be a potent dilator of human pulmonary arteries and its effects appear to involve the release of NO from the vascular endothelium.

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