Br. J. Pharmac. (1986), **89**, 157–162

Vasoactive intestinal peptide in bovine pulmonary artery: localisation, function and receptor autoradiography

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1 The role of vasoactive intestinal peptide (VIP) in the control of pulmonary vascular tone was investigated by functional response, immunocytochemical localisation and receptor autoradiography in bovine pulmonary arteries.

2 VIP-immunoreactive nerve fibres were present at the adventitial-medial junction and in the media of the vessels.

3 Exposure of precontracted bovine pulmonary artery segments to VIP *in vitro* resulted in almost complete (86 ± 3%; mean ± s.e.mean) relaxation, the concentration needed for 50% relaxation being $4.47 \pm 0.37 \times 10^{-9}$M. VIP effects did not depend on the presence of intact endothelial cells.

4 The distribution of VIP receptors was studied by autoradiography using [^{125}I]-VIP. A high density of VIP receptors was found in arterial vascular smooth muscle, with a gradient of density from adventitia to luminal surface. There were no receptors on endothelial cells.

5 These data show that VIP is a potent vasodilator of bovine pulmonary arteries, via direct activation of VIP receptors in vascular smooth muscle. VIP-immunoreactive nerves may influence pulmonary vascular tone directly and could, therefore, be important in regulating pulmonary blood flow.

Introduction

Non-adrenergic, non-cholinergic (NANC) nerves exist in many species. Although the neurotransmitters involved in this 'third' nervous system are not yet certain, there is some evidence that neuropeptides may be involved (Barnes, 1984). Non-adrenergic inhibitory nerves have been demonstrated in the lung, particularly in airway smooth muscle (Richardson, 1981) and there is evidence that vasoactive intestinal peptide (VIP) might be a neurotransmitter since VIP has been localised in these nerves by immunocytochemical methods (Dey *et al.*, 1981; Laitinen *et al.*, 1985) and VIP has been found to relax partially airway smooth muscle (Said, 1982; Cameron *et al.*, 1983; Palmer *et al.*, 1985). A NANC inhibitory response has also been demonstrated in the pulmonary artery of several species (Hamaski *et al.*, 1983b; Frank & Bevan, 1983). VIP is a potent vasodilator in the pulmonary circulation *in vitro*, and *in vivo* (Altiere & Diamond, 1983; Hamasaki *et al.*, 1983a; Hand *et al.*, 1984; Nandiwada *et al.*, 1985). The effects of VIP are mediated by specific cell surface receptors which have been identified in lung homogenates by direct binding techniques (Robberecht *et al.*, 1981). However some vasodilator responses are mediated by activation of endothelial cell receptors, rather than receptors on vascular smooth muscle, via the release of an endothelial-derived relaxant factor (Furchgott, 1983). In bovine pulmonary artery we examined the relationship between the localisation of VIP-immunoreactive nerves, the distribution of VIP receptors and the functional responses of isolated vascular smooth muscle to VIP in the presence and absence of endothelium.

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Methods

Tissue preparation

Fresh lung tissue was obtained from a local abattoir where young cattle were killed and exsanguinated. Tissue was immediately placed in ice-cold Krebs Henseleit (KH) solution, which had been pre-gassed with 95% O_2:5% CO_2 for transportation to the laboratory. The composition of KH (mM) was: NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, $NaH_2PO_4.H_2O$ 1.2, glucose 5.6 and $NaHCO_3$ 25.5. Intrapulmonary arteries 1–6 mm in diameter were carefully dissected free of adjacent tissue for functional and immunocytochemical studies. Small segments of lung tissue containing arteries of this size were also used for autoradiography.

Immunocytochemistry

Pulmonary arteries (PA) were fixed by immersion in 0.4% *p*-benzoquinone in 0.01 M phosphate buffered saline (PBS, pH;7.4) for 2 h (Bishop *et al.*, 1978). After fixation, the tissues were washed overnight in PBS containing 15% sucrose and 0.01% sodium azide. The PA segments were snap-frozen and cryostat sections (10 μm thick) were cut at −20°C. Sections were mounted on poly-L-lysine coated slides (Huang *et al.*, 1983) and air-dried for 1 h before incubation with primary antiserum. Immunohistochemistry was carried out according to the indirect immunofluorescence method (Coons *et al.*, 1955) and slides were examined using a Leitz fluorescence microscope.

Antibodies to natural porcine VIP conjugated to haemocyanin (Molluscan) were raised in New Zealand white rabbits by the glutaraldehyde method and used at a dilution of 1/2000. The specificity of VIP antiserum was confirmed by lack of staining in adjacent sections incubated with antiserum pre-absorbed with VIP soluble antigen in the dilution range 0.01–0.1 mmol ml^{-1} diluted antiserum. The antiserum did not show cross reactivity with other peptides known to be present in the lung (Bishop *et al.*, 1980; Ghatei *et al.*, 1982).

Functional studies

Rings 2–3 mm in width were cut from pulmonary arteries 3–5 mm in diameter. In half of these segments endothelial cells were removed by gently rotating a steel probe through the lumen for 30 s. Segments were suspended in a glass chamber containing 10 ml of KH by means of 2 gently curved stainless steel hooks. The lower hook was attached to a fixed support, while the upper was tied to a force transducer (Grass FT.03) mounted on a micrometer. The KH was continuously gassed with 95% O_2 : 5% CO_2 and maintained at 37°C. A preload tension of 6 g was applied to the tissues. This tension was found to be optimal for measuring changes in tension in this preparation. Preparations were equilibrated for 2 h and during this period, fluid in the baths was changed and the tension readjusted to 6 g every 15–30 min. Once equilibrated, preparations were used for 2–3 h. Changes in isometric tension were recorded on a polygraph (Grass 7D). Since pulmonary arteries of the size range used have little or no intrinsic tone, segments were precontracted to approximately 50% of maximal with either 5-hydroxytryptamine (5-HT) or phenylephrine (PE). Cumulative relaxation to adenosine triphosphate (ATP), acetylcholine (ACh) or VIP was then assessed by adding increasing concentrations of each agent to the bath. Each concentration was kept in contact with the tissue until the response reached a stable plateau. Maximal relaxation was defined as the greatest reduction of induced tone possible with a drug and EC_{50} as the concentration of drug required to produce 50% maximal relaxation.

The presence of endothelial cells was assessed histologically by a modification of the method of Poole *et al.* (1958). After functional studies were completed, tissue was removed from the bath and placed in the following solutions: 1% glutaraldehyde (5 min), 1% silver nitrate (2 min) and a mixture of 3% ammonium chloride and 5% cobalt bromide (2 min). Wet mount preparations were viewed *en face* at × 400 magnification.

Receptor autoradiography

Small pieces of lung tissue were snap-frozen in liquid dichlorodifluomethane (Arcton 12, ICI), cooled to −196°C by liquid nitrogen. Tissue sections (16 μm thick for binding studies and 10 μm thick for autoradiography) were cut on a cryostat at −16°C and thaw-mounted onto glass microscope slides coated with poly-L-lysine and stored at −80°C before use without loss of binding capacity. Binding of [^{125}I]-VIP to sections was characterised as previously described (Barnes & Carstairs, 1986).

Optimal specific binding of [^{125}I]-VIP (Amersham International; specific activity approximately 2000 Ci $mmol^{-1}$) was obtained by incubating sections at a radioligand concentration of 30 pM for 3h at 37°C in 50 mM Tris-HCl (pH 7.4), containing 5 mM $MgCl_2$, 2% polypep, 500 KI units ml^{-1} aprotinin and 0.5 mg ml^{-1} bacitracin followed by two × 5 min washes in ice-cold buffer lacking polypep.

Non-specific binding was determined on alternate sections by incubating in the presence of 1 μM unlabelled VIP. Under these conditions non-specific binding represented 15% of total binding (1227 ± 34 vs 182 ± 8 c.p.m.) in 4 separate experiments.

Autoradiograms were generated by the method of Young & Kuhar (1979). Slide-mounted sections (10 μm) were incubated as described and washed (2 × 5 min) in ice-cold buffer. The sections were then rinsed in distilled water to remove any buffer salts and rapidly air-dried and stored dessicated overnight. Glass coverslips that had been previously coated with stripping film (AR10, Kodak, UK) were then fixed to one end of the slides with cyanoacrylate adhesive and held in contact with the sections by butterfly clips. Slides were exposed to emulsion, dessicated in the dark at 4°C for 5 days. The coverslips were partially separated from the sections and the emulsion developed and fixed. Sections were stained with 1% cresyl fast violet and examined under a Zeiss microscope, equipped with light and dark-field illumination. Autoradiographic grain counts were performed using a calibrated eyepiece and a × 100 objective lens.

Drugs and chemicals

Chemicals were obtained commercially from the following sources: 5-HT, acetylcholine, ATP, poly-L-lysine, polypep, bacitracin, (±)-propranolol, and indomethacin (Sigma Chemicals, Poole); aprotinin (Bayer), VIP (Bachem, Torrance, California).

Results

Immunocytochemistry

VIP-immunoreactive fibres were seen in an annular formation around all pulmonary arteries, and particularly those which were 1–3 mm in diameter (Figure 1). VIP-immunoreactive nerves were tiny and rare in arteries smaller than 0.5 or larger than 5.0 mm in diameter. The immunoreactive nerve fibres were located either at the medial-adventitial junction or among the circularly arranged smooth muscle fibres in the tunica media of the arteries. No VIP-immunoreactive nerve fibres were seen in close contact with the tunica intima.

Functional studies

There were no significant differences in relaxation of arteries in response to VIP, whether they were precontracted with either 5-HT or PE; (maximum relaxation was 84 ± 3% after 5-HT and 86 ± 2% after PE; means ± s.e.mean, $n = 8$). The presence or absence of endothelial cells was determined both histologically and functionally by exposing segments to either ACh or ATP. Relaxation to ACh or to a low concentration ATP (< 10 μM) was seen in inrubbed segments only. Maximum relaxation of 78 ± 6% was seen with ACh (1 μM) in unrubbed segments, but this concentration

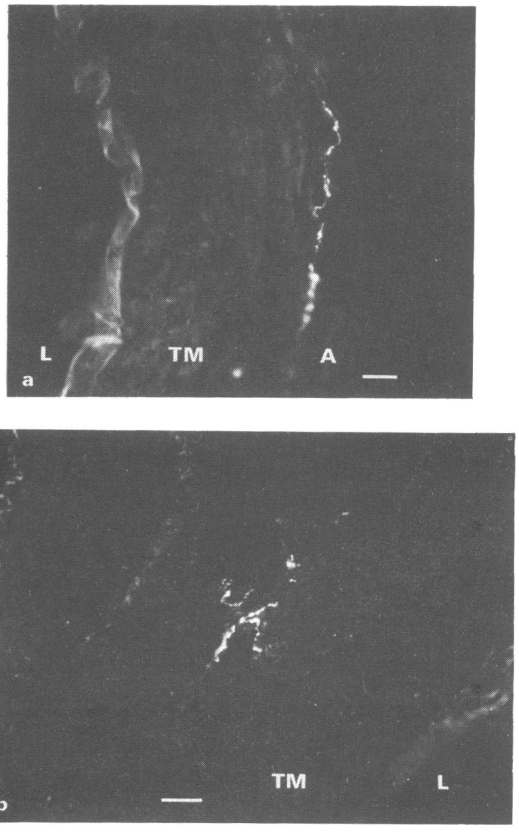

Figure 1 Vasoactive intestinal peptide (VIP)-immunoreactive nerves in bovine pulmonary artery: (a) longitudinal section of artery showing a brightly fluorescent nerve fibre at the junction of the media and adventitia. Magnification × 340; (b) cross section of artery showing fluorescent nerve fibre entering media. Magnification × 340. A: adventia; TM: tunica muscularis; L: lumen. Scale bar = 25 μm.

had no effect on rubbed segments. Higher doses of ATP, but not ACh, occasionally produced a small amount of relaxation in rubbed segments (maximum relaxation < 20% compared with relaxation of 80% in unrubbed vessels), which was easily distinguished from the response of unrubbed segments.

An isometric force of 3.0 ± 0.2 g was generated both in segments with and without endothelial cells. Dose-response curves for relaxation to VIP are shown in Figure 2. There were no significant differences in either maximal relaxation (85 ± 3% vs 87 ± 3%, $n = 16$) or in the EC_{50} ($4.47 \pm 0.37 \times 10^{-9}$M vs $5.12 \pm 0.35 \times 10^{-9}$M) between segments with or without endothelial cells. Neither indomethacin (10 μM) nor propranolol (1 μM) had any effect on VIP-induced

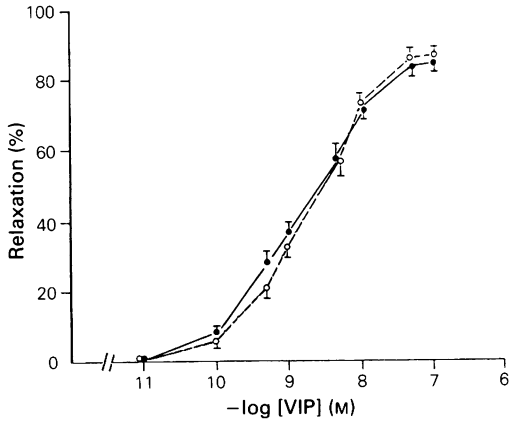

Figure 2 Inhibitory effects of vasoactive intestinal peptide (VIP) on bovine pulmonary artery rings. Arteries were precontracted with 5-hydroxytryptamine or phenylephrine. The response in segments with endothelial cells (●) was similar to that seen in segments without endothelial cells (○). Mean values of 28 arterial segments (3–5 mm diameter) are shown; s.e.mean indicated by vertical lines.

relaxation, indicating that neither prostaglandins nor β-adrenoceptor effects modulated the vascular response; the maximum relaxation in control segments was 86 ± 3%, after indomethacin 87 ± 7% and after propranolol 90 ± 2% (NS).

Autoradiography

Specific labelling was observed over the smooth muscle of all pulmonary arteries in the size range examined (1–5 mm in external diameter), although labelling was most dense in vessels 2–3 mm in diameter. An example of a 4 mm vessel is shown in Figure 3. A graph of specific grain counts at intervals throughout the thickness of the tunica media indicated that a density gradient exists with significantly higher counts at the adventitial surface compared with that at the luminal surface (171 ± 54 vs 73 ± 8 grains per 1000 μm; $P < 0.01$) (Figure 4). Endothelial cells did not appear to be labelled.

Discussion

We have demonstrated that VIP is a potent relaxant of bovine pulmonary arteries, which is in agreement with *in vitro* studies of pulmonary arteries from other species, including man (Altiere & Diamond, 1983; Hamasaki *et al.*, 1983a; Hand *et al.*, 1984; Greenberg *et al.*, 1985). Since many vasodilators are effective only in the presence of intact endothelial cells (Furchgott, 1983), we evaluated the effect of removing endothelial cells on the response to VIP. Present studies on the role of endothelieal cells on VIP-induced vasodilatation in systemic vessels have been conflicting, since in rat aorta relaxation induced by VIP is endothelial-dependent (Davies & Williams, 1984), whereas in canine carotid arteries it is not (D'Orleans-Juste *et al.*, 1985). In a preliminary study of human pulmonary artery we found that VIP-induced relaxation is unaffected by endothelial cell removal. Since, in the present studies, endothelial damage abolished the vasodilator response to ACh and ATP but left the response to VIP unaltered, we conclude that VIP receptors are present in bovine vascular smooth muscle, rather than on endothelial cells. This is supported by our autoradiographic studies, which showed a high den-

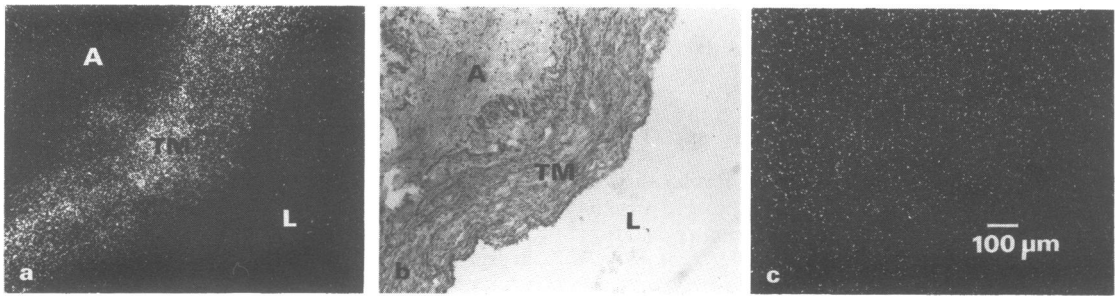

Figure 3 Distribution of vasoactive intestinal peptide (VIP) receptors in bovine pulmonary artery. Central panel (b) photomicrograph of a section of a 4 mm bovine pulmonary artery with lumen (L), tunica muscularis (TM), adventitia (A). The section is stained with 1% cresyl fast violet. (a) Dark-field photomicrograph of the same area showing the distribution of autoradiographic grains after incubation with [^{125}I]-VIP. (c) Non-specific labelling pattern in an adjacent section after the same incubation in the presence of an excess of unlabelled VIP (1 μM). Scale bar = 100 μm.

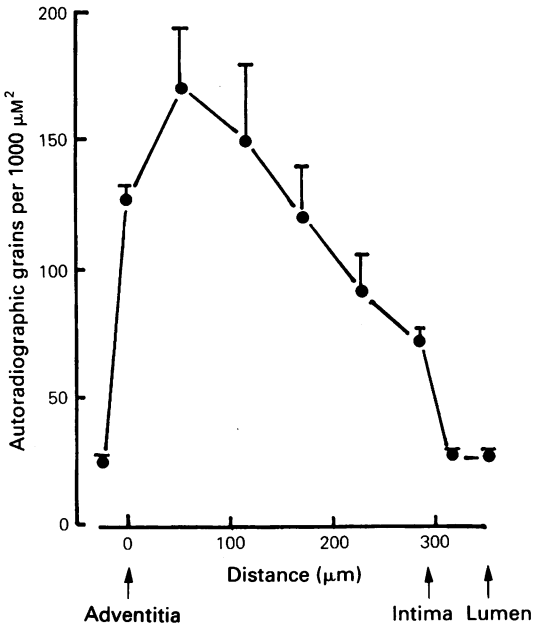

Figure 4 Distribution of autoradiographic grain density across pulmonary arterial wall from adventitia to lumen. Each point is the mean of 6 determinations; s.e.mean shown by verticals lines.

sity of VIP-receptors in bovine pulmonary artery. It is tempting to speculate that neurotransmitters, such as VIP, which are released from nerves in the adventitia of vessel walls vasodilate by a direct effect on smooth muscle, while vasodilatation due to agents, either blood borne or released from circulating cells, for example ATP, is mediated by a relaxant factor released from endothial cells. Acetylcholine-induced vasodilatation is endothelial-dependent, which is apparently inconsistent with this idea, since cholinergic nerves are also in the adventitia. However, recent studies have demonstrated that endothelial cells themselves may synthesize acetylcholine (Parnavelas *et al.*, 1985).

Bovine pulmonary artery is innervated by VIP-immunoreactive nerves which are located particularly at the adventitial/medial junction, with fine nerves penetrating the media. This is in agreement with findings for pulmonary vessels from other species, including rat, dog and human (Dey *et al.*, 1981). The demonstration of a VIP receptor density gradient, which is highest at the adventitial surface and diminishes towards the lumen, is of interest since VIP is presumably released predominantly at the adventitial surface. This finding is similar to the density gradient of muscarinic receptors in tracheal smooth muscle, in which the highest densities are localized away from the lumen where the cholinergic nerves enter the airway (Basbaum *et al.*, 1984).

Non-adrenergic, non-cholinergic neural vasodilatation has been demonstrated in blood vessels, including the pulmonary artery (Frank & Bevan, 1983; Hamasaki *et al.*, 1983b). Our results support the view that VIP is a possible neurotransmitter in the pulmonary artery, since in bovine artery VIP-immunoreactive nerves are present, VIP-receptors are found in the smooth muscle with a gradient related to the distribution of the nerves, and VIP is a potent direct relaxant. However, the role of VIPergic innervation in the regulation of vascular tone remains to be determined.

This work was supported by the Wellcome Trust.

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(Received February 4, 1986.
Revised April 9, 1986.
Accepted April 30, 1986.)