BRIEF COMMUNICATIONS

Vasoactive Intestinal Peptide Inhibitory Innervation in Bovine Mesenteric Lymphatics
A Histochemical and Pharmacological Study

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SUMMARY. The localization of vasoactive intestinal peptide-immunoreactive nerves innervating bovine lymphatic vessels was studied by an immunohistochemical technique. Nerve fibers containing vasoactive intestinal peptide immunoreactivity were present in the smooth muscle layers as well as in the adventitia of all mesenteric lymphatics that were examined. The effect of vasoactive intestinal peptide on isolated lymphatic vessels in vitro was studied. Vasoactive intestinal peptide caused a concentration-dependent relaxation of bradykinin-induced contractions of lymphatic vessels. The threshold and maximum relaxations were achieved with vasoactive intestinal peptide at concentrations less than $6 \times 10^{-9}$ M and $3 \times 10^{-7}$ M, respectively. The relaxant response to vasoactive intestinal peptide was not modified by atropine, propranolol, bretylium, or tetrodotoxin. These results suggest that vasoactive intestinal peptide may be a possible inhibitory neurotransmitter that causes relaxation of lymphatic vessels. (Circ Res 53: 535-538, 1983)

It is well known that the general function of the lymphatic system is to return fluid and protein that escape from the capillary blood vessels to the circulation. The mechanisms by which these functions are carried out depend upon passive and active driving forces, as well as upon the rate of lymph production in organs and tissues. It is clear that the architecture of lymphatics allows lymph transport to occur by external compression (passive driving forces) of the vessels by adjacent tissue. The lymphatic vessels also have the intrinsic ability to generate a pressure head by muscle contraction (active driving forces) which, in the presence of valves, could return lymph to the circulation without the aid of external forces (Yoffey and Courtice, 1970).

Lymphatic smooth muscle tone seems to play a major role in elastic behavior of the wall and in regulation of spontaneous contractions, thereby significantly affecting passive and active driving forces (Ohhashi et al., 1980a). Lymphatic smooth muscle tone is modified by nervous as well as humoral factors (Ohhashi et al., 1978; Ohhashi and Roddie, 1981). Both $\alpha$- and $\beta$-adrenoceptors are present in bovine mesenteric lymphatics (Azuma et al., 1977; Ohhashi et al., 1978). Selective stimulation of intramural nerves has been used to investigate the excitatory and inhibitory responses of the lymphatics (Ohhashi et al., 1980b; Ohhashi and Roddie, 1981). Contraction induced by transmural stimulation was due to the activation of $\alpha$-adrenoceptors via post-ganglionic sympathetic nerves, and transient relaxation following stimulation was mediated mainly by $\beta$-adrenoceptors. In contrast, transmural stimulation still induced a long-lasting relaxation of the lymphatic vessels, when the effects of sympathetic nerve stimulation were abolished by $\alpha$- and $\beta$-adrenoceptor antagonists. The results suggested that nonadrenergic inhibitory nerves may also be present in bovine mesenteric lymphatics. Histochemical studies also suggest that adrenergic and cholinergic fibers are present in bovine mesenteric lymphatics (Ohhashi et al., 1982).

Recently, a variety of polypeptides have been suggested as transmitter candidates of nonadrenergic and noncholinergic inhibitory nerves in the gastrointestinal tract (Jessen et al., 1980; Costa et al., 1980), urogenital tract (Alm et al., 1978; Wharton et al., 1981), cerebral arteries (Larsson et al., 1976; Edvinsson et al., 1981), and portal vein (Barja and Mathison, 1982). Some of the peptides coexist with classical transmitters (Lundberg et al., 1979; 1980). Among these, particular attention is currently being paid to vasoactive intestinal peptide (VIP) as a putative transmitter, which mediates relaxations of cerebral arterial smooth muscle, as this peptide is a powerful vasodilator substance in vitro and in vivo (Edvinsson et al., 1980; McCulloch and Edvinsson, 1980).

We undertook the present studies to attempt to identify and analyze further the mechanisms of...
potential transmitters contained in unknown nerves which innervate the bovine mesenteric lymphatics. For these purposes, immunocytochemistry and the isolated lymphatic vessel preparation were used to investigate the possible localization and in vitro effects of a variety of peptides.

## Methods

The preparation of bovine mesenteric lymphatics for pharmacological studies was described previously (Ohhashi et al., 1978, 1981). Longitudinal segments, 20 mm long, were fixed between silk strings in a muscle bath (5-ml capacity) filled with Krebs solution circulating through a heat exchanger kept at 36–37°C. The composition of the nutrient solution in mm was as follows: NaCl, 120.0; NaHCO3, 25.0; KCl, 5.9; NaH2PO4, 1.2; CaCl2, 2.5; MgCl2, 1.2; and dextrose, 5.5. The solution was equilibrated before and during the experiment with a gas mixture of 95% O2 + 5% CO2 to give a pH of 7.4. The string anchoring the upper end of the preparation was connected to a force-displacement transducer (Grass Instrument Co., FTO3C). The isometric tension detected by the transducer was amplified and recorded by an ink-writing oscillograph (Grass Instrument Co., 52-925-T5). The resting tension of each preparation was set at 0.4 g, which had been found to be optimal for obtaining a maximum contractile response in the lymphatic vessel (Ohhashi et al., 1981). All preparations were allowed to equilibrate for 60 minutes in the oxygenated bath medium before the start of the experiments. The preparations were partially contracted with 10−5 M bradykinin, which is known to be one of the most potent constrictors of lymphatic vessels (Ohhashi and Roddie, 1980); the contractions were in a range between 75 and 90% of the contraction induced by 120 mM K+. Vasoactive intestinal peptide (VIP), substance P (SP), neuropeptide Y (NPY), leu-enkephalin (leu-EK), and methionine-enkephalin (met-EK) were added directly to the bath media. Because rapidly developing tachyphylaxis resulted from repeated applications of these peptides, only one concentration was added in each series of experiments. The preparations then were perfused with fresh nutrient solution and equilibrated for 40 minutes. The concentration was raised stepwise to complete dose-response relationships. At the end of each series of experiments, 10−5 M isoproterenol hydrochloride (IS) was added to attain the maximum relaxation (Ohhashi et al., 1978); relaxations induced by the peptides relative to those induced by IS were studied. All peptides were obtained from Bachem Inc.

All data are reported as the means ± SEM (n = 4). Unpaired t-tests were performed for data at dose-relaxation curves of these peptides. Significance levels were set at P values <0.05.

## Immunocytochemical Method

Approximately 20–25 minutes after exsanguination, collecting lymph vessels (1–2 mm in outer diameter) from bovine mesentery were dissected free and immersed in fixative for 2 hours. The fixative was fixed either in 10% buffered formalin phosphate (pH 7.0) or 2% paraformaldehyde and 15% picric acid in 0.1 M sodium phosphate buffer (pH 7.3). Following fixation, the tissue was rinsed 48 hours in PBS with 20% sucrose at 4°C. The tissue was frozen on dry ice, cut into 20-μm sections, mounted on chrome-alum-coated slides, and processed for immunohistochemistry. Cryostat sections were incubated 1 hour at 4°C in rabbit anti-VIP (Immuno Nuclear Co.). The antisera was diluted 1:1000 in PBS with whole goat serum and 0.3% Triton X-100. The sections were washed in PBS with 0.2% Triton X-100 and then incubated for 30 minutes in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel) diluted 1:300 in PBS with 0.3% Triton X-100. Sections were washed, rinsed in PBS, mounted in glycerin-PBS (3; pH 8.3), and examined under a Leitz Orthoplan fluorescence microscope. No immunoreactivity was demonstrated when the antisera were replaced with normal rabbit serum.

## Results

Figure 1 shows typical recordings from one of four experiments designed to investigate the dose-response relationship of VIP. In longitudinal preparations of bovine mesenteric lymphatics contracted with bradykinin (10−7 M), VIP at concentrations of 6 × 10−9 M, 1.5 × 10−8 M, 6 × 10−8 M, and 3 × 10−7 M caused a concentration-dependent relaxation. Mean absolute relaxations of each contraction were 52 ± 22, 108 ± 40, 315 ± 84, and 568 ± 97 mg (n = 4), respectively, from the tension raised by bradykinin of 841 ± 48 mg in the lymphatic preparations. Similar relaxation was obtained when the lymphatics were contracted with 5-hydroxytryptamine. As illustrated in Figure 1D, when the preparation was treated for 30 minutes with a β-adrenoceptor antagonist (5 × 10−6 M propranolol hydrochloride), VIP (6 × 10−8 M) caused a marked relaxation in the lymph vessel, the relaxation being reduced only slightly compared with the initial response to the same amount of VIP (Fig. 1C). The slight reduction of relaxation may have been related to the local anesthetic action of the high concentration of propranolol or tachyphylaxis. The response to VIP was not affected significantly by 10−4 M atripine, 10−5 M bretylium, or 10−5 M tetrodotoxin. These findings indicate that VIP may act via a specific receptor on the lymphatic smooth muscle.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Relaxant responses of a strip of bovine mesenteric lymph vessel to vasoactive intestinal peptide (VIP) before (A, B, C, D) and after (E) treatment with 5 × 10−5 M propranolol. The same preparation responded with contractions to bradykinin (10−7 M). ISP, isoproterenol, 10−5 M.
The other peptides, NT (6 × 10⁻⁸ - 6 × 10⁻⁷ M), SP (7 × 10⁻⁸ - 7 × 10⁻⁷ M), met-EK (3 × 10⁻⁷ - 3 × 10⁻⁶ M), and leu-EK (3 × 10⁻⁷ - 3 × 10⁻⁶ M) also produced a concentration-dependent relaxation (Fig. 2). Maximum relaxations were attained by 3 × 10⁻⁷ M VIP (568 ± 97 mg from the tension raised by bradykinin of 841 ± 48 mg), 6 × 10⁻⁷ M NT (308 ± 80 mg from 852 ± 34 mg), 7 × 10⁻⁷ M SP (268 ± 74 mg from 821 ± 29 mg), 3 × 10⁻⁶ M met-EK (344 ± 72 mg from 798 ± 59 mg), and 3 × 10⁻⁶ M leu-EK (153 ± 80 mg from 834 ± 42 mg). Mean values of the effective concentration of 30% relaxation from the maximum tension raised by bradykinin (ED₃₀) of NT (4.5 ± 2.2 × 10⁻⁷ M), SP, met-EK, and leu-EK were significantly higher than the value for VIP (3.2 ± 1.8 × 10⁻⁸ M). The decreasing order of the relaxant response was as follows: VIP > NT > SP > met-EK > leu-EK. The addition of VIP (5 × 10⁻⁸ M) produced a profound relaxation of the lymphatic preparation; however, additional application of the same concentration of VIP failed to alter the tension. Despite such a remarkable tachyphylaxis, the relaxations induced by VIP did not alter action of the other peptides, or cause tachyphylaxis, in the same preparations.

Immunohistochemical observations of the lymphatic vessels revealed a small number of fine varicose nerve fibers containing VIP immunoreactivity in the media, the smooth muscle layers, as well as in the adventitia of the vessels (Fig. 3). The fine VIP-ergic fibers sometimes reached as far as the subendothelial layer. Most of the VIP-positive fibers were longitudinally oriented.

Discussion

VIP was first isolated from porcine intestine (Said and Mutt, 1970). It is a peptide of twenty-eight amino acid residues structurally related to glucagon and secretin (Said and Mutt, 1970), and has a wide variety of actions. In the cardiovascular system, it causes relaxation of smooth muscle of the feline lingual, renal, and femoral arteries (Uddman et al., 1981), the canine mesenteric arteries (Toda, 1982), and the feline cerebral arteries and veins (Edvinsson et al., 1982). Immunohistochemical studies have revealed a VIP-like immunoreactive substance associated with nerves to the smooth muscle of blood vessels in several vascular beds (Larsson et al., 1976; Edvinsson et al., 1980; Barja and Mathison, 1982). Stimulation of autonomic nerves supplying a number of organs results in an increase in the amount of VIP-like immunoreactive substance in the venous effluent. In the cat colon, vasodilation resulting from nerve stimulation was associated with increased release of VIP-like immunoreactive material (Fahrenkrug et al., 1978). Thus neuronal release of VIP may cause relaxation of the smooth muscle of blood vessels.

The present results provide the first demonstration that lymphatic vessels are innervated by nerve fibers containing VIP. Furthermore, VIP was shown to induce a concentration-dependent relaxation of isolated bovine mesenteric lymphatics. The magnitude of the maximum relaxation produced by 3 × 10⁻⁷ M VIP was significantly larger than those induced by NT, SP, met-EK, and leu-EK. The minimum effective concentration of VIP in eliciting relaxations is considerably lower than those of mono-
amines examined under similar conditions (Ohhashi et al., 1978). Pharmacological studies using transmural stimulation have also suggested that a non-adrenergic, non-cholinergic inhibitory innervation may be present in bovine mesenteric lymphatics (Ohhashi and Roddie, 1981). The relaxant effect of VIP does not appear to be mediated by cholinergic or β-adrenergic receptors, since the response to VIP was not significantly affected by the presence of atropine or propranolol. Thus, the presence of VIP-containing nerve fibers, together with the demonstration of its potent lymphatic action, provides evidence that VIP may be a nonadrenergic inhibitory transmitter in the lymphatic vessel.

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