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PEPTIDERGIC INNERVATION OF MESENTERIC LYMPHATICS IN GUINEA PIGS: AN IMMUNO-CYTOCHEMICAL AND PHARMACOLOGICAL STUDY

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ABSTRACT

By immunocytochemistry, substance P immunoreactive (SP-IR) and vasoactive intestinal peptide immunoreactive (VIP-IR) nerve fibers were examined in guinea pig mesenteric lymph collectors. The immunoreactive nerve fibers, located in the adventitia of lymphatics, were few and were irregularly distributed along the vessel wall. These fibers appeared to be more numerous and more evenly distributed along the corresponding artery and vein walls within the same area. SP immunoreactivity in the vascular nerves was depleted in guinea pigs injected with capsaicin but was unaffected by the injection of 6-hydroxydopamine. By contrast, VIP-IR nerve fibers were unaffected by both treatments. It is concluded that SP-IR nerve fibers in the lymphatics are likely to be of sensory origin and that VIP containing nerves in the lymph collectors are distinct from SP-containing and noradrenergic nerves. It is also suggested that lymph collectors possess a complex although limited innervation pattern not only of autonomic nerve fibers containing classic neurotransmitters but also of peptidergic nerve fibers of a different origin with a vasomotor and/or sensory action.

The smooth muscle of the lymphatic wall plays an important role in both the elastic properties and in the regulation of spontaneous contractility upon which the active mechanism of lymph propulsion is based (1). Whereas the regulation of lymphatic contractility is mainly myogenic, it also depends on humoral and neuronal factors (2) and on the action of neurotransmitters located in nerve fibers within the lymphatic wall (3). In this respect previous ultrastructural studies carried out in guinea pigs and in humans have shown the presence of unmyelinated nerve fibers located in the adventitia of the lymph collectors at 200-300nm from the outermost smooth muscle cells of the media. These fibers are usually made up of two or three axons which sometimes have characteristic synaptic vesicles of a heterogeneous content (4). In humans these fibers are more numerous and are located closer to the smooth muscle cells of the media (5).

Various histochemical studies have shown the presence of catecholaminergic and acetylcholinesterase (AChE) positive fibers in the mesenteric lymph vessels in guinea pigs (4) and in the mesenteric lymph vessels of cattle (6). It has also recently been shown that apart from classic noradrenaline (NA) and acetylcholine (ACh) neurotransmitters other substances have a transmitter function in the autonomic nervous system (7). Pharmacological and immunocytochemical studies have demonstrated that adenosine 5'-triphosphate (ATP), 5 hydroxytryptamine (5-HT),

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dopamine and various peptides may behave like transmitters in nerves associated with blood vessels (8). Whereas many studies in blood vessels have been carried out on this subject, information on the presence of peptides in nerve fibers within the lymph vascular wall is rare (9,10). On the other hand, the presence of AChE positive fibers in the lymphatic wall raises the possibility of nerves containing SP (P Substance) and VIP (vasoactive intestinal peptide) in the lymph wall, as both VIP and ACh can apparently coexist in peripheral nerve fibers (11). Furthermore, it is well known that AChE is a hydrolytic enzyme for some peptides including SP (12). Therefore, AChE positive nerve fibers may contain not only ACh but also SP or other peptides.

The aim of this study, therefore, was to determine whether immunoreactive nerve fibers for SP (SP-IR) and for VIP (VIP-IR) exist in mesenteric lymph collectors, to examine the distribution and density of these fibers in the lymphatic wall and to compare the findings with those of the arterial and venous vascular wall in the same area. SP, a transmitter widely distributed in the central nervous system (CNS) and in the peripheral nervous system (PNS) (13) is also present in primary afferent neurons and in almost all their central and peripheral branches (14). In order to determine the nature and origin of the immunoreactive SP fibers in the blood vascular and mesenteric lymphatic vessels the effect of capsaicin on these fibers was also tested. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a constituent of various species of red peppers, and causes a selective depletion of the SP content in primary sensory neurons and in related fibers in adult animals but not in other types of neurons and fibers containing SP localized in the CNS and PNS (15,16). To investigate the possible coexistence of VIP and SP in noradrenergic nerves supplying mesenteric lymphatic vessels, several guinea pigs were subsequently treated with 6-hydroxydopamine (6-OHDA), a neurotoxin which causes selective degeneration in the noradrenergic fibers of adult animals.

MATERIALS AND METHODS

The study was carried out on 24 female guinea pigs weighing between 150 and 200g. The guinea pigs were killed by exsanguination after cutting the carotid artery. The mesentery was removed as soon as the abdomen was opened and was laid out on balsa-wood, fixed and processed as a whole mount according to Costa's technique (17). For the immunocytochemical reaction, a monoclonal antibody against SP produced in rats (clone n° NCl/34 HL, Sera Lab, Oxford, UK) was used. Its characteristics regarding specificity and cross-reactivity have previously been described (18). A polyclonal antibody against VIP produced in rabbits (Immuno-nuclear Co. Stillwater, MN, USA) and indirect immuno-fluorescence were also used in order to localize the two peptides immuno-cytochemically (19). The preparation stretched out on clean slides was incubated in a humid atmosphere at 4°C for 48-72h with the primary antibodies (dilution 1:80 for anti-SP, dilution 1:200 for anti-VIP) followed by incubation with the secondary antibodies: fluorescein isothiocyanate (FITC) labeled goat antirabbit IgG (1:20) (Sigma Chemicals) and FITC labeled goat anti-rat IgG (1:30) (Sigma Chemicals) for 2h at room temperature. The preparations were then mounted in glycerine: phosphate buffered saline (3:1 v/v) and observed under a Zeiss Axiomat microscope equipped with excitation and barrier filters for FITC. Controls consisted of incubating the antibodies in excess of antigen, by omitting the first antiserum and using non-immune serum instead. No immunostaining of nerve fibers was observed in these controls. A group of guinea pigs was injected daily under the skin with capsaicin (Sigma Chemicals) in increasing doses (25mg/kg the first day; 50mg/kg the second day; 100mg/kg the third day; 200mg/kg the fourth day; 400mg/kg the fifth day). The capsaicin was dissolved in a mixture of 5:1 ethanol and polyoxyethylene sorbitan mono-oleate (Tween 80-BDH Chemicals, UK). Seven days after the last treatment the

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guinea pigs were killed and the mesentery was removed and treated in the same way as described above. A second group of guinea pigs was treated with 6-OHDA (Sigma Chemicals). The drug was dissolved in distilled water containing ascorbic acid (1mg/ml) and injected under the skin: 200mg/kg the first day; 300mg/kg the seventh day). The tissues were removed on the ninth day and processed as above. Control groups for capsaicin and 6-OHDA were treated only with the solvents. The capacity of 6-OHDA to induce degeneration of the noradrenergic nerves was tested by examining the localization of the noradrenaline in tissue by using a histochemical technique with glyoxylic acid (20).

RESULTS

There are few SP-IR and VIP-IR nerve fibers in the adventitia of the lymphatic collectors. The VIP-IR fibers often contain numerous highly fluorescent varicosities and generally run longitudinally along the vessel wall *(Fig. 1)*. Long tracts of the lymphatic wall do not seem to have any immunoreactive fibers. Some nerve fibers are seen in proximity to a lymphatic valve without, however, preferential innervation of the perivalvular tracts *(Fig. 2)*. SP-IR fibers course irregularly along the wall of the lymphatic collectors. They mainly run longitudinally but also cross the lymphatic transversely *(Fig. 3)*. These fibers seem to

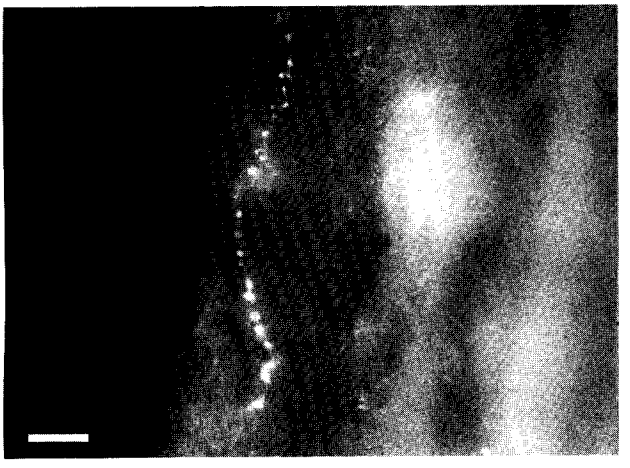

Fig. 1. Guinea pig mesenteric lymph collector. A VIP-IR nerve fiber is seen coursing longitudinally to the lymphatic containing numerous intensely fluorescent varicosities. Bar=40μm.

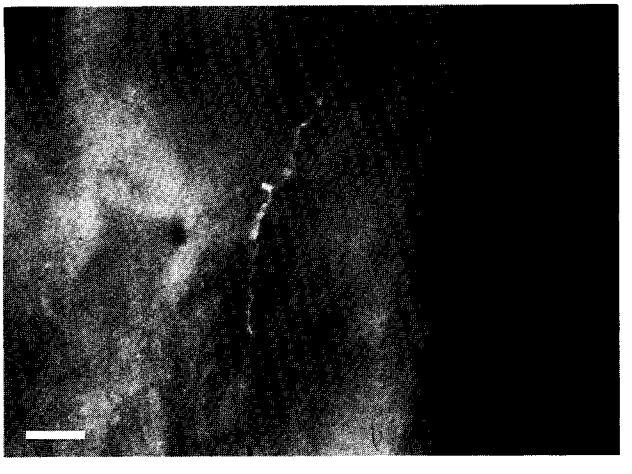

Fig. 2. Mesenteric lymph collector. Near a valve a VIP-IR fiber running vertically is seen. Bar=40 μm.

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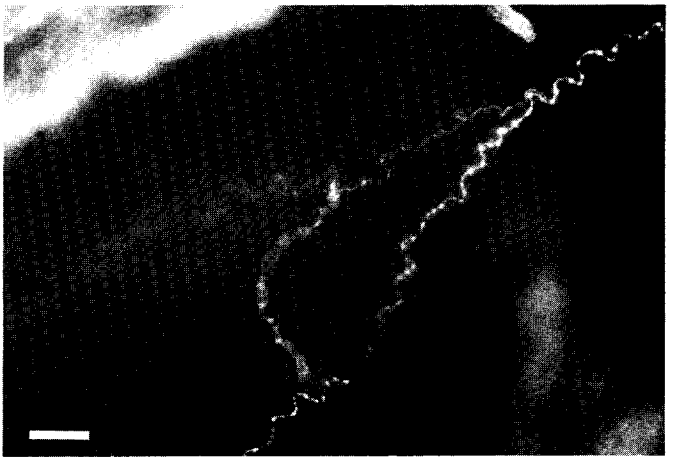

Fig. 3. Lymph collector wall. SP-IR nerve fibers with both a vertical and transverse course to the axis of the lymphatic are seen. Bar=40μm.

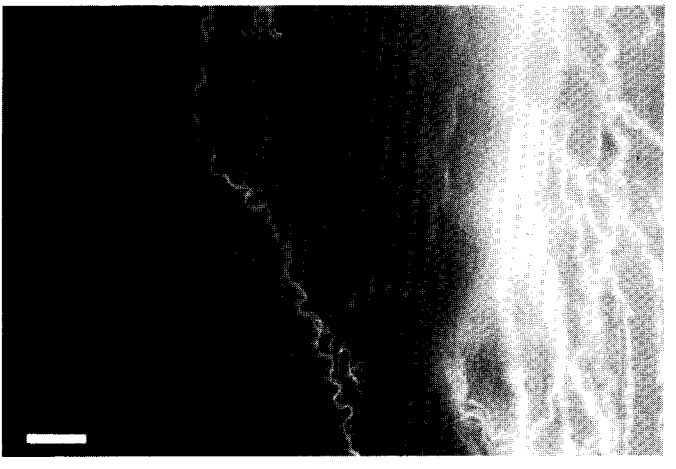

Fig. 4. Mesenteric lymph collector adjacent to an artery. The arterial wall (right) has a rich network of SP-IR nerve fibers whereas in the lymph collector wall there are axon terminations emanating from a nerve trunk running parallel to the lymphatic. Bar=40μm.

derive from large nerve trunks located near or between the lymphatic collectors and the adjacent arteries. The SP-IR fibers arising from the nerve trunks, in some instances, seem to terminate with axon varicosities in the lymphatic wall *(Fig. 4)*. Other times after crossing the lymphatic wall they terminate in the mesentery at a distance from the lymphatic.

Mesenteric arteries and veins, on the other hand, show a dense network of immunoreactive nerve fibers which is denser and more uniform in the arteries and looser and more irregular in the veins *(Fig. 5)*. As the arteries become smaller, the nerve density gradually decreases. The veins also show a gradual reduction of the immunoreactive fibers. The smaller mesenteric veins show few or no associated nerve fibers. In the blood vessel wall, SP-IR fibers seem more numerous than VIP-fibers *(Fig. 5,6)*. This quantitative difference is also observed in the lymphatic vessels. However, it is less noteworthy because of the small number of immunoreactive fibers present in the lymphatic collectors.

Effects of drugs

Treatment with capsaicin totally depleted the SP-IR fibers of the blood and lymph vessels and of the immunoreactive fibers present in the mesentery without altering the density of the VIP-IR fibers. Treatment with 6-OHDA was associated with complete disappearance of the

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Fig. 5. Mesenteric artery and vein. A denser and more regular network of SP-IR nerve fibers is seen in the arterial wall (left) as compared to the vein wall (right). Bar=40μm.

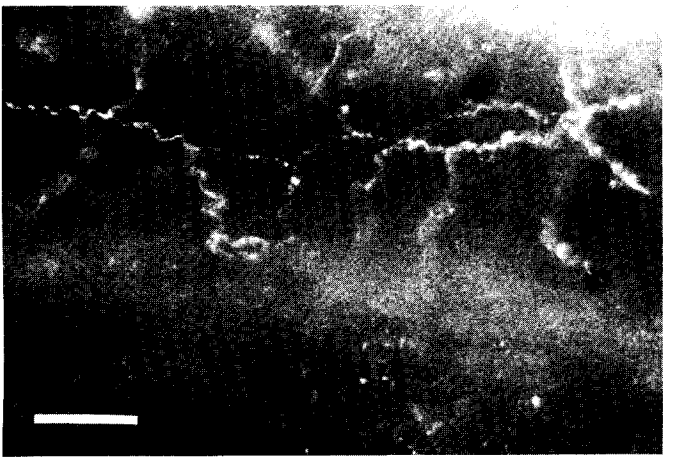

Fig. 6. Mesenteric artery. VIP-IR fibers appear less numerous and more irregular than SP-IR fibers. Bar=40μm.

glyoxylic acid induced fluorescence of noradrenergic nerves of the blood and lymph vessels. However, there was no effect on fibers showing immunoreactivity for SP and for VIP.

DISCUSSION

Our results reveal the presence of few SP-IR and VIP-IR nerve fibers in the adventitia of the mesenteric lymph collector wall. The use of whole mount preparations shows long tracts of the lymphatic wall without IR nerve fibers and a lack of preferential innervation between vessel segments with and without intraluminal valves. Only a slight difference was noted in the density of the IR fibers for the two peptides. These fibers seem more numerous and more regularly distributed along the corresponding arterial and vein walls. The rare presence, however, of VIP-IR and SP-IR nerve fibers in the lymphatic vessel wall raises questions about their functional role. It is known that VIP, a peptide with 28 amino acid residues isolated in pig intestine, is present in CNS and PNS neurons and nerve fibers (21). Histochemical studies have shown the presence of VIP-IR nerve fibers in the blood vessel wall of various vascular beds (22,23). Most likely, VIP's presence in vascular nerve fibers helps regulate systemic and local blood flow (22). In fact, this peptide is a powerful vaso-dilator capable of relaxing the smooth muscle of the arteries of various areas (22).

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In particular, VIP mediates the atropine resistant vasodilator reaction observed after parasympathetic nervous stimulation in some vascular areas (24). *In vitro*, VIP is also capable of inducing an atropine resistant relaxation of a mesenteric lymphatic isolated in cattle and precontracted with bradykinin (9). The lack of effect of capsaicin on the VIP-IR fibers within the lymph collector wall shows that these fibers belong to a different neuronal population than SP-IR fibers. On the other hand, treatment with 6-OHDA does not eradicate the VIP-IR fibers. Therefore, the VIP-IR fibers associated with mesenteric lymphatics represent a population distinct from the other two groups of fibers: the sensory one containing SP and the autonomic one containing noradrenaline.

Evidence that VIP coexists with ACh in perivascular nerves in some areas (11) suggests that VIP-IR nerves associated with mesenteric lymphatics also contain ACh. Nonetheless, the rare number of VIP-IR fibers, compared to the high number of AChE positive fibers within the mesenteric lymph collector wall (4), suggests that only some of these fibers also contain VIP. These results taken together with earlier pharmacological data (9), suggest that VIP present in autonomic nerve fibers exerts a vasomotor function on mesenteric lymphatic collectors.

SP is also widely distributed in the nerve fibers of various arteries and veins (25,26). The nerve fibers of the blood vasculature which contain SP are sensitive to capsaicin but not to 6-OHDA and are therefore probably of a sensory nature (26). The SP-IR sensory fibers in blood vessels are thought to carry out a dual function: transporting sensory information to the CNS about the state (i.e., "tone") of the blood vascular and the perivascular environment and a vasomotor function (25). Indeed, *in vitro* SP vasodilates various arteries and veins (27). In mesenteric lymphatics isolated *in vitro* SP is also a vasodilator even if this action is less marked than that of VIP (9). Thus, SP may be released following stimulation of sensory nerve terminals. In fact, in some tissues such as the iris, the skin and the bowel, this peptide (SP) may be released through axon reflexes which are locally generated by noxious stimuli or by antidromic nerve stimulation (28). In turn, these peripheral axon reflexes produced by SP neuron stimulation may induce a reflex vasodilation in lymph collectors (29). The depletion of SP-IR nerve fibers in lymph collectors by capsaicin and the lack of effect by 6-OHDA suggests that these fibers are also of a sensory nature. When taken in conjunction with previous pharmacological data, the findings suggest that, as in blood vessels, SP-IR nerve fibers have a sensory and/or vasomotor function in modulating smooth muscle tone in lymphatics.

In conclusion, our results support that lymph collectors, like blood vessels, possess a complex, although limited, innervation pattern not only of autonomic nerve fibers containing classic neurotransmitters with vasomotor action, but also of peptidergic nerve fibers of a different origin with both a vasomotor and/or sensory action.

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