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SUBENDOTHELIAL NERVE FIBERS IN BOVINE MESENTERIC LYMPHATICS: AN ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

In the lymphatic vessels of man and most animals the nerve fibers are confined to the adventitia. However, immunohistochemical studies suggest that acetylcholinesterase-positive and monoamine-containing fibers reach as far as the endothelium in bovines. The aim of this study was to verify the presence of subendothelial nerve fibers by transmission electron microscopy (TEM) in bovine mesenteric lymphatics and to determine whether typical sensory neurotransmitters such as Substance P (SP) and calcitonin gene related peptide (CGRP) could be detected in these fibers. TEM revealed numerous unmyelinated nerve fibers in the subendothelial connective environment in close association with endothelial cells. Their axons were devoid of Schwann cell sheath on the endothelial side and contained small clear vesicles and large vesicles with a dense core. Subendothelial nerve fibers were demonstrated to be SP and CGRP-immunoreactive with mouse monoclonal antibodies against SP and rabbit polyclonal antibodies against CGRP. It is hypothesized that these fibers act as mechanoceptors capable of detecting intraluminal pressure and vessel wall tension variations and of locally releasing SP and CGRP. Since SP, potentiated by CGRP, is known to be a vasoconstrictor in lymphatics, we propose that the contraction of bovine mesenteric lymphatics may also be neurogenic.

It is generally believed that the vessel wall of lymphatics is less innervated than that of blood vessels. In man (1) and most animals, nerve fibers are confined to the adventitia where they run longitudinal to the major axis of the vessel. In guinea pig mesenteric lymphatics, these fibers, scant and irregularly distributed, are localized at a distance of at least 200-300nm from the basal surface of the more external smooth muscle cells, and they have been demonstrated by histochemical techniques to be acetylcholinesterase (AChE)-positive and to contain monoamines (2). An immunocytochemical study subsequently revealed substance P (SP) and vasoactive intestinal peptide (VIP) immunoreactive (IR) fibers in the adventitia of the same vessels (3).

In bovine mesenteric lymphatics, light microscopy shows that AChE-positive, catecholamine-containing (4) and VIP-IR nerve fibers (5) are located not only in the adventitia but also in the smooth muscle layers and reach as far as the subendothelial layer. Moreover, in canine lacteals, an intimate association between peptidergic (SP and calcitonin gene related peptide, CGRP-IR) nerve fibers and the endothelium has been demonstrated by immunohistochemical and immunocytochemical techniques. Their presence in vessels devoid of smooth muscle suggests that these nerve fibers may be sensory in nature (6).

To our knowledge, no ultrastructural evidence of subendothelial nerve fibers has yet

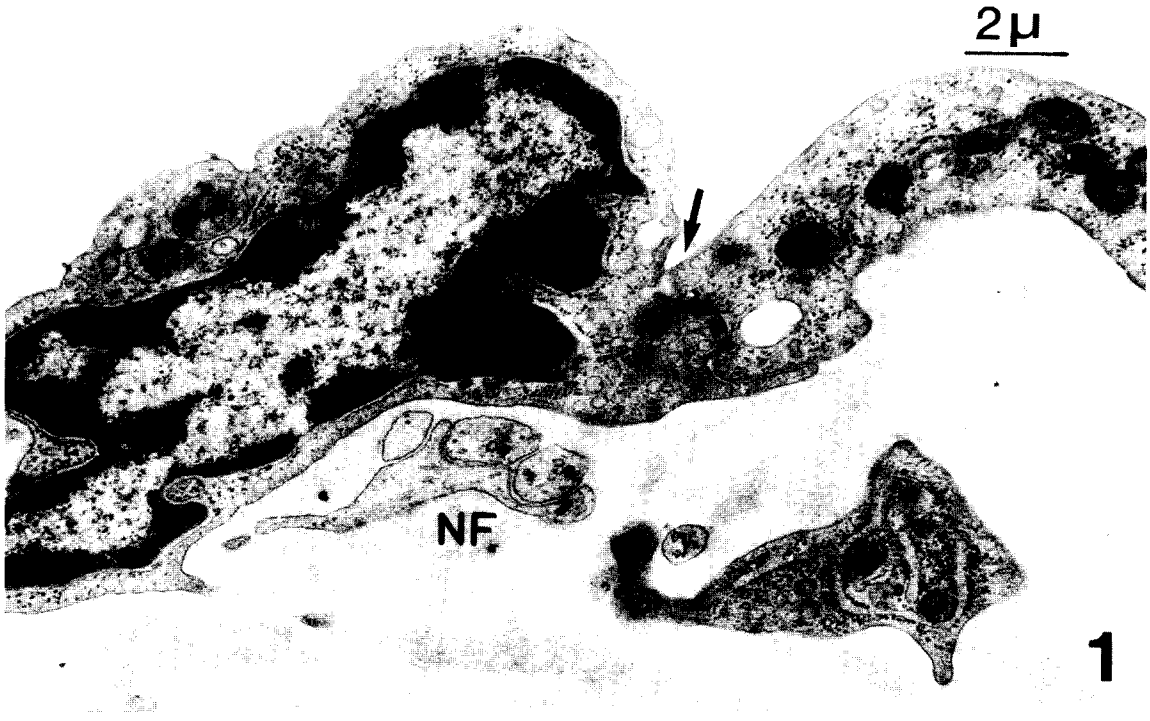

Fig. 1. Unmyelinated nerve fiber (NF) partially surrounded by the basal surface of a bovine mesenteric lymphatic endothelial cell, situated near an interendothelial junction (arrow). The nerve fiber consists of three axons devoid of their Schwann cell sheath on the endothelial side. The distance between the nerve fiber and the endothelium is approximately 50 nm.

been provided in lymphatic collectors. The aim of this study was to verify by transmission electron microscopy (TEM) the presence of subendothelial nerve fibers in bovine mesenteric lymphatics and to determine their relationship to endothelial cells. We also performed an immunohistochemical study to determine whether typical sensory neurotransmitters such as SP and CGRP could be detected in these fibers.

MATERIALS AND METHODS

Transmission Electron Microscopy

Bovine mesenteries were obtained at the local slaughterhouse and kept in PBS (Phosphate Buffer Saline) at 37°C until used. Postnodal lymphatics were visualized by injecting 0.1% Evans blue (7) into lymph nodes, excised free of fat and cannulated at one end. The vessels were then infused with PBS to remove lymph, pressure perfusion fixed with 2.5% glutaraldehyde at the physiologic pressure of 5cm of H_2O and further fixed by immersion in the same fixative for 2h at 4°C. The vessel segments were then postfixed in 1% OsO_4 in 0.1M cacodylate buffer pH 7.35, dehydrated in ethanol and embedded in Epon 812. Ultrathin sections obtained with a diamond knife were stained with uranyl acetate and lead citrate and observed under a Philips EM 201 electron microscope operating at 60kV.

Immunohistochemistry

Mesenteric lymphatics were pressure perfusion fixed with Zamboni's fixative (8) (2% paraformaldehyde in a 15% saturated solution of picric acid in 0.1M phosphate buffer pH 7.35), further fixed in the same

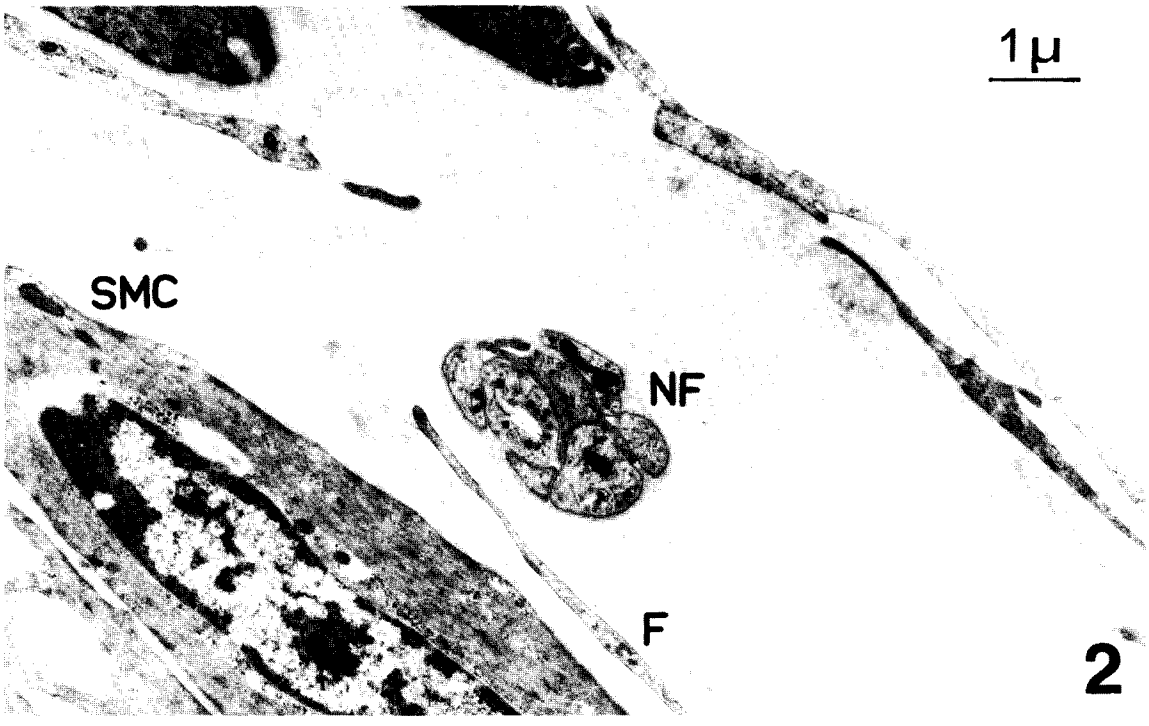

Fig 2. Nerve fiber (NF) in the bovine mesenteric lymphatic subendothelial connective tissue, separated from the underlying smooth muscle cells (SMC) by a fibroblast (F) expansion. The axons contain a heterogenous population of vesicles and mitochondria.

fixative for 6-7h at 4°C, washed 3 times for 30 minutes with PBS containing 15% sucrose and kept refrigerated overnight in the same buffer. The vessel segments were then frozen in melting nitrogen-cooled isopentane. Cryostat sections, collected on poly-D-lysine (Sigma)-coated glasses, were incubated overnight at 4°C in a humidified chamber with mouse monoclonal antibodies against SP (Chemicon) at a dilution of 1:200-1:1000, or with rabbit polyclonal antibodies against CGRP (Boehringer, Mannheim) at a dilution of 1:50-1:200. The antibodies had previously been biotinylated with biotin-N-hydroxide succinamide (Boehringer, Mannheim) using the procedure suggested by the manufacturer. The antibodies were removed by washing 3 times for 15 minutes in PBS and the sections incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Vector) at 1:50 dilution for 45 minutes at room temperature, washed in PBS, mounted under coverslips with Vectashield mounting medium (Vector) and observed under a Zeiss Axioplan fluorescence microscope.

RESULTS

Transmission Electron Microscopy

In pressure perfusion fixed lymphatic vessels a subendothelial layer containing fibroblasts, amorphous material, collagen and elastic fibers was evident by TEM. In this subendothelial connective environment unmyelinated nerve fibers were frequently encountered, especially near interendothelial contacts *(Figs. 1-4)*. The distance between nerve fibers and the basal surface of the endothelial cells was variable, sometimes very reduced, even less than 50nm *(Fig. 1)*. In these instances, endothelial cell expansions partially surrounded the nerve fibers in intimate association. These subendothelial nerve fibers

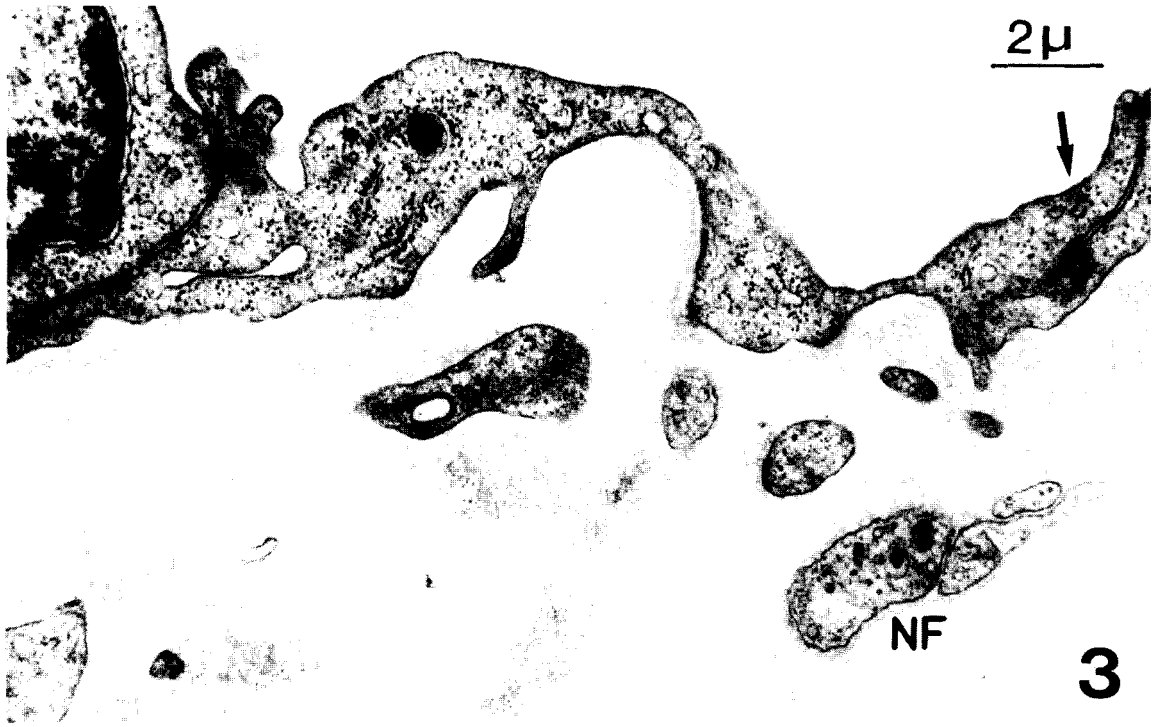

Fig 3. Subendothelial nerve fiber (NF) near an interendothelial junction (arrow) of a bovine mesenteric lymphatic. An axon devoid of Schwann cell sheath contains small clear vesicles and large vesicles with a dense core.

consisted of 3-4 axons devoid of Schwann cell sheath on the endothelial side *(Fig. 4)*. Fibroblasts often separated these fibers from the underlying smooth muscle cells *(Fig. 2)*. The axons contained mitochondria *(Fig. 2)*, small clear vesicles and large vesicles with a dense core *(Figs. 2-4)*.

Immunohistochemistry

Both SP *(Fig. 5)* and CGRP *(Fig. 6)*-immunoreactive (IR) fibers were detected by fluorescence microscopy. The bright green fluorescence of FITC was clearly distinguishable from the yellowish autofluorescence of elastic fibers.

Most SP and CGRP-IR fibers were located subendothelially, less frequently in the adventitia, rarely in the muscle layer.

All nerve fibers were caught in transverse section, confirming the ultrastructural findings and suggesting that they are prevalently longitudinal. No quantitative differences between SP-IR and CGRP-IR fibers were detected. It is well known, however, that these two peptides are frequently colocalized (9).

DISCUSSION

Our results show that SP and CGRP-IR nerve fibers are present in the subendothelial connective tissue of bovine mesenteric lymphatics. SP immunoreactivity had previously been detected by radioimmunoassay in these vessels but not in lymph, indicating that it originated in the lymphatic wall (10). Their prevalent localization in the subendothelial connective tissue and the immunohistochemical demonstration that they contain SP and CGRP, two neurotransmitters that are characteristic of primary sensory neurons (11), suggest that subendothelial nerve fibers are sensory in nature.

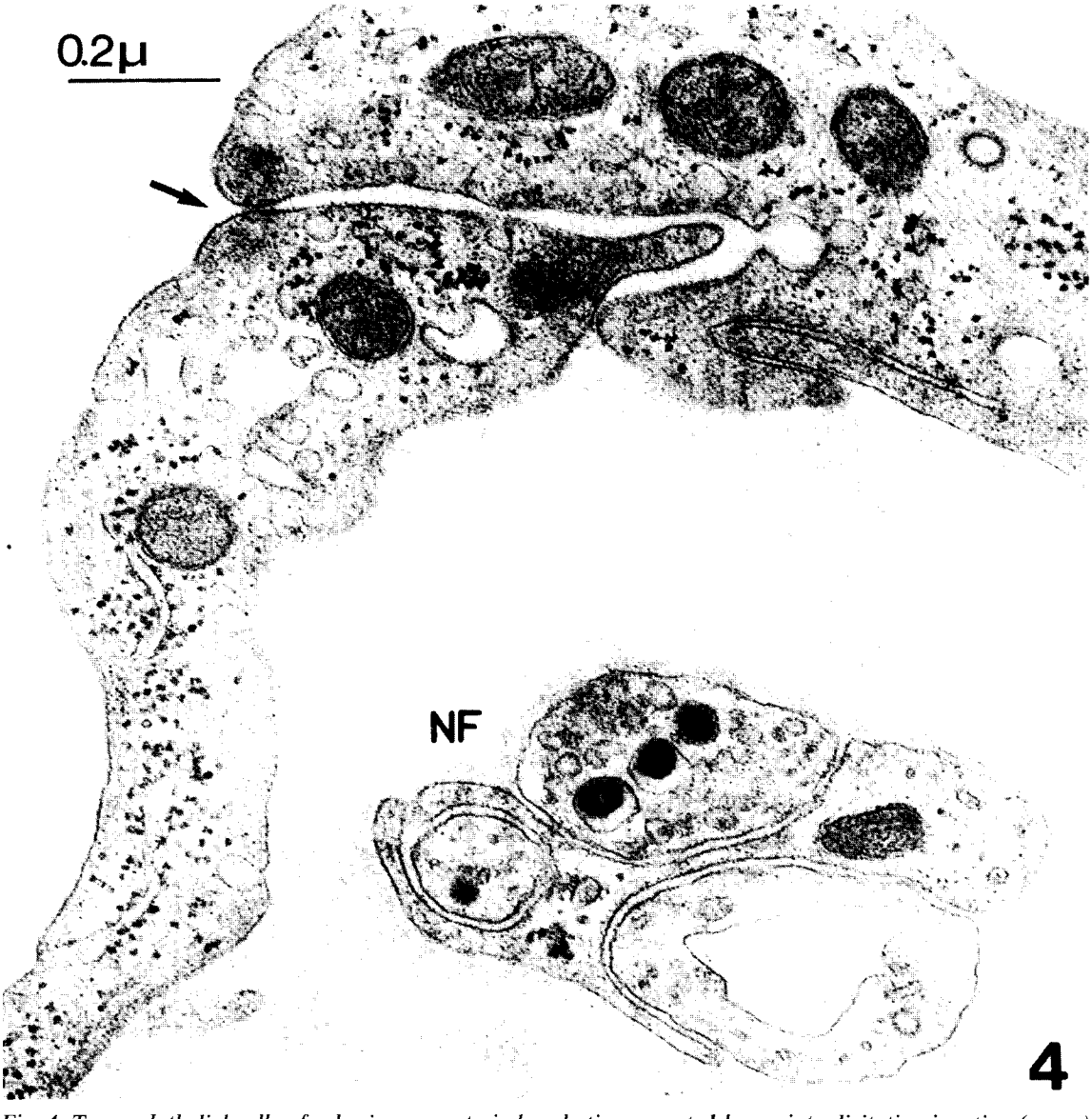

Fig. 4. Two endothelial cells of a bovine mesenteric lymphatic connected by an interdigitating junction (arrow) partially surrounding a nerve fiber (NF). An axon, containing small clear vesicles and large dense core vesicles, is devoid of Schwann sheath on the endothelial side.

It may be hypothesized that these nerve fibers act as mechanoceptors capable of detecting intraluminal pressure and variations in lymphatic vessel wall tension. They may also act as thermoceptors or chemoceptors. Upon stimulation these fibers presumably release SP and CGRP locally.

SP is a vasodilator in blood vessels (12) and increases the frequency of contraction in lymphatics (10). CGRP has been demonstrated to potentiate SP-induced effects via inhibition of neuropeptide degradation (13). Therefore an increase in intraluminal pressure or appropriate thermal or chemical stimuli by activation of these nerve fibers would cause the local release of SP and CGRP and induce

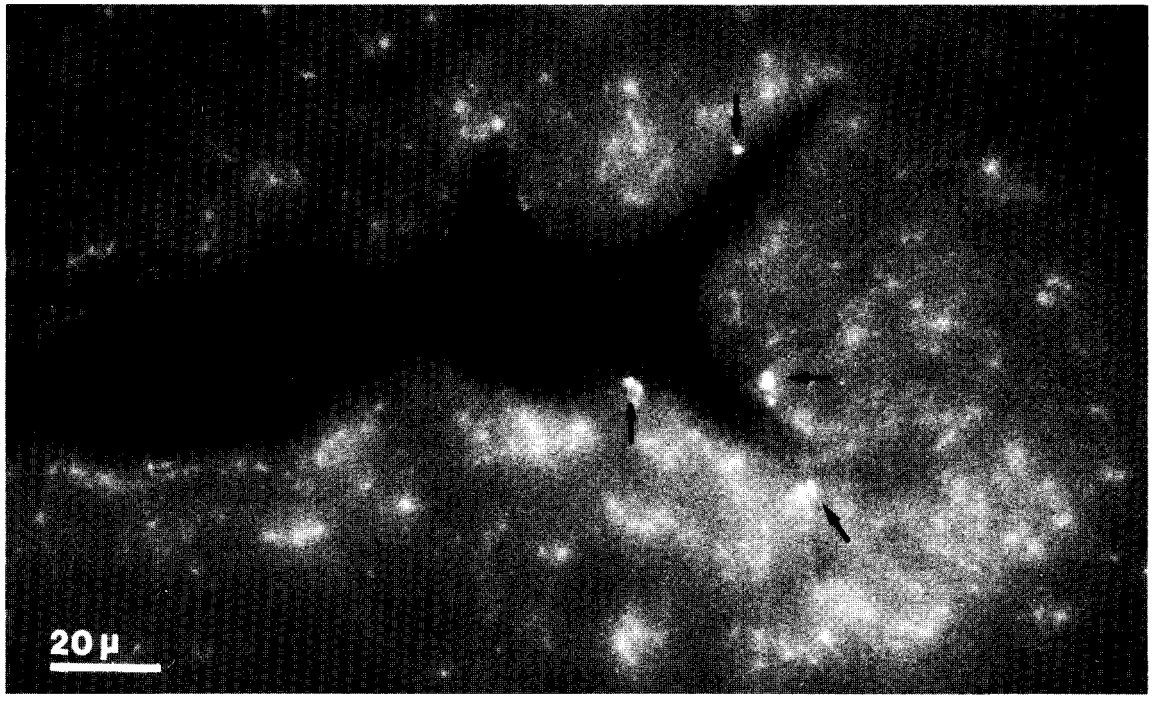

Fig. 5. SP immunofluorescence reaction in a cryostat transverse section of a bovine mesenteric lymphatic. SP-IR nerve fibers (arrows) are localized subendothelially. Some of them contain 2-3 SP-IR axons.

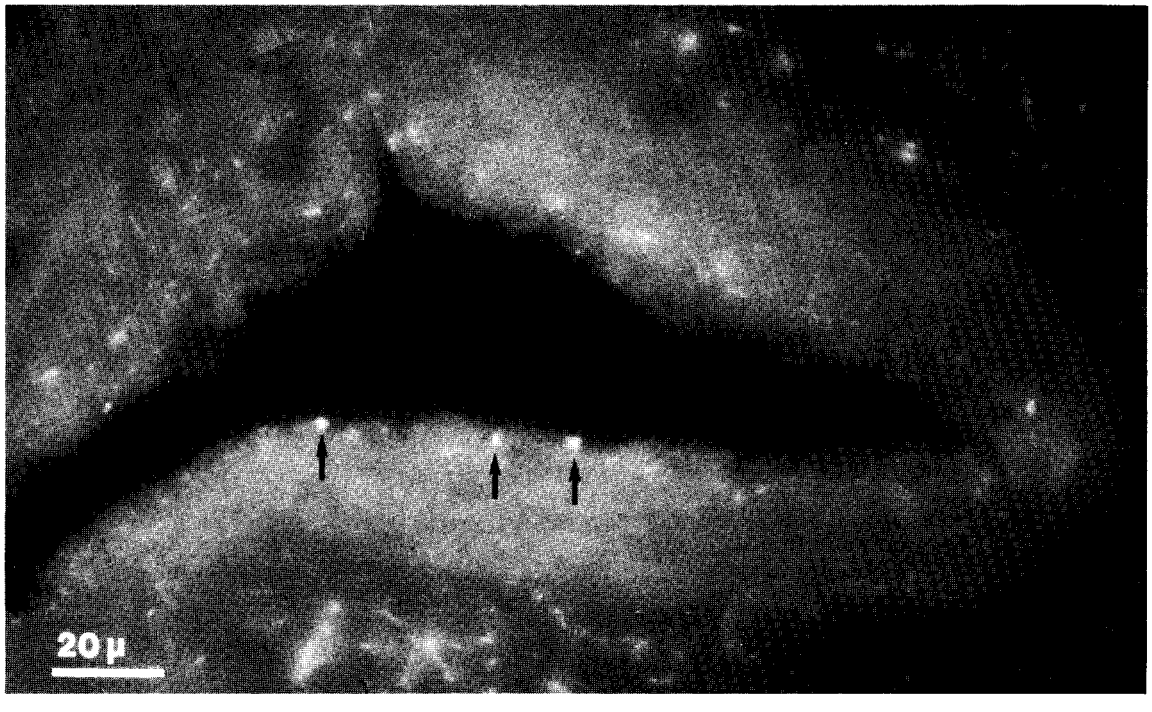

Fig. 6. CGRP immunofluorescence reaction in a cryostat transverse section of a bovine mesenteric lymphatic. CGRP-IR nerve fibers (arrow) are localized subendothelially.

lymphatic vessel wall contraction and propulsion of lymph.

It is generally believed that the contraction of the lymphatic vascular wall, and particularly of its morphofunctional unit, the lymphangion, is exclusively myogenic (14), the role of innervation being limited to a modulation of the intrinsic contractile activity. On the basis of our data, and subject to its physiological and pharmacological confirmation, we propose that in bovine mesenteric lymphatics vessel wall contraction may also be modulated by neurogenic mechanisms. This hypothesis is supported by the observation of Hanley (15) that contractile activity is completely abolished in bovine mesenteric lymphatics that have been chemically deendothelialized by collagenase or dispase treatment while it is retained when deendothelization is achieved mechanically by passing a silk suture through the lymphatic vessel lumen. According to these authors, enzymatic digestion not only removes endothelial cells but also disrupts "other more subtle elements essential for contraction". Perhaps these elements are subendothelial nerve fibers.

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