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Neuropeptide-Containing Nerve Fibers in the Pharynx of the Rabbit

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Abstract. The distribution of peptide-containing nerve fibers in the pharyngeal region of rabbits was studied by immunocytochemistry. Neuropeptide Y (NPY)-containing fibers were numerous around blood vessels and moderate in number among bundles of striated muscle fibers. A few NPY-containing fibers were seen around seromucous glands and beneath the epithelium. Nerve fibers containing vasoactive intestinal peptide (VIP) were numerous around seromucous glands and moderate in number around blood vessels, bundles of muscle, and in the subepithelial layer. A few nerve fibers containing substance P (SP) were seen around blood vessels, seromucous glands, among bundles of muscle, and in the subepithelial layer. Nerve fibers containing calcitonin gene-related peptide (CGRP) were numerous. They were distributed close to blood vessels, among bundles of muscle, in the subepithelial layer, and within the epithelium. A conspicuous finding was the occurrence of CGRP within motor end plates of striated muscle.

Key words: Pharynx, neuropeptides – Immunocytochemistry, pharynx.

The pharynx has a wide variety of functions pertinent to swallowing, respiration, and perception [1, 2]. Basic knowledge about the nervous control of the pharynx and cervical esophagus is not well documented. Vascular tone, exocrine secretion, and other epithelial functions are thought to be regulated by sympathetic, parasympathetic, and sensory nerve fibers. The occurrence of nerve fibers containing "classic" neurotransmitters, such as noradrenaline and acetylcholine, and neuropeptides is well established in the upper respiratory tract and lower esophagus [3, 4]. Less attention has been given to a possible peptidergic nerve supply of the pharyngeal region. We have recently found that the nerve-mediated contractions of pharyngeal muscle are partially resistant to autonomic receptor blocking agents, suggesting the presence of other potential transmitters [5]. We therefore thought it of interest to examine the occurrence and distribution of peptide-containing nerve fibers in the pharynx and cervical esophagus of rabbits.

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Materials and Methods

Five rabbits weighing 1.7–3.0 kg were used. The animals were killed by an overdose of pentobarbitone. Specimens, including the surface epithelium, were dissected from four regions: the middle pharyngeal constrictor muscle, the inferior pharyngeal constrictor muscle, and the cricopharyngeal muscle. In addition, specimens from the cervical part of the esophagus were removed.

The specimens were fixed by immersion in an ice-cold mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) for 12 h. They were then rinsed in a Tyrode solution containing 10% sucrose for 48 h, frozen on dry ice, and sectioned at 10 μm thickness in a cryostat. The specimens were processed for the immunocytochemical demonstration of neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), substance P (SP), and calcitonin gene-related peptide (CGRP) using the indirect immunofluorescence method [6]. Details on the antisera are given in Table 1. The sections were exposed to the antisera for 24 h at 4° C in a moist chamber. The site of the antigen-antibody reaction was revealed by application of fluorescein isothiocyanate-labeled antibodies against immunoglobulin G (Milab, Malmö, Sweden) in a dilution of 1:320 for 1 h at room temperature. Control sections were exposed to antiserum that had been preabsorbed with an excess amount of the antigen (10–100 μg of synthetic or pure natural peptide per ml diluted antiserum). The antisera did not cross-react with the other peptides examined. However, cross-reaction with other peptides or proteins containing amino acid sequences recognized by the different

Table 1. Details of the antisera used for immunocytochemistry

Antigen	Code	Raised against	Directed against	Raised in	Working dilution	Source
NPY[a]	G 26	Protein-conjugated porcine NPY	–	Goat	1:640	T. Schwartz, Copenhagen, Denmark
VIP[b]	8701	Unconjugated pure	N-terminus	Guinea pig	1:640	Milab, Malmö, Sweden
SP[c]	N-Cl/34	Protein-conjugated SP	C-terminus	Monoclonal	1:80	Seralab, Oxford, UK
CGRP[d]	8513	Protein-conjugated rat CGRP	–	Guinea pig	1:640	Milab, Malmö, Sweden

[a] No cross-reaction with peptide YY or pancreatic polypeptide.
[b] No cross-reaction with PHI.
[c] The SP antiserum cross-reacts with physalaemin but not with any other known neuropeptide.
[d] The CGRP antiserum does not cross-react with any other known neuropeptide.

antisera cannot be excluded. The immunoreactive material should therefore appropriately be referred to as NPY-like, VIP-like, and so on. For brevity, however, the shorter terms (NPY, VIP) are often used.

Results

On the whole, the supply of peptide-containing nerve fibers in the pharynx was moderately dense. Clear differences in the innervation density were, however, noted both between different peptides and different regions and tissue components (Table 2).

Middle Constrictor

A rich supply of NPY-containing fibers surrounded small blood vessels and a moderate number of NPY-containing fibers was seen among bundles of striated muscle. Only a few NPY-containing fibers were seen among the seromucous glands. VIP-immunoreactive fibers were numerous in the glandular area. In addition, such fibers were seen in moderate numbers around blood vessels, in between bundles of striated muscle, and in the subepithelial layer. CGRP-containing fibers were numerous in the striated muscle. They were seen scattered among the muscle bundles and often terminated in motor end plates. CGRP-containing fibers also occurred around blood vessels and in nerve bundles passing through the area. In the subepithelial layer a rich supply of CGRP-containing nerve fibers was encountered, and a few fibers were seen to penetrate into the epithelium. A few SP-containing fibers were seen in the supepithelial layer and in between the muscle bundles (see Fig. 4B). SP-containing fibers were not seen in association with motor end plates.

Table 2. Relative frequency of peptide-containing nerve fibers in the pharynx and cervical esophagus of the rabbit

Area	Cell type	NPY	VIP	CGRP	SP
Middle pharyngeal constrictor	Epithelium	0	0	+	+
	Glands	+	+ + +	0	0
	Muscle	+ +	+	+ + +	+
	Blood vessels	+ + +	+	+ +	0
Inferior pharyngeal constrictor	Epithelium	0	0	+ +	+
	Glands	+	+ + +	0	0
	Muscle	+ +	+	+ +	+
	Blood vessels	+ + +	+	+ + +	+
Crico-pharyngeal region	Epithelium	0	0	+ + +	+
	Glands	+ +	+ + +		0
	Muscle	+	+ +	+ +	+
	Blood vessels	+ +	+ +	+ +	0
Cervical esophagus	Epithelium	0	0	0	0
	Glands	0	+ + +	+	+
	Muscle	+	+ +	+ +	0
	Blood vessels	+ +	+ +	+ +	0

The relative frequency was graded arbitrarily: 0, no fibers; +, few; + +, moderate number; + + +, numerous.

Inferior Constrictor

NPY-containing fibers were numerous around blood vessels. In addition, a moderate supply of NPY-containing fibers was seen among bundles of striated muscle (Fig. 1A). NPY-containing fibers were sparse among the mucous glands and in the subepithelial layer. VIP-containing fibers were numerous among the seromucous glands in the submucosa and in the subepithelial layer. A few fibers were seen among the muscle bundles. A moderate supply of CGRP-containing fibers was seen among bundles of muscle and in small nerve bundles passing through the area. The CGRP-containing fibers often terminated on motor end plates (Fig. 2B). A rich supply of CGRP-containing

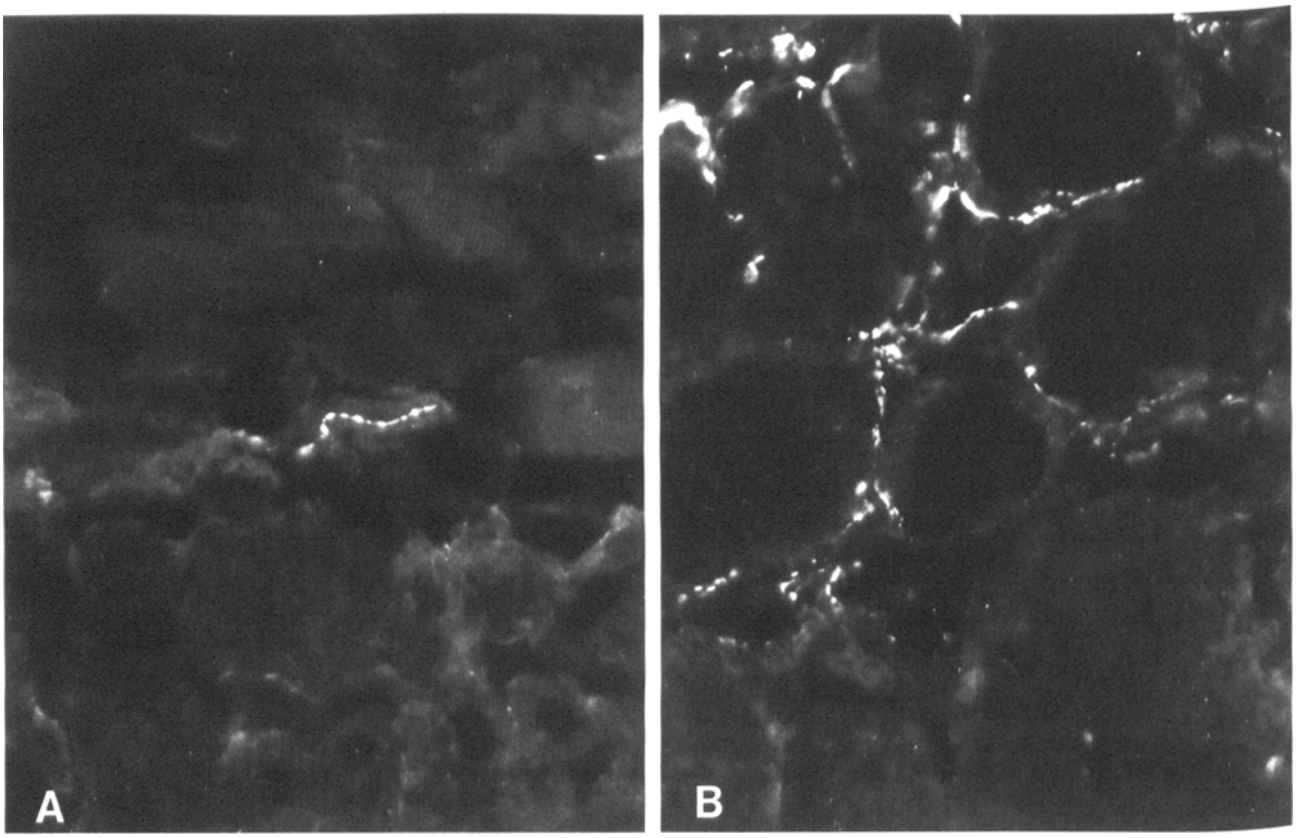

Fig. 1. A An NPY-immunoreactive nerve fiber between bundles of skeletal muscle in the inferior constrictor region. **B** NPY-containing nerve fibers distributed among small seromucous glands in the cricopharyngeal region. (× 225).

fibers was seen among blood vessels, in the subepithelial layer, and within the epithelium. SP-containing fibers were few and occurred around blood vessels and in between bundles of muscle.

Cricopharyngeal Area

A moderate supply of NPY-containing fibers was seen among blood vessels, seromucous glands (Fig. 1 B), and in the subepithelial layer. Only few fibers were seen among bundles of muscle. VIP-containing fibers were numerous among the submucous glands and in the subepithelial layer. They were moderate in number around blood vessels and among bundles of muscle. Numerous CGRP-containing fibers were seen in the surface epithelium (Fig. 2A) and in the subepithelial layer. CGRP-containing fibers also occurred around blood vessels and between bundles of muscle. A proportion of these latter fibers was seen in association with motor end plates. SP-containing fibers were few in the subepithelial layer and among the muscle bundles.

Cervical Esophagus

NPY-containing fibers were seen in moderate numbers around blood vessels and in the subepithelial layer. A few fibers occurred between the muscle bundles and in small plexus formations located between the longitudinal and circular muscle layers. VIP-containing fibers were particularly numerous among the seromucous glands and in the subepithelial layer (Fig. 3A). A moderate supply was seen around blood vessels and between the muscle layers. Occasionally, VIP-immunoreactive nerve cell bodies and nerve fibers could be seen in small plexus formations between the muscle layers (Fig. 3B). Also CGRP-containing fibers were numerous in such plexus formations. In addition, CGRP-storing fibers were seen between bundles of striated muscle. Some of the fibers terminated on motor end plates. In addition, CGRP-containing fibers were seen around blood vessels and among seromucous glands. A rich supply of CGRP-containing fibers was encountered in the subepithelial layer. SP-containing fibers were few in the subepithelial layer and among seromucous

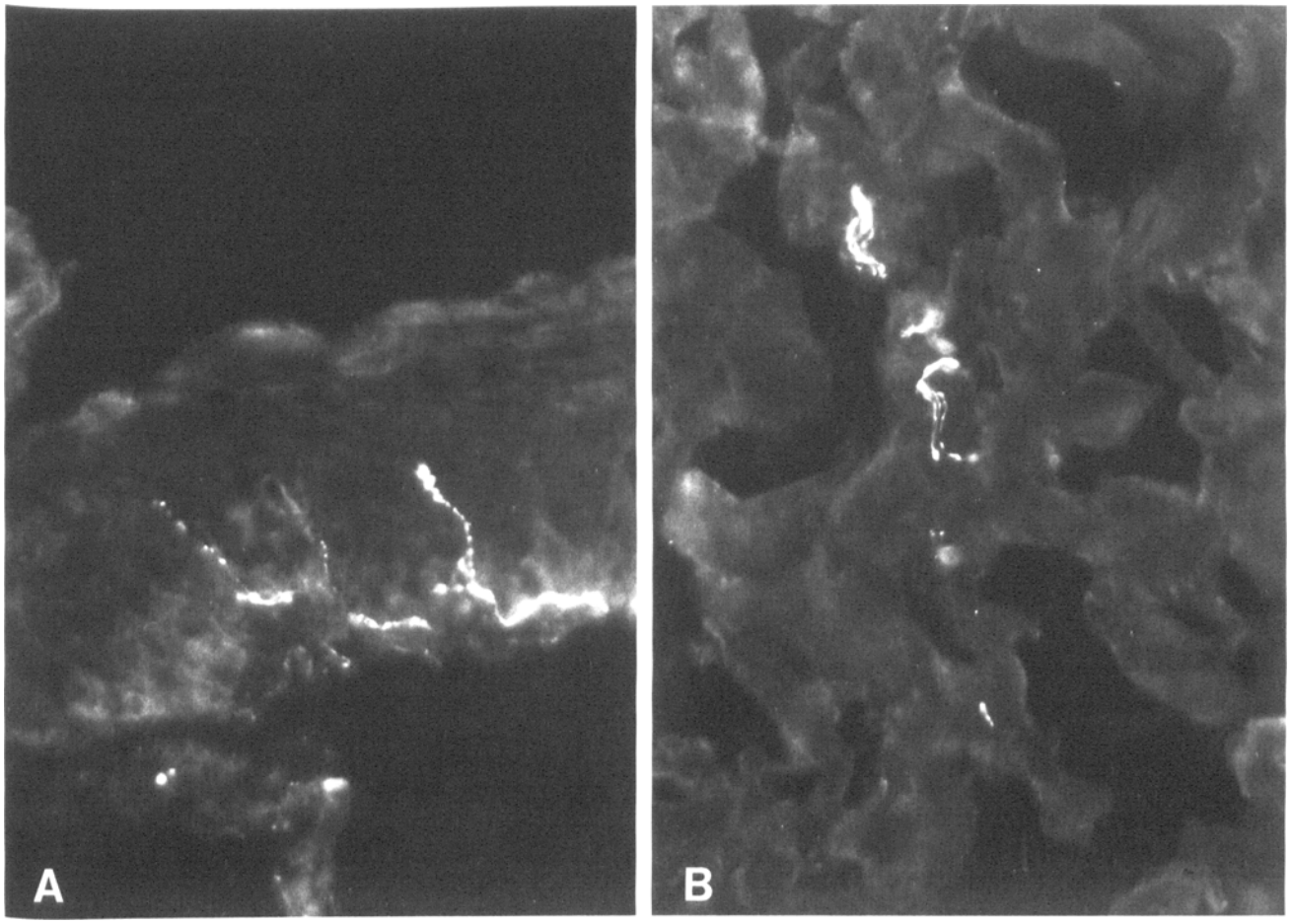

Fig. 2. **A** CGRP-immunoreactive nerve fibers in the surface epithelium of the cricopharyngeal area. **B** Motor end plates containing CGRP in the inferior constrictor area (× 225).

glands. Numerous SP-containing fibers could be seen in association with small plexus formations (Fig. 4A).

Discussion

The anatomy of the pharynx differs markedly from the esophagus: the intramural ganglia are inconspicuous in the pharynx while they are regularly seen and more prominent in the wall of the esophagus. Recent in vitro findings have shown that nerve-mediated contractions of pharyngeal muscle segments of the rabbit may be partially resistant to blockade of the cholinergic (nicotinic) receptors, and that these persisting contractions are not affected by autonomic receptor blocking agents [5]. It is interesting that the present study has shown that motor end plates of the pharyngeal striated muscle are immunoreactive to CGRP. Thus, CGRP is a potential transmitter involved in the nerve-evoked contractions of the rabbit pharynx that were not blocked.

In the rabbit, the pharyngeal constrictors are supplied with a nerve plexus formed on the surface of the middle constrictor by branches of the vagal and glossopharyngeal nerves and the sympathetic trunk [7]. Noradrenaline-containing nerve fibers in the esophagus are distributed in the lamina muscularis mucosa, submucosa, muscle layers, and around myenteric ganglion cells [8]. The present study revealed that the distribution of peptide-containing nerve fibers was similar in the constrictors and the cricopharyngeal area. In general, a rich supply of NPY-containing fibers was seen around blood vessels and a moderate number occurred among the muscle bundles. Previous double immunostaining experiments have revealed that a major population of the NPY-containing nerve fibers contains also noradrenaline (sympathetic nerve fibers), whereas a minor population of NPY-containing fibers stores VIP (nonadrenergic fibers) [9,

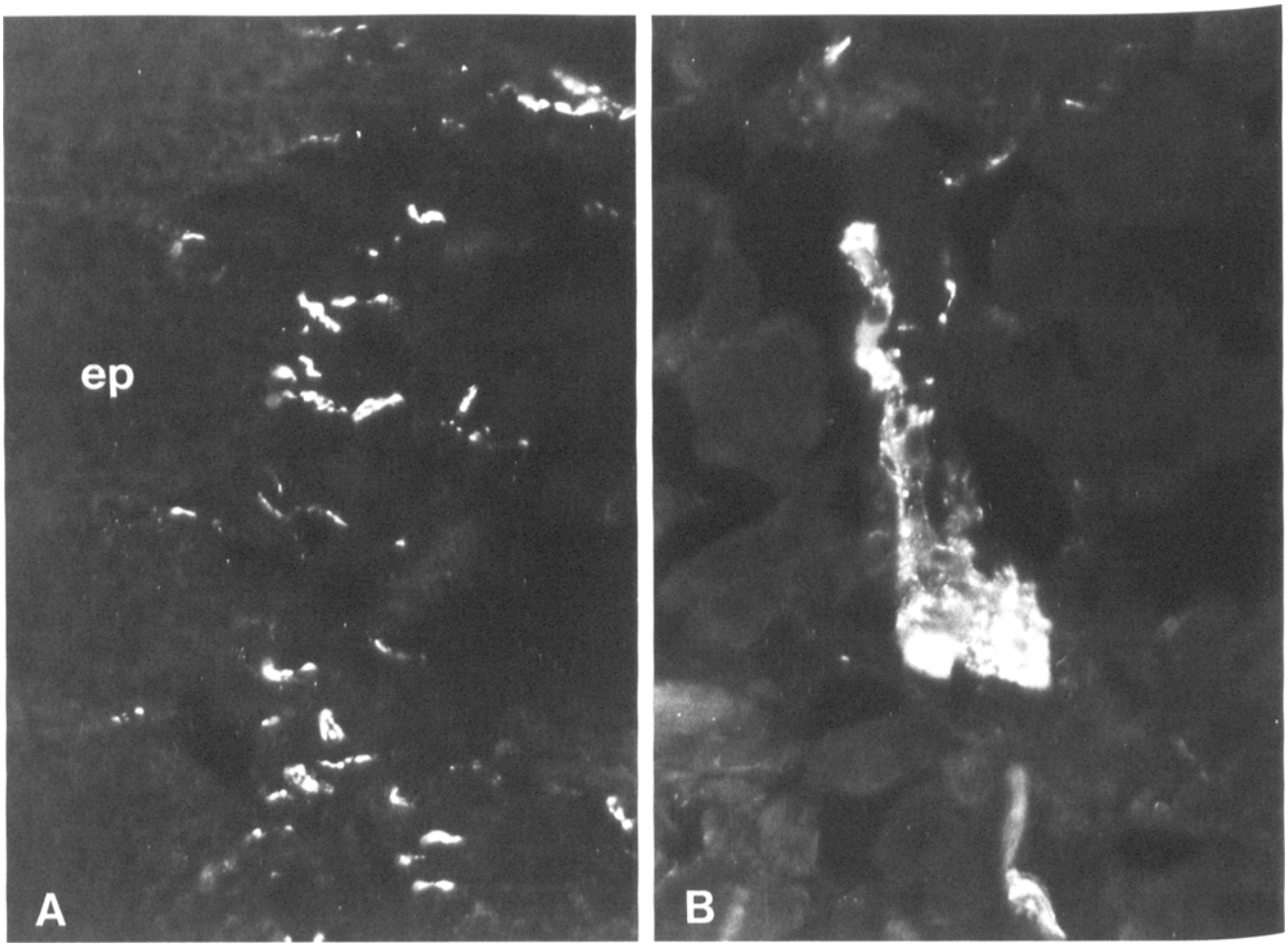

Fig. 3. A A rich supply of VIP-containing nerve fibers in the subepithelial layer of the cervical esophagus. **B** A small ganglionic formation displaying VIP-immunoreactive nerve cell bodies and nerve fibers between the bundles of muscle in the cervical esophagus. *ep*, epithelium (× 225).

10]. The physiological significance of this coexistence has been evaluated on isolated blood vessels and an enhancement of the vascular response to electrical stimulation and to exogenous adrenaline has been reported in the presence of NPY [9]. A possible consequence of this enhancement is a reduction of the noradrenaline demand, with an improvement of the noradrenaline "economy" at the neuroeffector junction [11]. It is conceivable that the majority of NPY-containing fibers in the rabbit esophagus are sympathetic and emanate from cervical sympathetic ganglia.

A particularly rich supply of VIP-containing fibers was seen among the seromucous glands. VIP-containing fibers were regularly seen also around blood vessels and in the subepithelial layer. This distribution fits well with the known actions of VIP: stimulation of secretion from seromucous glands and potent vasodilation [12].

CGRP-containing fibers were numerous in the rabbit pharynx. They were seen close to blood vessels, among bundles of muscle, in the subepithelial layer, and within the epithelium. A conspicuous finding was the occurrence of CGRP within the motor end plates. It has previously been shown that CGRP occurs with acetylcholine in motor-neurons and that CGRP modulates the action of acetylcholine on motor end plates [13]. It has further been suggested that CGRP may cause an increase in the number of acetylcholine receptors [14]. In the esophagus of rat, cat, and monkey a rich supply of CGRP-containing nerve fibers has been observed; also here CGRP can be seen in the motor end plates of striated muscle [15]. In a subpopulation of sensory neurons, CGRP is colocalized with SP [16, 17]. Treatment with the sensory neurotoxin capsaicin has been found to reduce markedly the number of CGRP-containing fibers in all layers of the esophageal wall, which suggests that these fibers are of sensory origin. However,

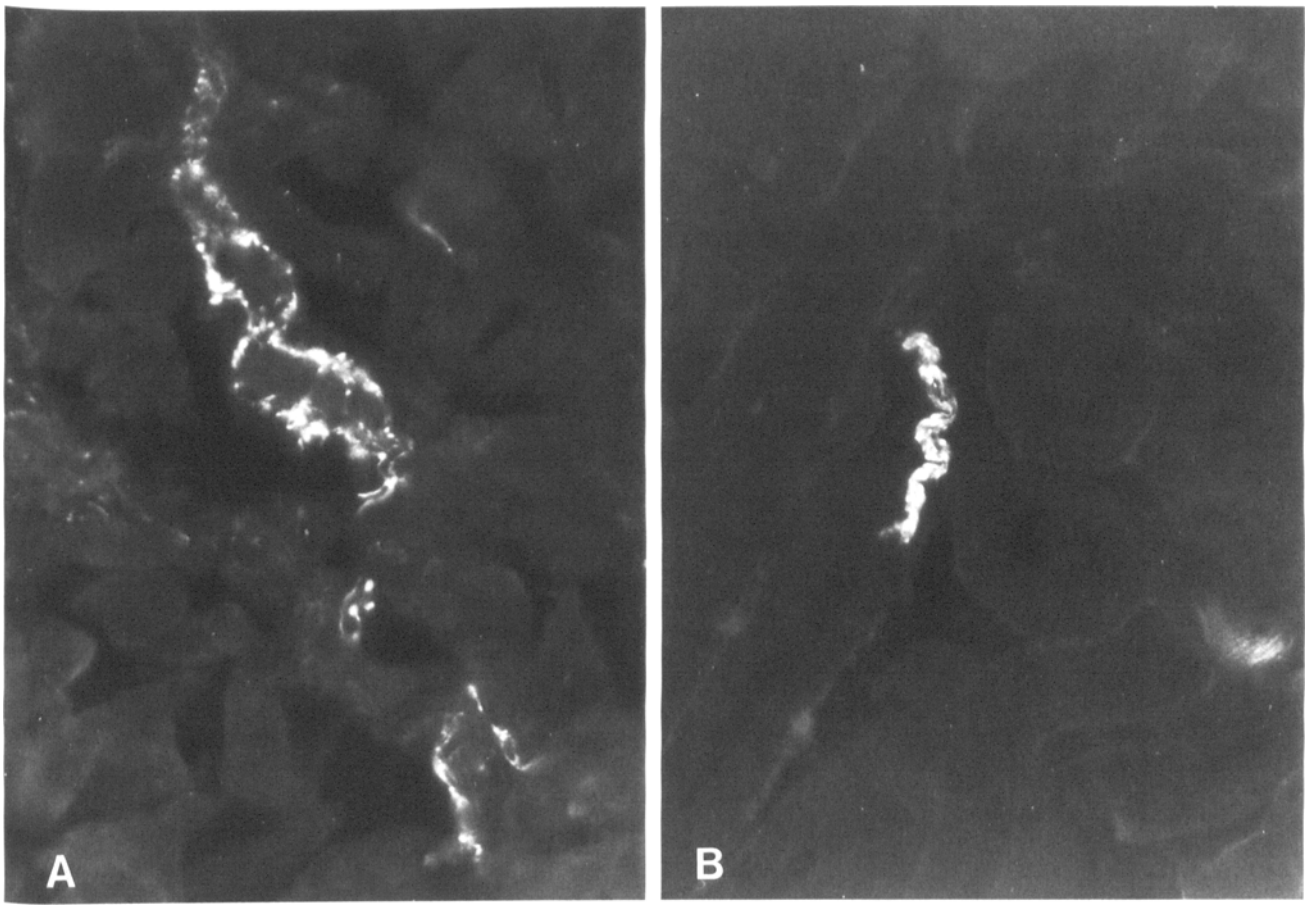

Fig. 4. **A** A ganglionic formation between the muscle layers in the cervical esophagus surrounded by SP-immunoreactive nerve fibers. **B** A nerve bundle displaying SP-immunoreactivity in the skeletal muscle layer of the middle constrictor area (×225).

no change was seen in the frequency of CGRP-immunoreactive motor end plates [15]. A portion of the CGRP-containing fibers originated in the nodose ganglion, as shown by a marked loss of intra- and subepithelial fibers after extirpation of this ganglion. No effect could be detected in the frequency of CGRP-immunoreactive motor end plates. Also, no effect could be seen on the CGRP fibers that were associated with the myenteric plexus [15].

In the present study, SP-containing fibers were mainly distributed close to blood vessels and seromucous glands. In contrast to the rich supply of CGRP in the subepithelial layer and in the epithelium, only few SP-containing fibers were present in these areas, and SP was not observed in association with the motor end plates. Thus, the SP as well as the CGRP fibers, besides having a nociceptive role, may mediate antidromic vasodilatation and enhance secretion from seromucous glands. In the cervical part of esophagus, SP-containing nerve cell bodies were found in the myenteric plexus, which indicates a local origin of such fibers.

On the whole, the distribution of peptide-containing fibers as studied here agrees with findings made in the rat [18; unpublished observations]. The pharyngeal region and upper esophagus of the rabbit contained a rich supply of peptide-containing nerve fibers innervating blood vessels and seromucous glands. In addition, such fibers were seen among the muscle bundles and in small plexus formations. Conspicuous findings were the numerous CGRP-immunoreactive motor end plates and the rich supply of CGRP-containing fibers in the surface epithelium.

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