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Localization of galanin immunoreactivity in the opossum esophagus

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Abstract

Galanin-like immunoreactivity was studied at 7 levels of the opossum esophagus, lower esophageal sphincter (LES) and adjacent portion of the stomach by indirect immunofluorescence; it was restricted to nervous structures. The majority of myenteric and submucous neurons were galanin-positive and received positive axo-somatic terminations. They also sent out axons staining positively. Galanin-positive fibers and a few atypically located neurons formed a mucous plexus at the bases of mucous glands. Varicose galanin fibers innervated the muscularis mucosae, circular and longitudinal muscle layers, while thick fascicles traversed the muscularis mucosae and circular muscle, possibly interconnecting the myenteric, submucous and mucous plexuses. Galanin-positive fibers did not supply blood vessels. There was no obvious gradient of innervation density along the esophagus, but the sphincter appeared to be more densely innervated than the esophageal body. There was no galanin-positive input to striated muscle. In view of its widespread distribution, this neuropeptide may serve multiple functions in the esophagus.

Introduction

Galanin, a recently discovered [14] 29 amino-acid peptide, is known to cause smooth muscle contraction in the rat gut [14] and inhibit it in the esophagus [9]. It is now known to be present in the enteric plexuses of the intestines of man, pig, rat, guinea pig, and mouse [2,7,10,11], but the localization of galanin in the esophagus is not known.

The smooth muscle portion of the esophagus exhibits regional differences in its physiological behavior which are responsible for peristalsis in the esophageal body and relaxation of the sphincter in response to swallowing [8]. Some of these regional physiological differences may be related to regional differences in the innervation of the esophagus [8].

The purpose of the present investigation was to study the distribution of galanin immunoreactivity in the esophagus and to determine regional differences, if any, along the esophagus and lower esophageal sphincter in the opossum.

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Materials and Methods

Three opossums (*Didelphis virginiana*) of either sex were anesthetized with sodium pentobarbital (40 mg/kg i.p.). Isoproterenol was administered i.v. (20 μg/kg) to relax the sphincteric musculature, which otherwise goes into tonic contraction. The esophagus and a part of stomach were extirpated and immersion-fixed overnight in Zamboni's fluid [13]; the tissue was opened flat and divided into the following segments: stomach, lower esophageal sphincter (LES), and esophageal body segments 1 cm, 5 cm, 7 cm, 9 cm and 13 cm above the LES. The LES was known from our previous study [12] to extend from the last ridge of the rugose mucosa (corresponding quite closely to the squamocolumnar junction) cephalad to 5–7 mm caudal to this line. Tissues were cryosectioned at 16 μm in the plane of the muscle (planar) and sagittally (longitudinal).

Following blocking of non-specific binding sites, sections were incubated for 24 h at 4° C in the primary antiserum (rabbit anti-galanin RAS-7153N, Peninsula Labs, Belmont, CA, Lot no. 008504-2; the serum had 100% cross-reactivity for galanin and 0% for secretin, PHM-27 and VIP) at 1 : 350. Sites of antigen–antibody binding were visualized with FITC-conjugated goat anti-rabbit IgG (ICN ImmunoBiologicals, Lisle, IL, Lot no. E446) at 1 : 32.

Parallel controls were employed using the same procedure after preabsorption of the antiserum with an excess of the antigen (100 μg/ml galanin from Sigma, St. Louis, MO, Lot 126F-01021). Slides were viewed under fluorescence epi-illumination with an Olympus BH-2 microscope. For histological corroboration of fluorescent structures, particularly neurons, many slides were re-stained in 0.1% thionin after careful marking of stage coordinates and removal of the coverslip, and later rephotographed under brightfield illumination.

Colchicine treatment was attempted with a starting dose of 5 mg/kg i.p. [6] but this was fatal, as was the subsequent dose of 3.75 mg/kg; the dose finally used in one animal was 1 mg/kg and was sufficient to produce diarrhea.

Since antibodies recognize only certain amino acid sequences, all descriptions that follow apply to *galanin-like immunoreactivity*.

Results

Immunoreactivity in nerve fibers. Galanin-immunoreactive fibers were seen at the bases (deepest parts) of the mucous glands, in the muscularis mucosae, the submucous connective tissue and fascicles of the submucous plexus, the circular muscle, the myenteric fascicles, and the longitudinal muscle (Fig. 1A). The histological topography of the fluorescent structures can be identified from Fig. 1B, which shows the same field after post-staining in thionin.

The mucous glands were innervated at their bases by fine non-varicose galanin-immunoreactive fibers (Fig. 2A and B) of the mucous plexus. The cytoplasm of the glands themselves showed non-specific fluorescence. An occasional neuron was found as high up as the bases of the mucous

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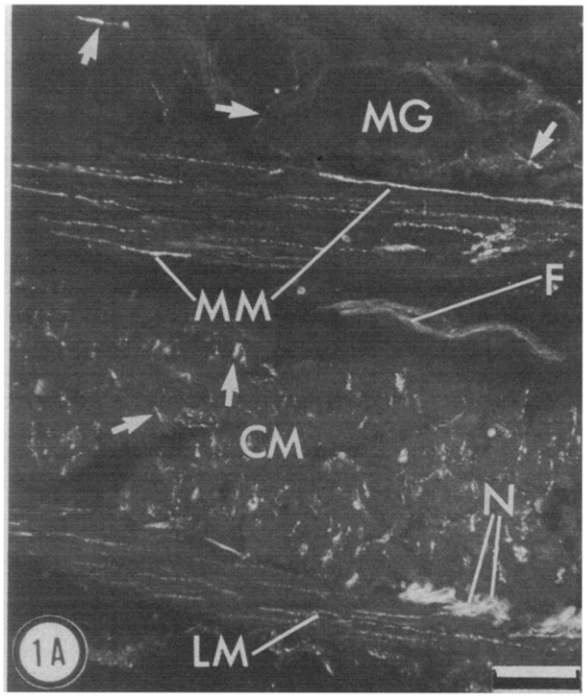
Fig. 1. A: longitudinal section through the 1-cm esophageal body segment showing widespread distribution of galanin-immunoreactive fibers at the bases of the mucous glands (MG) (arrows), in the muscularis mucosae (MM), circular (CM) and longitudinal (LM) musculature. A prominent fascicle (F) is seen in the submucosal connective tissue, and also two myenteric neurons (N) surrounded by variously oriented fascicles. Indirect immunofluorescence. B: the same field as in A post-stained with thionin for identification of histological entities. Abbreviations as in A. Note the large submucosal space (X) between the muscularis mucosae and circular muscle. Bar = 100 μm (A + B).

Fig. 2. A: planar section through esophageal body showing details of the mucous plexus, which consists of fine fibers around the bases of the glands (arrows). The pale fluorescence of the glands is artifactual. A single immunoreactive neuron (N) and several interganglionic fascicles (F) are visible. Indirect immunofluorescence. B: the same field as in A post-stained with thionin to confirm the location of the neuron (N), which is clearly identifiable by its vesicular nucleus (at the end of the guideline) with a prominent nucleolus. Bar = 100 μm (A + B).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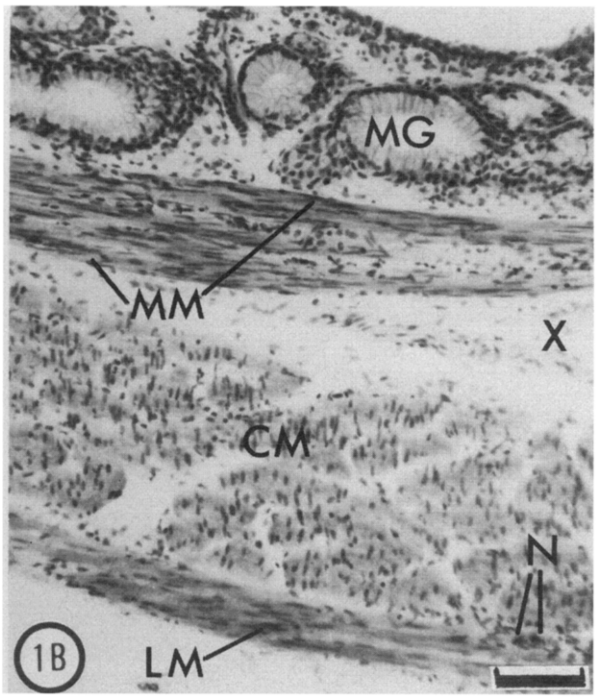
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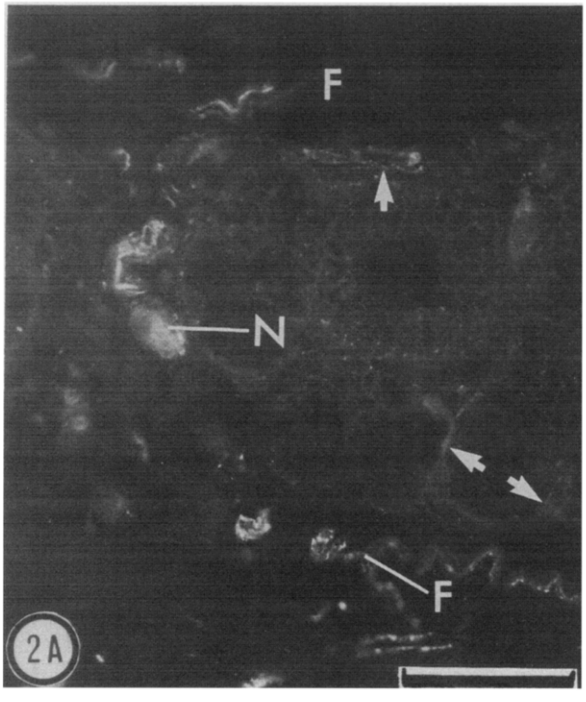
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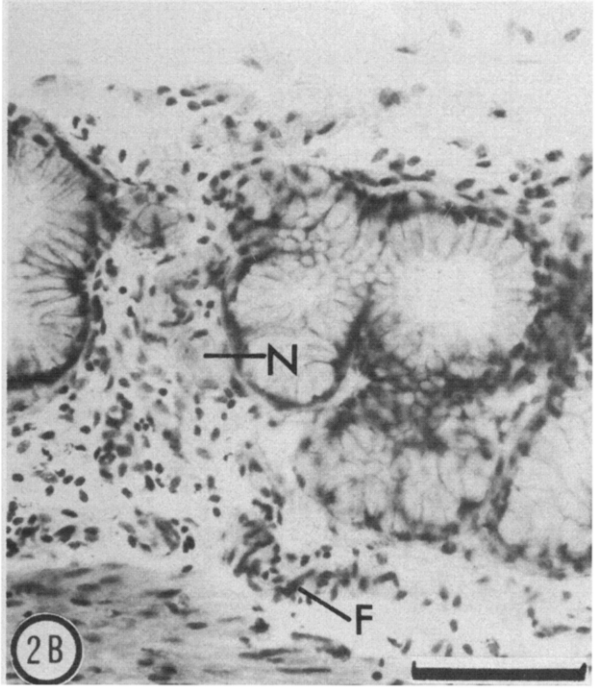
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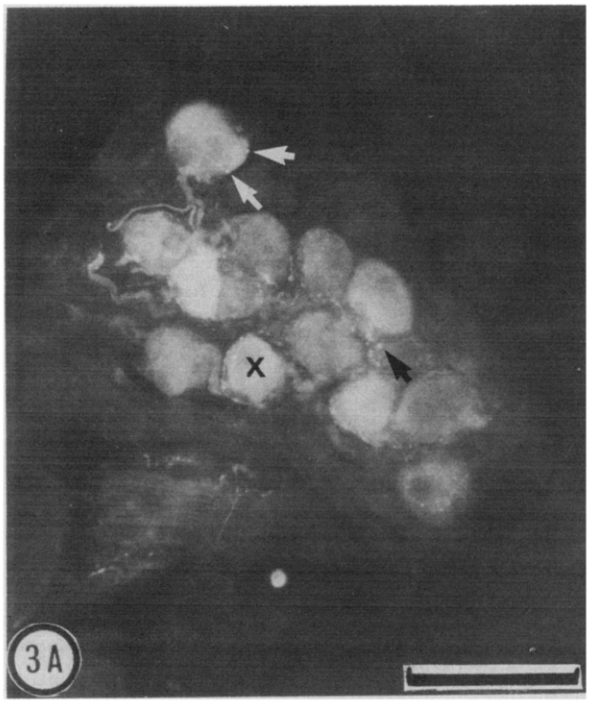
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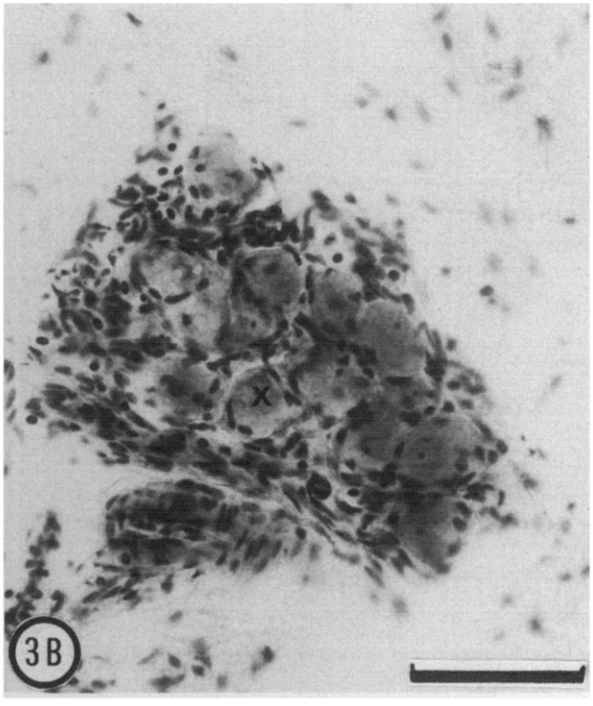
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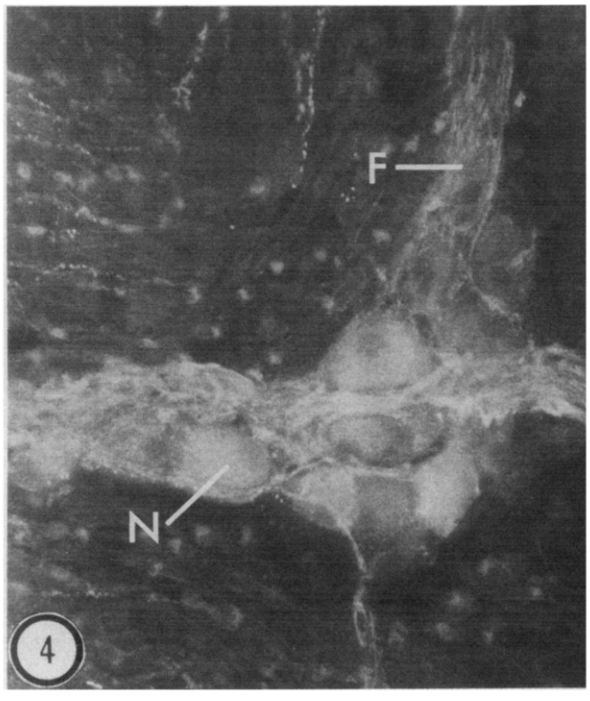
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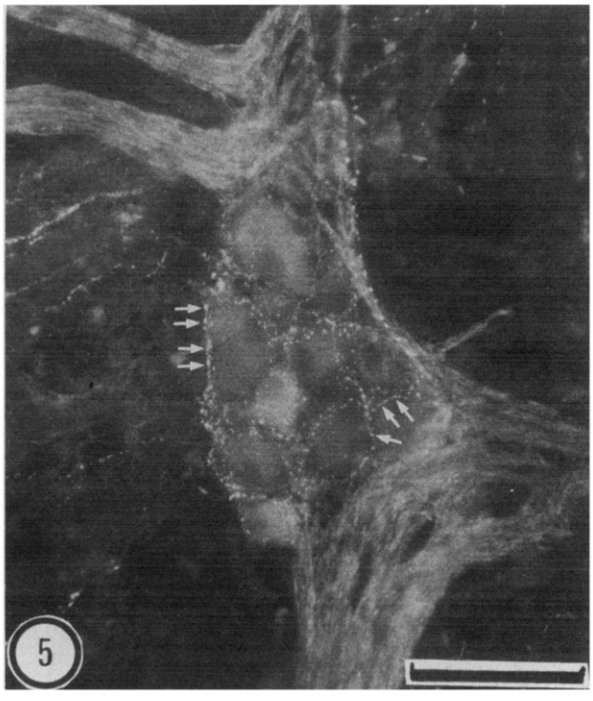
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glands, thus becoming part of the mucous plexus (Fig. 2A and B). Also evident in the same field are the interganglionic fascicles supplying terminations to the mucous glands.

The muscularis mucosae (Fig. 1A, B) was richly innervated by galanin-immunoreactive fibers which ran mainly longitudinally. It appears that the muscularis mucosae was penetrated and traversed by radial fibers on their way luminally to the mucous glands or neurons at the bases of those glands, and that it was supplied from the peripheral direction by fibers emanating from the submucous plexus, whose thick fascicles could be seen to course along the muscularis itself (Fig. 1A).

The submucous space between the muscularis mucosae and circular muscle was fairly wide (Fig. 1B), containing loose connective tissue fibers and blood vessels. Numerous galanin-positive fibers were seen here, but none occurred in association with any blood vessel. However, the intima of arterioles yielded a bright, sharply defined nonspecific fluorescence.

The circular musculature was also richly innervated by galanin-positive fibers. As with all endings on smooth musculature, this consisted of fine, bead-like varicosities connected by delicate strands. Additionally, the circular muscle was traversed radially by thick galanin-immunoreactive fascicles running across it towards the submucous plexus. The origin and termination of these fascicles is not known, but they appeared to be identical to the interganglionic fascicles of the submucous and myenteric plexuses.

The longitudinal muscle was also richly galanin-innervated (Fig. 1A), but to a lesser extent than the circular muscle or muscularis mucosae.

Interganglionic fascicles in all the strata of the esophagus were brightly positive.

Immunoreactivity in neurons. There are not very many submucous neurons in the opossum esophagus, but all of them appeared to be positive for galanin (Fig. 3A and B); overall, over 50% of the neurons were intensely positive. The cell bodies of many of these neurons received brightly galanin-immunoreactive terminations (Fig. 3A), though these were not as numerous as those on some myenteric ganglia.

Most of the neurons of the myenteric plexus were galanin-positive with or without colchicine pretreatment. More than half the total number of myenteric neurons were brightly galanin-positive (Fig. 4), but some showed brightness only slightly above control levels (Fig. 5), making their positivity a matter of judgement. The proportion of brightly galanin-positive neurons varied from ganglion to ganglion and there was no fixed topographical localization of these neurons within the ganglia. As all the galanin-positive fibers were brightly stained, they could be seen to provide input to less brightly galanin-positive neurons (Fig. 5).

Galanin output in the form of positively staining axons from myenteric ganglia was also evident as positive fibers in fascicles and in cases where a neuron's axonal hillock was sectioned (Fig. 6). In no instance was any galanin termination found upon striated muscle.

Regional and sphincter-body comparisons. This study examined tissues from 7 sites: the stomach, LES and 5 locations on the esophageal body. The density of galanin-positive innervation based upon

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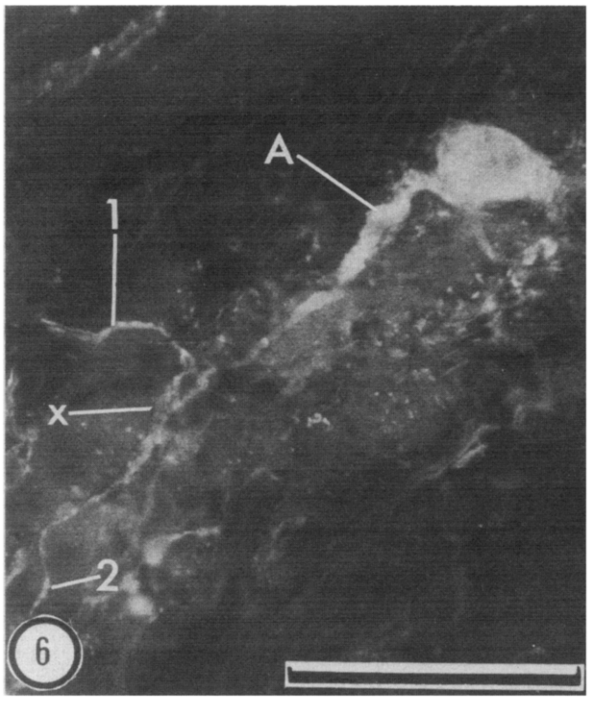
Fig. 3. A: submucous ganglion in the esophageal body seen in planar section; 14 galanin-immunoreactive neurons are evident. The small arrows point to the brightly immunoreactive terminations upon the neuronal bodies; in particular the neuron labelled X can be seen to be surrounded by them. Indirect immunofluorescence. B: the same field as in A post-stained with thionin for corroboration of neuronal number. No more neurons become visible, showing thereby that all neurons in the ganglion were positive for galanin; X marks the previously labeled neuron for reference. Bar = 100 μm (A + B).

Fig. 4. Myenteric ganglion in esophageal body in planar section. The neurons (N) are about as bright as the fascicles (F). Indirect immunofluorescence.

Fig. 5. Myenteric ganglion in esophageal body; neurons are less bright than the fascicles, but many beadlike galanin-immunoreactive terminations (arrows) are evident upon the somata. Indirect immunofluorescence. Bar = 100 μm.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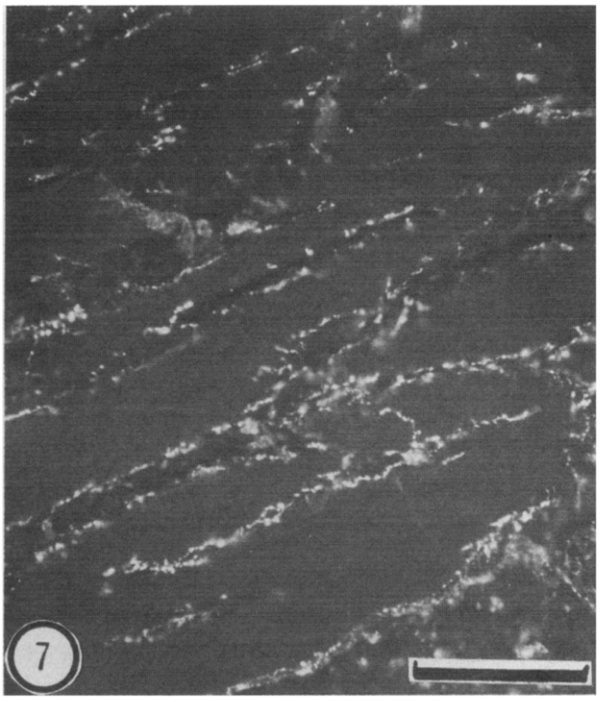
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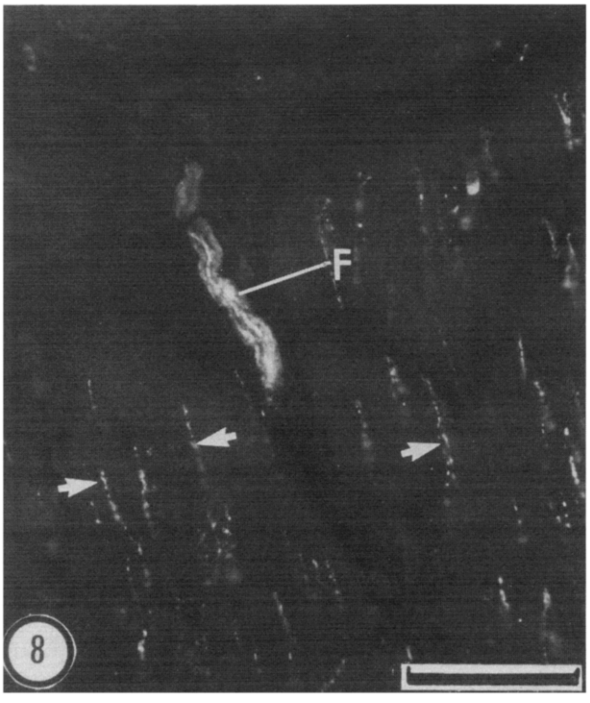
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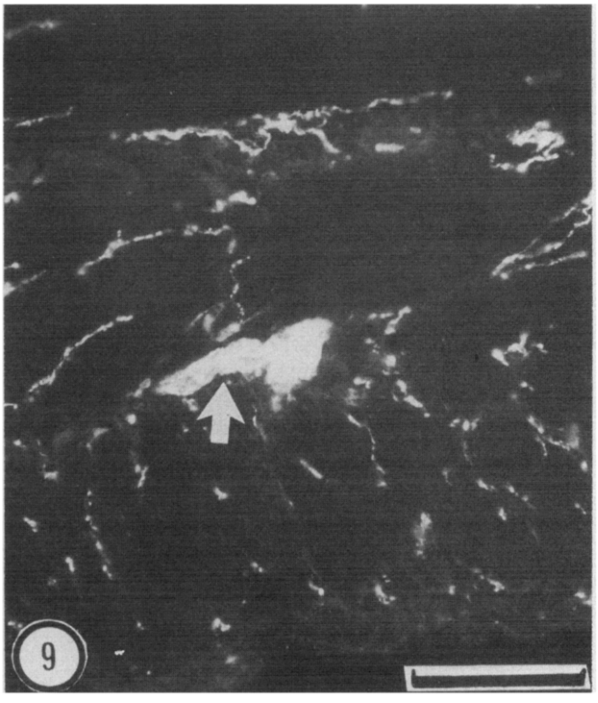
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visual assessment alone appeared to be the highest in the LES (Fig. 7), declining both cephalad in the esophageal body (Fig. 8) and caudad in the stomach (Fig. 9).

Discussion

The localization of galanin as shown in this study closely parallels that of VIP in the smooth-muscle portion of the opossum esophagus as shown by Christensen et al. [5]. The principal difference between the localization of the two substances is that galanin does not occur in association with blood vessels or striated muscles, whereas VIP does [5].

The similarities between the distribution of galanin immunoreactivity in the present study and that of VIP [5] are, however, more striking than the differences. This is more relevant in the light of the work of Bishop et al. [2], who found co-localization of VIP and galanin in the submucous neurons of man, pig and rat.

With reference to specific sites of localization, we find that the opossum esophageal mucosa is well innervated with galanin-containing fibers. This contrasts with the reported [2] poor galanin innervation of the mucosa of the stomach of man, pig and rat. Pig duodenum and large and small bowel of rat, like the opossum esophagus, may be more densely innervated than stomach [2,7]. The importance of the regional distribution of galanin immunoreactivity in the different segments of the gut is not known.

The submucous plexus in the esophagus is not well developed (37–84 neurons/cm^2 compared to a peak of 2234/cm^2 in the jejunum). Despite the small numbers, it has a large proportion of intensely galanin-positive neurons, a situation similar to that in other parts of the gut. The latter have previously been shown to be galanin-positive in man, pig and rat [2,7].

The occurrence of isolated neuronal cell bodies (perikarya/somata) near the bases of the mucous glands has not been reported before. The importance of these isolated, galanin-positive neurons is not known.

The muscularis mucosae is well innervated by galanin-containing fibers and the visual pattern is reminiscent of that found for VIP fibers by Christensen et al. [5]. The pattern of innervation of the circular and longitudinal muscle is also similar to VIP innervation in that study.

This study found no supply of galanin to the vasculature of the esophagus. This finding is similar to those in the stomach and intestine of man, pig and rat [2]. Contrarily, Ekblad et al. [7] have reported galanin-positive innervation of blood vessels in the stomach and intestine of rats. The reason for these reported differences is not known.

The majority of myenteric neuronal bodies seen by us were immunoreactive for galanin, though to varying extents. The large number of unambiguously positive neurons in the opossum esophagus is interesting in the light of Bishop et al.'s [2] findings to the effect that there are no galanin-positive myenteric neurons in the human and porcine stomach and intestine. In the esophagus, galanin-immunoreactive output from myenteric neurons terminates clearly upon muscle as varicosities. Additionally, the brightly positive axosomatic endings may represent terminations of other myenteric neurons. Some authors [7] believe that this dual pattern of termination may be indicative of an integrative function in the control of motor activity.

The radially oriented interganglionic fascicles which traverse the circular muscle and muscularis

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Fig. 6. A single myenteric neuron from the esophageal body showing the emergence of an immunoreactive axon (A), which becomes progressively attenuated and bifurcates at point x into two rami, one of which (2) becomes varicose at the point where the guideline terminates. Indirect immunofluorescence. Bar = 100 μm.

Figs. 7–9. Comparative galanin innervation of lower esophageal sphincter (Fig. 7), esophageal body (Fig. 8), and cardiac stomach 1 cm behind sphincter (Fig. 9). Note the radially disposed interganglionic fascicle (F) in addition to terminal varicosities (arrows) in Fig. 8. The large arrow in Fig. 9 points to a myenteric ganglion. Bar = 100 μm.

mucosae may interconnect the myenteric, submucous and mucous plexuses. Therefore the possibility arises of a third ending for myenteric neurons: communication with the submucous neurons, which we have observed to possess axosomatic endings.

Based upon visual estimations, this study showed the greatest density of innervation to lie in the sphincter and to decline both cephalad in the esophageal body and caudad in the stomach. The position regarding sphincteric galanin innervation corresponds to another study on VIP innervation [1] and to our observations on myenteric neuronal density, which is highest in the LES and declines both ahead of and behind it [12]. However, another study [3] found the sphincter to have the lowest neuronal density.

The physiological role of galanin in the esophagus is not known, but its widespread occurrence may indicate diverse functions.

Acknowledgements

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