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Peptidergic Innervation of the Human Esophageal Smooth Muscle

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Studies were performed to define the peptidergic nature of intramural nerves in the human esophagus. Cryosections of uninvolved surgically resected tissues from 14 individuals were studied by immunofluorescence for the localization of 10 neuropeptides. Myenteric neurons showed bombesin–, calcitonin gene-related peptide–, galanin–, substance P–, vasoactive intestinal polypeptide–, leucine-enkephalin–, methionine-enkephalin–, neuropeptide Y–, and somatostatin–like immunoreactivity. Submucous neurons had all the above except neuropeptide Y, methionine-enkephalin, leucine-enkephalin, and bombesin. Both groups of neurons received nerve terminations positive for calcitonin gene-related peptide, galanin, neuropeptide Y, substance P, and vasoactive intestinal polypeptide. Myenteric neurons additionally received terminations positive for neuropeptide Y, methionine-enkephalin, and somatostatin. All muscle layers had varicose fibers that reacted for calcitonin gene-related peptide, galanin, neuropeptide Y, and substance P. Longitudinal and circular muscle received few nerves reactive for leucine-enkephalin, whereas methionine-enkephalin was localized in a few nerve endings in the circular muscle. Somatostatin- and bombesin-reactive nerves occurred in longitudinal muscle. No cholecystokinin-reactive nerves were found. This study extends the results of previous studies and shows the previously undescribed presence of calcitonin gene-related peptide– and galanin–reactive nerves in the human esophagus and identifies neuropeptides that may serve as motor, sensory, and modulatory neurotransmitters of esophageal nerves.

The nature of the nerves controlling esophageal function is not fully understood (1). The morphological demonstration of a chemical substance in the neurons is an important criterion for identification of putative neurotransmitter candidates. Studies have shown that apart from cholinergic (2) and adrenergic (3) nerves, a variety of peptidergic nerves (1) may play important roles in esophageal function. Immunocytochemical studies have shown the existence of substance P (SP)- and vasoactive intestinal polypeptide (VIP)-reactive nerves that innervate various components of esophageal smooth muscle in animals (4–7) and humans (8–10). Moreover, a small number of bombesin (BOM), leucine-enkephalin (L-ENK)-, and somatostatin (SOM)-reactive nerves have been shown to be present in the human esophagus (8,11,12).

Calcitonin gene-related peptide (CGRP)- (13,14) and galanin (GAL)- (15,16) immunoreactive nerves have been described in the esophagus of animals, but their presence in the human esophagus is not known. The presence of methionine-enkephalin (M-ENK)-reactive nerves in the esophagus has not been examined.

The purpose of the present study was to systematically examine the presence and distribution of neurons and nerve fibers that are reactive for BOM, cholecystokinin (CCK), L-ENK, M-ENK, neuropeptide Y (NPY), SOM, SP, and VIP as well as CGRP, GAL, and M-ENK in the human esophagus.

Materials and Methods

Esophageal tissues were obtained from 14 individuals (9 male and 5 female), aged 44–76 years, who underwent resections for various malignancies. These patients had no esophageal motility disorders and were also free of diabetes, scleroderma, and other degenerative neurological diseases.

Abbreviations used in this paper: **BOM, bombesin; GAL, galanin; L-ENK, leucine-enkephalin; M-ENK, methionine-enkephalin; SOM, somatostatin.**

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Tissues were obtained from sites at least 4 cm away from areas showing the presence of tumor cells as seen by immediate cryosectioning and rapid staining. More than one sample of tissue was taken from each individual; in 8 patients, upper, middle, and lower thoracic esophageal specimens including the lower esophageal sphincter were obtained; in 4, specimens came from the middle and lower thoracic areas; and in 2, specimens were taken from the upper and lower thoracic esophageal regions. Specimens were rinsed in cold saline and immersion-fixed in buffered picroformaldehyde (17) at 4°C for 18–24 hours. Not more than 20 minutes elapsed between surgical resection and immersion in fixative. After fixation, tissues were rinsed in phosphate-buffered saline (PBS) repeatedly, and each tissue piece was divided in two, one for cryosectioning and the other for paraffin. The latter was dehydrated, cleared, infiltrated in paraffin wax, and sectioned for routine histology, whereas the former was placed in PBS enriched with 15% sucrose (wt/vol) and cryosectioned at 16 μm first in one orientation and then perpendicular to the former direction, so that all muscle layers, longitudinal, circular, and muscularis, could be seen in longitudinal section. This was performed to permit meaningful comparisons of visual estimates of innervation density.

Cryosections were incubated overnight at 4°C in polyclonal rabbit antisera against BOM, CCK, CGRP, GAL, L-ENK, M-ENK, NPY, SOM, SP, and VIP and subjected to the procedure of indirect immunofluorescence (18). Details of primary antisera and their dilutions are as reported elsewhere (19); all were raised in rabbits against the 10 peptides named above and were purchased from Peninsula Laboratories (Belmont, CA). Specificities and cross-reactivities of these antisera are defined by the manufacturer, and we have listed them previously (19); anti-SP was specific against SP and not a general marker for tachykinins. Sites of antiserum binding were visualized with fluorescein isothiocyanate–conjugated affinity-purified goat anti-rabbit immunoglobulin G (IgG) (1:60, 1 hour at room temperature; Calbiochem, La Jolla, CA) under epifluorescent illumination on an Olympus BH2 research microscope (Olympus Optical Co., Tokyo, Japan). When required, nonspecific fluorescence was suppressed with pontamine sky blue (20). In many cases the coordinates of a given field of interest, particularly one containing neurons, were marked, and preparations were poststained with thionin to corroborate histological details and neuronal identity.

Three kinds of controls were performed. In omission controls, the primary antibody was either omitted (PBS was used instead) or substituted with nonimmune rabbit serum at the same dilution. In preadsorption controls, the antiserum was preincubated with its particular antigen (10–100 μg antigen/mL working strength antiserum; 24 hours at 4°C) and applied to sections in place of regular antiserum. A site was construed as being positive only if fluorescence was eliminated in these controls. Because the sharing of immunodeterminant sequences by tissue components cannot theoretically be ruled out, in the present account a term such as CGRP-positive, immunoreactive for CGRP, or CGRP-reactive means that the site showed CGRP-like immunoreactivity.

This protocol, entitled "use of discarded tissues from the gastrointestinal tract," was approved by the Committee on Clinical Investigations, Beth Israel Hospital.

Results

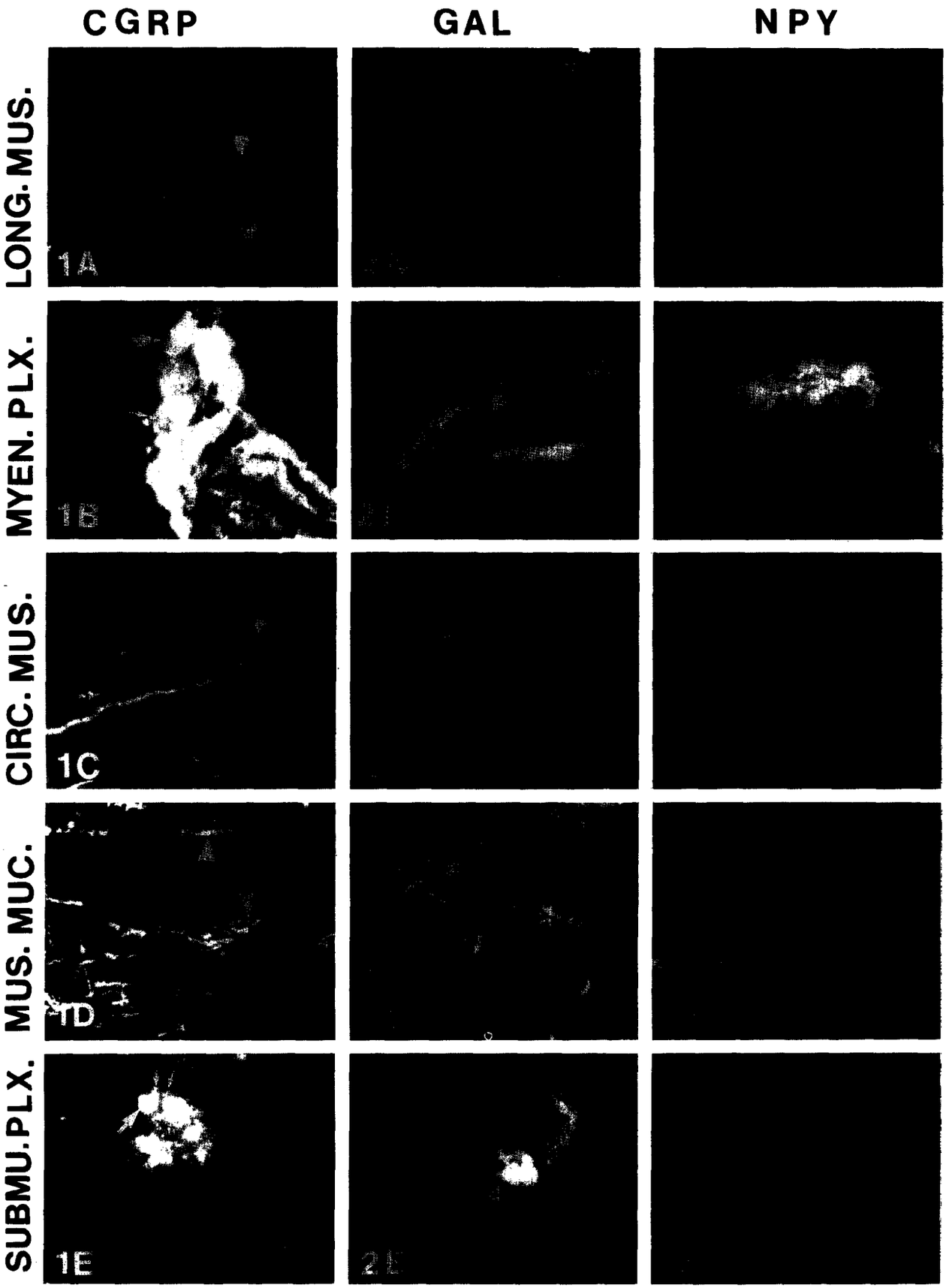
Longitudinal muscle

The longitudinal muscle is thicker in the upper esophagus and thins down caudally. Varicose CGRP- (Figure 1*A*), GAL- (Figure 2*A*), and VIP-containing (Figure 5*A*) fibers were abundantly present both singly and in bundles. There was no observable difference in the distribution of these fibers in the upper, middle, and lower parts of the thoracic esophagus. In contrast, fibers showing immunoreactivity for NPY (Figure 3*A*) and SP (Figure 4*A*) were present in moderate numbers. Many of them were long, varicose, and ran singly, with occasional branching. Only a few

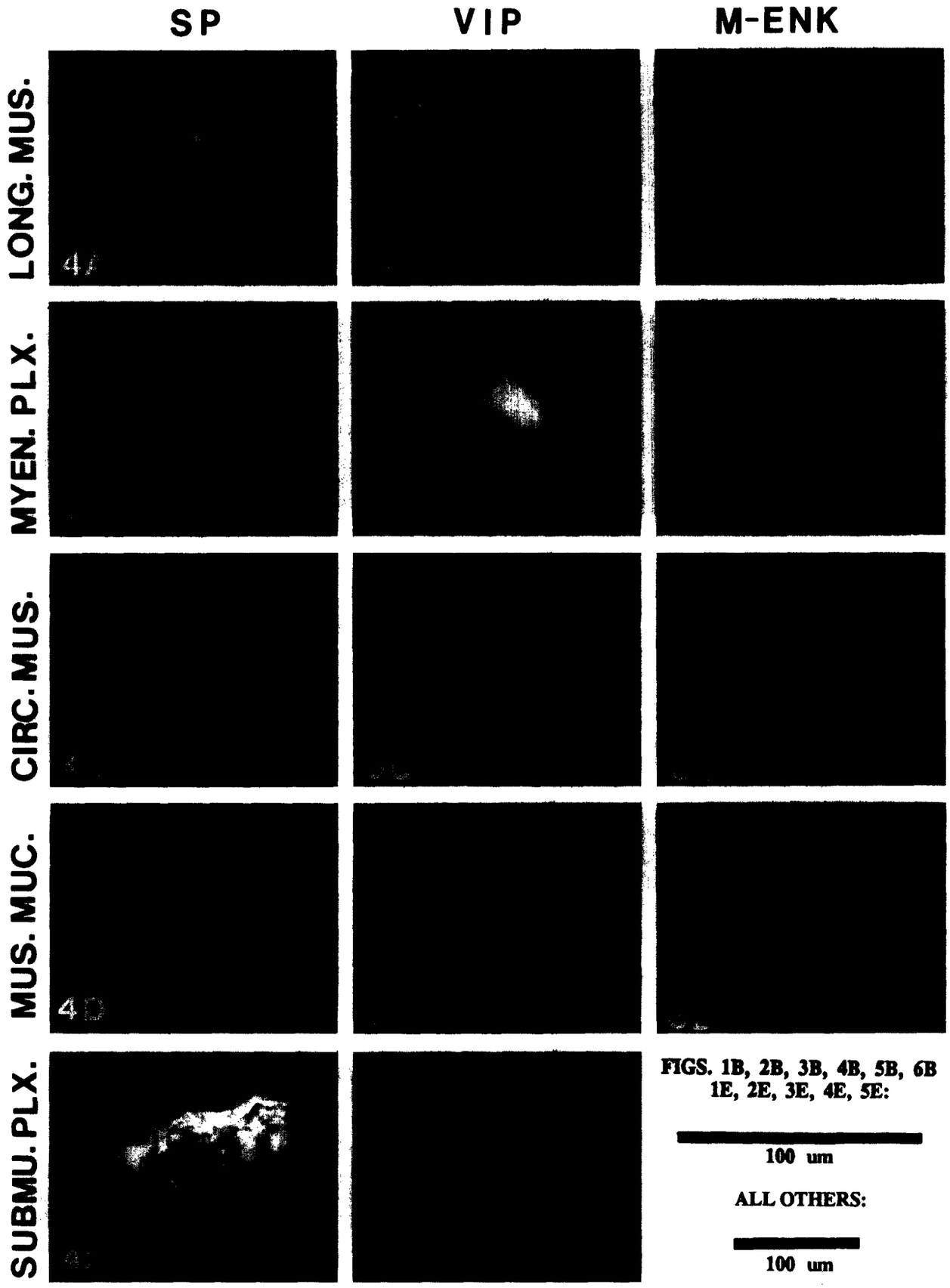
Figure 1. Calcitonin gene-related peptide. (*A*) Varicose CGRP-positive fibers in the longitudinal muscle (*arrowheads*). (*B*) Myenteric ganglion showing several CGRP-positive neuronal somata (one bearing *large arrow*) with numerous terminations on them (one with *small arrow*). A fascicle descends to *bottom right*. (*C*) Calcitonin gene-related peptide positive fibers (one with *arrowhead*) innervating circular muscle. (*D*) Calcitonin gene-related peptide innervation of the muscularis mucosae; two fibers bear *arrowheads*. (*E*) Submucous ganglion showing CGRP-like immunoreactivity. Both somata (one with *large arrow*) and terminations on them (two, *small arrows*) are positive. Tissues were variously oriented so that all muscle layers were sectioned longitudinally. *Magnification bars* for all figures appear after Figure 6*D*.

Figure 2. Galanin. (*A*) Galanin innervation of longitudinal muscle (one fiber with *arrowhead*). (*B*) Two myenteric ganglia showing GAL-like immunoreactivity in somata (one with *large arrow*) and terminations (two with *small arrows*). (*C*) Galanin in the circular muscle; *arrowheads* point to two fibers. (*D*) Galanin in muscularis mucosae; two prominently varicose fibers (*arrowheads*). (*E*) Galanin positivity in submucous neurons (one *arrowed*). Although terminations are also positive, they are not well differentiated in this figure. Part of a fascicle is also visible at *upper right*.

Figure 3. Neuropeptide Y. (*A*) Longitudinal muscle showing NPY innervation. One fiber bears an *arrowhead*. (*B*) Neuropeptide Y in a myenteric ganglion. Both somata (one with *large arrow*) and terminations (two prominent ones with *small arrows*) are positive. (*C*) Neuropeptide Y innervation in circular muscle; *arrowheads* point to two varicose fibers. (*D*) Neuropeptide Y–immunoreactive fibers in the muscularis (one with *arrowhead*). (*E*) Submucous neurons showing absence of NPY in the somata but strong positivity in terminations (*small arrows*).


CGRP
GAL
NPY
LONG. MUS.
MYEN. PLX.
CIRC. MUS.
MUS. MUC.
SUBMU. PLX.
1A
1B
1C
1D
1E

Figures 1–3.


SP
VIP
M-ENK
LONG. MUS.
MYEN. PLX.
CIRC. MUS.
MUS. MUC.
SUBMU. PLX.
4A
4D
FIGS. 1B, 2B, 3B, 4B, 5B, 6B
1E, 2E, 3E, 4E, 5E:
100 um
ALL OTHERS:
100 um

Figures 4–6.

L-ENK fibers were present in the longitudinal muscle; most sections showed one or two. They were long, varicose, and thin, and generally ran singly. Because of the small numbers of these fibers, it is not possible to make comparisons of regional density. There was no M-ENK in the longitudinal muscle (Figure 6*A*) of the human esophagus. Bombesin fiber distribution was spotty among the sections observed. This is indicative of small and widely spaced fibers, not easily intercepted. Fibers containing BOM and SOM were also seen. It is also noteworthy that BOM was seen additionally only in myenteric neurons and occasionally in fibers innervating mucous glands but was absent in all other layers. The distribution of SOM-reactive fibers was sporadic, and fibers were short and far apart. Cholecystokinin-reactive fibers were not seen.

Circular Muscle

To permit meaningful comparison with longitudinal muscle, blocks were reoriented (see Materials and Methods) so that circular muscle was also sectioned longitudinally. As in longitudinal muscle, CGRP- (Figure 1*C*), GAL- (Figure 2*C*), and VIP-reactive (Figure 5*C*) nerve fibers occurred abundantly in the circular muscle. In most fields, the density of NPY- (Figure 3*C*) and SP-reactive (Figure 4*C*) nerve fibers was lower than that of the preceding three peptides, as it was in the longitudinal muscle. However, Figure 4*C* shows a higher than usual density. Leucine-enkephalin fibers were sparsely present in circular muscle. Methionine-enkephalin fibers, which were absent in the longitudinal muscle, were present in the circular muscle (Figure 6*C*) in generally small numbers. Somatostatin- and BOM-reactive fibers, which were sparsely present in the longitudinal muscle, were absent in the circular muscle, as was CCK.

Myenteric Plexus

The well-developed myenteric plexus lay between the longitudinal and circular muscle layers. Immunofluorescence observations on the neuronal somata were followed by poststaining with thionin to ascertain their histological identity. Immunocytochemical observations on neurons/ganglia were tabulated under two heads: for neuronal somata and for nerve terminations on them. A given peptide may be present in somata alone, terminations alone, in both, or be absent altogether. Neuronal somata of the myenteric plexus contained CGRP (Figure 1*B*), GAL (Figure 2*B*), NPY (Figure 3*B*), SP (Figure 4*B*), and VIP (Figure 5*B*). Whereas most somata in these illustrations are positively fluorescent, the actual distribution of positive somata was variable from ganglion to ganglion. Negative somata in a largely positive ganglion could be discerned as dark areas that later stained with thionin or as dark areas containing bright, positive terminations. The preceding five peptides occurred in terminations as well as in somata, and neurons positive for these five peptides were numerous. Methionine-Enkephalin– (Figure 6*B*) and SOM-containing neurons occurred less frequently, but they also had terminations positive for these peptides. There were few L-ENK– and BOM-positive neurons in the myenteric plexus, and these peptides did not occur in any terminations on neurons. No CCK-containing myenteric neurons were seen.

Muscularis Mucosae

The innervation of the muscularis was somewhat similar to that of the other two muscle layers in that it had moderate to abundant supplies of CGRP (Figure 1*D*), GAL (Figure 2*D*), NPY (Figure 3*D*), and VIP (Figure 5*D*) fibers. Fibers immunoreactive for SP (Figure 4*D*) were fewer than those of the preceding

Figure 4. Substance P. (*A*) Longitudinal muscle showing a particularly long SP fiber (*arrowhead*). (*B*) Substance P in a myenteric ganglion showing positive somata (one with *large arrow*) and small, bright terminations (*small arrows*). (*C*) Substance P in the circular muscle; *arrowhead* points to one fiber. (*D*) Substance P innervation of muscularis mucosae; one fiber bears an *arrowhead*. (*E*) Substance P–like immunoreactivity in a submucous ganglion. Both neuronal somata (one with a *large arrow*) and numerous terminations (two, *small arrows*) are brightly positive.

Figure 5. Vasoactive intestinal polypeptide. (*A*) Vasoactive intestinal polypeptide innervation of the longitudinal muscle. Tissue was not exactly flat. Numerous fibers can be seen; *arrowheads* point to two. (*B*) Vasoactive intestinal polypeptide in the myenteric plexus; a single neuron (*arrow*) and part of a fascicle adjacent to it are sectioned. Varicosities, although present, have not recorded in this plane of focus. (*C*) Vasoactive intestinal polypeptide innervation of circular muscle. The *arrowhead* points to one of numerous fibers. (*D*) Vasoactive intestinal polypeptide fibers in the muscularis mucosae (one with *arrowhead*). (*E*) Submucous neurons showing somata (one with a *large arrow*) and terminations (two, *small arrows*) brightly positive for VIP.

Figure 6. Methionine-enkephalin. (*A*) Absence of M-ENK in the longitudinal muscle. (*B*) Two groups of myenteric neurons showing numerous brightly M-ENK–positive terminations (*small arrows*) upon somata, which are themselves not as brightly fluorescent. (*C*) Circular muscle showing the presence of M-ENK–positive fibers (*arrowheads*). (*D*) Muscularis mucosae showing absence of M-ENK immunoreactivity. The bright spots show nonspecific fluorescence.

four peptides. There were no L-ENK–, M-ENK–, (Figure 6*D*), BOM-, SOM-, and CCK-containing fibers in the muscularis mucosae.

Submucous Plexus

The human esophageal submucous plexus is far less developed than the myenteric plexus. Ganglia were seen to be smaller and more widely scattered. It was possible to examine many serial sections without encountering a single submucous neuron. However, when these were seen, their identity was never in doubt because of their location and metachromatic staining with thionin. Calcitonin gene-related peptide– (Figure 1*E*), GAL- (Figure 2*E*), SP- (Figure 4*E*), and VIP-like (Figure 5*E*) immunoreactivity occurred abundantly in terminations and somata of submucous neurons, whereas NPY (Figure 3*E*) did not. However, numerous NPY-positive terminations (Figure 3*E*) were seen upon neuronal somata not positive for NPY. A few SOM-containing neurons were present in the submucous plexus. There were no L-ENK–, M-ENK–, BOM-, or CCK-containing neurons.

These observations are summarized in Table 1.

Discussion

This study shows that terminal innervation in all the muscle layers of the human esophagus is immunoreactive for CGRP, GAL, NPY, SP, and VIP. However, the neuronal origin of the nerve endings cannot be determined by these studies.

Calcitonin gene-related peptide–reactive fibers have been identified in longitudinal and circular muscle and myenteric and submucous plexuses of the esophagus in the cat (13), rat (13,21), opossum (14), and monkey (13). Calcitonin gene-related peptide–reactive nerve fibers have also been found in the small intestine in the cat, dog, guinea pig, pig (22), and rat (21,23). The present studies show abundant CGRP-reactive nerves also in the human esophagus. Studies using chemical or surgical ablation in animals have shown that many of the CGRP-reactive endings belong to neurons located outside the gut (24). The present study shows that in the human esophagus there are CGRP-reactive intramural neurons, raising the possibility that some CGRP-reactive nerve terminals may represent a noncholinergic nonadrenergic innervation from the intramural nerves to the esophageal smooth muscle. Intrinsic CGRP-immunoreactive neurons have been found in the esophagus in monkeys (13) and opossums (14) and in the small intestine of experimental animals (22). Pharmacological studies have shown that CGRP has a direct inhibitory action on the lower esophageal sphincter in the opossum (14). Moreover, CGRP is 400 times more potent than VIP, which is another potential inhibitory neurotransmitter in the lower esophageal sphincter (14).

Vasoactive intestinal polypeptide–immunoreactive nerves to both muscle layers of the esophagus have been found in rat (6), opossum (4), and humans (10) and in the small intestine of many animal species examined (25–28). Vasoactive intestinal polypeptide is contained in nerves with extrinsic cell bodies as well as in endings of intrinsic neurons. However, there are no studies on the effect of extrinsic denervation on VIP-reactive nerve endings. Vasoactive intestinal polypeptide is considered to be an important inhibitory neurotransmitter and causes relaxation of the lower esophageal sphincter. Antagonism of VIP has been shown to cause antagonism of neurally mediated LES relaxation, suggesting that VIP may be one of the inhibitory neurotransmitters (29). However, the effect of VIP in the esophageal body may be excitatory (30). Vasoactive intestinal polypeptide–immunoreactive nerves have been shown to be decreased in esophageal specimens in achalasia (10). However, it is not known if such a reduction is selective for VIP. Endings positive for VIP were also found on myenteric and submucous neurons, which is consistent with the effects of VIP via intramural neurons (31).

Substance P–reactive nerve endings have been reported in the longitudinal and circular muscle layer of esophagus and small intestine of all the animal species studied. Substance P–reactive nerve endings have been reported in the myenteric plexus in the esophagus of the guinea pig and opossum and myenteric and submucous plexuses in dog, pig, and rat small intestine (5,7,24,26–28,32,33). The present study shows that SP-reactive endings are also present on the myenteric and submucous neurons, consistent with the neurally mediated actions of SP (31). Substance P

Table 1. Summary of Observations

	Neurons		Nerve terminations				
Peptides	MN	SN	LM	MN	CM	MM	SN
CGRP	++	++	+++	++	+++	+++	++
GAL	++	++	+++	++	+++	+++	++
NPY	++	0	++	++	++	+++	++
SP	++	++	++	++	++	+	++
VIP	++	++	+++	++	+++	+++	++
M-ENK	+	0	0	+	+	0	0
L-ENK	+	0	+	0	+	0	0
SOM	+	+	+	+	0	0	0
BOM	+	0	+	0	0	0	0
CCK	0	0	0	0	0	0	0

0, Absent; +, ++, and +++, increasing degrees of subjective positivity.

MN, myeuteric neuron; SN, submucous neuron; LM, longitudinal muscle; CM, circular muscle; MM, muscularis mucosae.

also exerts a direct excitatory effect on the opossum esophageal smooth muscle and may serve as a noncholinergic excitatory neurotransmitter in the esophagus (34,35). Other studies have shown that SP is an important transmitter of afferent nerves, particularly the primary unmyelinated afferents, because extrinsic denervation is associated with reduction in SP-reactive nerve terminals (24).

Galanin-reactive fibers have been reported in the esophagus of the guinea pig, pig, and opossum (15,16) and in the small intestine of the guinea pig, pig, rat, and human (15,36,37). The present study shows that galanin-reactive fibers are also present in all muscle layers of the human esophagus.

Galanin is present in about 15% of the neuronal population of pig nodose ganglia, and the ganglion's extractable galanin content is only one ninth that of the ileum (38). In the guinea pig ileum, all the galanin is intrinsic, as shown by ablation studies (37). The galanin-positive endings on intramural neurons may represent endings of interneurons. Galanin-reactive nerve endings may have excitatory motor effects on the smooth muscle. In the opossum esophagus, galanin causes dose-dependent contraction of the lower esophageal sphincter (39,40). Galanin has been shown to modify vagally stimulated contraction in the esophagus (41) and may act by modulatory release of other neurotransmitters (42).

Neuropeptide Y–reactive fibers have been reported in all muscle layers and in the myenteric and submucous plexuses in the small intestine of pig and rat (25). In the opossum esophagus it is present only in the submucous plexus (19). In contrast, NPY was widely distributed in nerves to all muscle layers of the human esophagus. It is known that cell bodies of neurons reacting for NPY are present in sympathetic ganglia and enteric ganglia, but only few NPY neurons are present in the afferent neurons in the dorsal root and nodose ganglia (31). There are no reported studies of extrinsic denervation on NPY-reactive endings in the esophagus. However, NPY-reactive intrinsic neurons are present in animals (28) and humans (26). Thus, NPY-containing nerves in the human esophagus may arise from intrinsic neurons. The effects of NPY on the smooth muscle are mainly indirect. In the feline lower esophageal sphincter, NPY causes neurally mediated excitation followed by inhibition. The NPY excitation is mediated by stimulation of adrenergic neurons and relaxation by stimulation of inhibitory neurons (43). Neuropeptide Y is also known to act by inhibiting cholinergic neurons. Studies in the human esophagus have shown that there is complete colocalization of NPY in the VIP-reactive nerves (26). Thus, it is possible that the role of NPY endings is to presynaptically modulate the release of VIP from the same nerves. Neuropeptide Y has also been shown to augment α-adrenergic contractions of the LES (43).

Only a few nerves showing immunoreactivity for somatostatin, L-ENK, and BOM are present in the human esophageal longitudinal muscle. Methionine-enkephalin and L-ENK nerves are present in the circular muscle. The possible role of these nerves in the esophageal longitudinal muscle is not clear. Leu-enkephalin has been shown to inhibit neurotransmitter release and may act as a modulatory peptide (44) for esophageal circular muscle contraction.

In summary, CGRP, galanin, NPY, SP, and VIP are abundantly present in the human esophageal nerves. These peptides may be present in different afferent and efferent nerves and may serve several neurotransmitter functions. Calcitonin gene-related peptide and VIP are candidates as inhibitory and SP as an excitatory transmitter. Neuropeptide Y, the enkephalins, and galanin may serve as modulatory neurotransmitters. These morphological studies show no profound differences in the nature of innervation of longitudinal and circular muscle layers and the muscularis mucosae despite the reported major functional differences in the neurally mediated responses of these muscles (1).

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