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PAPERS

Nitrinergic and peptidergic innervation of the human oesophagus

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Abstract

The distribution, colocalisation, and interconnections of nitrinergic and peptidergic neurons and nerves in the human oesophagus were examined. Cryosections of surgically resected tissues from eight subjects were studied with indirect immunofluorescence for the presence of 11 neuropeptides and neuron specific enolase. After immunohistochemistry, nitric oxide synthase was shown on the same sections with the β nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemical reaction. The histochemical findings were verified immunohistochemically on other sections with an antiserum against nitric oxide synthase. Most myenteric neurons (55%) were nitrinergic. Most (96%) received terminations positive for vasoactive intestinal polypeptide (VIP), calcitonin gene related peptide (CGRP) (80%), and galanin (59%). The neuronal somata of 14% also contained VIP, while 10% had galanin. Of the NADPH-diaphorase containing fibres seen in the muscle layers, many had closely associated VIP and galanin, but only rarely CGRP and substance P. Thus, despite abundant representation of both peptidergic and nitrinergic systems in oesophageal smooth muscle, only VIP and galanin colocalised to any significant extent with the nitrinergic elements. These findings provide morphological support for the role of nitric oxide as the non-adrenergic non-cholinergic inhibitory mediator in the human oesophagus and for its possible interactive role with the peptidergic system.

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The chemical nature of the nerves responsible for relaxation of the oesophageal smooth muscle, lower oesophageal sphincter, latencies of oesophageal peristaltic contractions, and oesophageal 'off' contractions is not known. As the mediator of these nerves is neither an adrenergic nor a cholinergic substance, they are categorised as non-adrenergic non-cholinergic (NANC) nerves.[1] Functional studies have suggested that the peptides, vasoactive intestinal polypeptide (VIP), and calcitonin gene related peptide (CGRP) may be candidate NANC inhibitory neurotransmitters in the oesophagus.[2–4] Morphological studies in the human oesophagus and in experimental animals show abundant VIP and CGRP in the myenteric plexus, and VIP and CGRP positive fibres in the muscle layers.[5–12]

It has now been shown that nitric oxide or a related product of the L-arginine-nitric oxide-synthase pathway may participate in the oesophageal smooth muscle relaxation, latencies of oesophageal contraction, and oesophageal off contraction in the opossum as well as in humans.[13–16] Furthermore, the involvement of the nitric oxide synthase pathway in swallow induced peristaltic contractions in the opossum have been shown.[17] Also, electrophysiological studies support the role of nitric oxide as the inhibitory mediator in the oesophageal smooth muscle.[18–23] In sharp contrast, there is no morphological information on the nitrinergic innervation of the human oesophagus.

It is possible that the inhibitory neurotransmitters VIP and CGRP may act in concert with nitric oxide in the oesophageal smooth muscle. In some tissues neurally released VIP is thought to exert its effect by releasing nitric oxide from the smooth muscle.[24] Inhibition of nitric oxide synthase has been shown to enhance CGRP induced relaxation in vascular smooth muscle.[25 26] It is known, however, that at least in the oesophageal smooth muscle, the inhibitory action of VIP or CGRP does not involve nitric oxide.[14] Recently nitric oxide synthase activity has been reported to be colocalised in the VIP reactive neurons in the guinea pig ileum.[27]

The aims of this study are to characterise the nitrinergic innervation of the human oesophagus by (a) identifying nitric oxide synthase containing neurons and nerve fibres using the NADPH diaphorase reaction as well as a specific antiserum against nitric oxide synthase; (b) to investigate the colocalisation of NADPH diaphorase and various peptides in myenteric neurons; and (c) to evaluate the presence and colocalisation of nitrinergic fibres with various peptides in the external muscle layer of the human oesophagus.

Methods

Tissues were obtained from the lower oesophagus of eight subjects (five male and

three female) aged 38–66, who had surgical resections for various malignancies. The tissue samples were obtained from between five and 15 cm proximal to the squamocolumnar junction. These patients had no motility disorders and were also free of diabetes, scleroderma, and other degenerative neurological disorders. Tissues were taken from sites at least 4 cm away from areas showing the presence of tumour cells as shown by cryosectioning and rapid haematoxylin and eosin staining. The mucosa delaminated or was removed by peeling in most cases. Fixation was by immersion in cold buffered picro-formaldehyde followed by thorough rinsing in phosphate buffered saline and overnight immersion in 15% sucrose enriched phosphate buffered saline. Tissues from each patient were freeze blocked in OCT Tissue-Tek (Miles Diagnostics, Elkhart, IN) in two orientations so as to permit cross sections of both longitudinal and circular muscle layers. Tissues from five of these eight patients have also been used in a previous study on oesophageal peptidergic innervation.[12]

Several sets of slides were prepared from each patient. Each set contained about 15 slides and each slide bore at least five 16 μm thick cryosections. One slide from each set was initially incubated at 4°C for four hours with polyclonal rabbit antiserum samples raised against cholecystokinin, CGRP, galanin, gastrin releasing peptide, leucine enkephalin (L-ENK), methionine enkephalin (M-ENK), neuropeptide Y, peptide YY, somatostatin, substance P, and VIP (all from Peninsula Labs, Belmont, CA). Details of antiserum samples and their dilutions are as reported previously.[28] Rabbit anti-neuron specific enolase (Polysciences, Warrington, PA) was used similarly at 1:1000. After thorough rinsing, the second incubation was done in goat antirabbit fluorescein isothiocyanate (Calbiochem, La Jolla, CA) (1:60, one hour, room temperature). All immunostaining was carried out with a Shandon 'Cadenza' automated immunostainer (courtesy Shandon, Pittsburgh, PA). Sites of antigenic binding were visualised under epifluorescence with an Olympus BHT-2 research microscope (Olympus, Tokyo, Japan).

After immunocytochemistry, the sections were rinsed and subjected to a histochemical examination of NADPH diaphorase. After thorough rinsing in phosphate buffered saline, they were incubated with 0·25 mg/ml nitro blue tetrazolium+1 mg/ml β nicotinamide adenine dinucleotide phosphate, reduced, tetrasodium salt (NADPH) (both from Sigma Chemical, St Louis, MO)+0·5% Triton X-100 in 0·1 M TRIS buffer, pH 7·6, for 15–20 minutes at 37°C.[27] The reaction was stopped by washing in tap water. The method stains sites of NADPH diaphorase, indicative of nitric oxide synthase, a dark blue.[29–31] Sections were then coverslipped in carbonate buffered glycerol, and enzyme and antigen positive structures were counted (respectively under brightfield and fluorescence) and photographed.

Several sections from three oesophaguses were incubated with an antiserum against nitric oxide synthase (Gift of Snyder and Dawson of John Hopkins University). This polyclonal antiserum was raised against the C terminal 14 amino acid sequence to neuronal nitric oxide synthase and has been shown previously to be specific for all neuronal nitric oxide synthase including that in the gastrointestinal tract. The lyophilised antiserum was diluted 1:50 and incubated with 4% paraformaldehyde fixed sections for 24 hours at 37°C. This was followed by an antirabbit fluorescein isothiocyanate conjugate. Most of these sections were then stained for NADPH diaphorase as already described to find out if both methods stained the same structures.

As the sharing of immunodeterminant sequences by more than one moiety cannot theoretically be excluded, a statement in this paper such as 'CGRP immunoreactive' or 'CGRP positive' means that tissue showed CGRP like immunoreactivity. Except for the colocalisation experiments with NADPH diaphorase and the nitric oxide synthase antiserum, all other findings were made using the NADPH diaphorase histochemical reaction. NADPH diaphorase histochemistry was chosen over nitric oxide synthase antibody in colocalisation studies because the bright blue reaction particle could easily be studied along with peptide immunofluorescence in quantitative studies with both small amount of bright light as well as the ultra violet light and only occasionally was there a need to shut one or the other light completely off.

QUANTITATION

After the myenteric plane was identified, the total number of NADPH diaphorase positive neurons was counted per 20× field using brightfield transillumination. Fluorescence epi-illumination was then used on the same field to enumerate the peptide (or enolase) positive neurons. Because the blue stain for NADPH diaphorase was visible against the faint fluorescence background, it was possible to count neurons negative for NADPH diaphorase but positive for various peptides. Another field was then selected until a count of one 'unit' (100 or more NADPH diaphorase positive neurons) was reached. The peptide neuronal counts were then normalised to 100 NADPH diaphorase neurons. At least five 'units' (500 nitrinergic neurons) were counted from each slide, and at least four slides were counted from each patient for each peptide examined. A simple mean was calculated from each patient and the standard deviation and means from eight patients were calculated by the single Student's *t* test on a Minitab statistical program (State College, PA).

CONTROLS

A series of controls were carried out. The sequence was reversed for a few experiments (enzyme reaction first, immunohistochemistry second), and counts taken as before to rule out

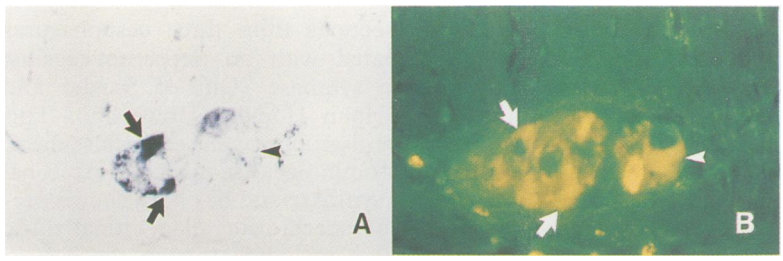

Figure 1: The same field showing the human oesophageal intermyenteric plane under brightfield illumination after staining histochemically for NADPH diaphorase (A) and immunohistochemically for neuron specific enolase (B). All neurons that stained for NADPH diaphorase also stained for neuron specific enolase (some arrowed). Enolase stained showed additional neurons that did not stain for NADPH diaphorase (arrowheads).

the theoretical objection that immunohistochemistry for peptides may compromise the subsequent enzymatic reaction. In these experiments identical results were obtained regardless of the sequence.

Several sections 'kissing sections' from two oesophaguses were studied for the theoretical objection that the NADPH diaphorase staining may be interfering with the number of peptide containing neurons. On one of these mirror image sections only NADPH diaphorase reaction was performed and the other section underwent both NADPH diaphorase as well as immunohistochemistry reaction to VIP. Several areas from each section was carefully evaluated for the number of VIP positive neurons. These studies showed that there is no decrease in the number of peptide neurons when the sections are simultaneously stained for NADPH diaphorase.

One step controls for the enzyme reaction were performed (elimination of NADPH or nitro blue tetrazolium salt), which yielded negative results, confirming the validity of the enzymatic localisation.

The substitution of NADH for NADPH totally abolished the staining.[5]

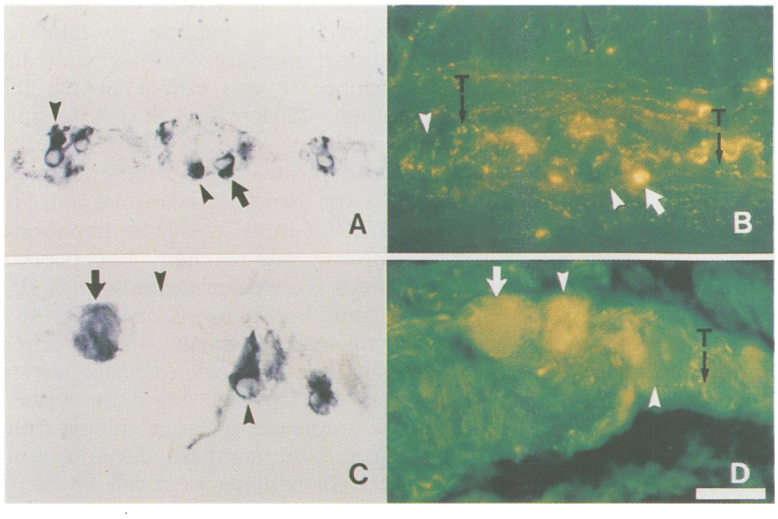

Figure 2: The same field showing NADPH diaphorase staining (A) and immunofluorescent localisation of VIP (B). Arrows point to neurons that show both kinds of positivity, while arrowheads point to those that are only nitrinergic. T-peptide terminations on NADPH diaphorase positive, VIP negative neurons. (C) and (D) The same field from oesophageal intermyenteric plane stained histochemically for NADPH diaphorase (C) and immunohistochemically for galanin (D). Arrows point to neurons containing both substances, arrowheads to those that have only NADPH diaphorase or galanin. Note also the galanin containing terminations (T) on some nitrinergic neurons (D). Bar=50 μm and applies to all parts of Figures 1 and 2, except for 1(A), 2(A), and 2(B), where it equals 100 μm.

Testing for specific time and temperature for the NADPH histochemical reaction showed that the specific staining of the neurons that colocalises with nitric oxide synthase antibody staining was seen only between 15–30 minutes at 37°C. Incubation longer than 30 minutes or other temperatures tested resulted in non-specific staining of several neurons and other non-neuronal structures.

All immunohistochemistry results were subjected to omission of the first antibody that yielded negative reactions except for non-specific background.

All immunohistochemistry reactions for peptides were confirmed by preabsorption of their antiserum samples with their respective antigens (10 and 100 μg/ml working strength primary antiserum), with negative results.

The research protocol has been approved by the committee on clinical investigations, Beth Israel Hospital, and the human subject committee of the University of Wisconsin Hospitals.

Results

MYENTERIC NEURONS

NADPH diaphorase positive myenteric neurons were clearly identifiable by their sharply defined dark blue cytoplasmic stain with almost no background; the nuclei appeared as colourless 'holes'. They were abundantly present in the oesophageal myenteric plexus (Fig 1). For every 100 neurons counted with neuron specific enolase (total neuronal population), mean (SEM) 55·8 (14·8) were NADPH diaphorase positive. Experiments for colocalisation with peptides yielded clearly differentiable populations of NADPH diaphorase positive, NADPH diaphorase and peptide positive (Fig 2) and only peptide positive neurons.

For every 100 NADPH diaphorase neurons, there were 27·4 (11·6) VIP, 34·9 (9·3) galanin, 21·6 (7·5) CGRP, and 13·9 (6·4) substance P neurons. Of every 100 NADPH diaphorase neurons counted, 14·1 (3·5) also contained VIP, while 10·5 (1·6) contained galanin (Fig 3). Only VIP and galanin colocalised to any significant extent with NADPH diaphorase positive neurons, while other prominently present peptides such as CGRP, substance P, and neuropeptide Y did not. Other peptides such as M-ENK and somatostatin colocalised with less than 1% of the nitrinergic neurons.

TERMINATIONS ON MYENTERIC NEURONS

Many NADPH diaphorase positive neurons received terminations containing peptides. For every 100 NADPH diaphorase positive neurons counted, 92·4 (7·3) received terminations containing VIP (Fig 2), 79·3 (4·8) received terminations with CGRP, and 64·4 (16·5) received galanin immunoreactive terminations (Fig 4). These were seen as bright fluorescent points attached to the somata of the neurons in question, and often

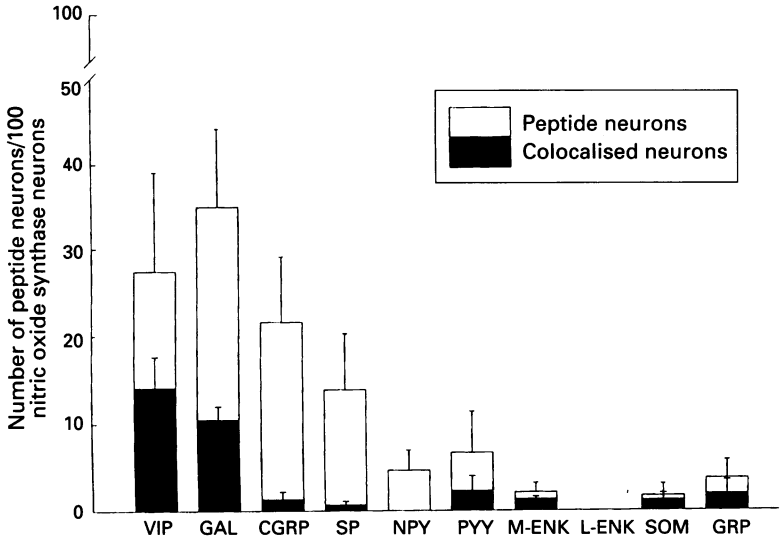

Figure 3: Numbers of neurons containing various peptides in human oesophageal myenteric plexus and numbers of peptide containing neurons colocalised with NADPH diaphorase positive neurons per area containing 100 NADPH diaphorase positive neurons. GAL=galanin, SP=substance P, NPY=neuropeptide Y, PYY=peptide YY, SOM=somatostatin, GRP=gastrin releasing peptide.

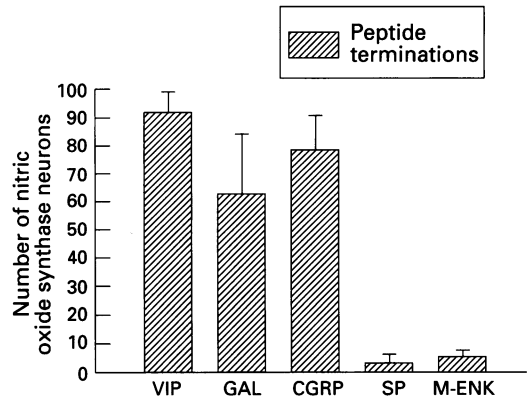

Figure 4: Numbers of NADPH diaphorase positive neurons, per 100 counted, which received terminations from various peptides. Note that many nitrinergic neurons received terminations containing several peptides. Abbreviations the same as in Figure 3.

their fibres were visible for a short distance (Figs 2B and 2D). Substance P (0·9 (0·8)) and M-ENK (0·7 (0·3)) also sent terminations to somata of nitrinergic neurons, but neuropeptide Y, peptide YY, L-ENK, somatostatin, gastrin releasing peptide, and cholecystokinin provided very few, if any, terminations on NADPH diaphorase positive neurons.

The peptidergic neurons in turn received NADPH diaphorase positive terminations, but these were difficult to study with as much clarity because of the presence of a bright 'halo' effect of immunofluorescence seen around the brightly stained neurons. It seemed subjectively that most peptidergic neurons received NADPH diaphorase positive fibres and terminations upon their somata.

FIBRES IN CIRCULAR MUSCLE

The human oesophageal circular muscle showed abundant NADPH diaphorase innervation in the form of varicose fibres. Several of these fibres were associated with VIP (Fig 5) and galanin. CGRP and substance P, even though significantly present in the circular muscle, were only rarely seen in close association with the nitrinergic fibres.

FIBRES IN LONGITUDINAL MUSCLE

The density of fibres containing NADPH diaphorase positive fibres seemed to be less than in circular muscle. Consistent with our earlier findings very few fibres containing L-ENK, somatostatin, and gastrin releasing peptide were found in the longitudinal muscle. CGRP, galanin, substance P, and neuropeptide Y were found but none of them were seen to be in any close association with the NADPH diaphorase positive fibres in all oesophaguses studied. Several of the VIP and galanin positive fibres similar to that seen in the circular muscle were once again found to be closely associated with the nitrinergic fibres.

SUBMUCOSAL NEURONS AND FIBRES

The submucosal region could not be included in this report as it tends to delaminate from the muscularis externa before fixation, and in many cases was separated to permit good sectioning of the thick oesophageal musculature. From fragments attached to the luminal face of the circular muscle, however, it can be said that submucous neurons in general tended to be less frequently NADPH diaphorase positive

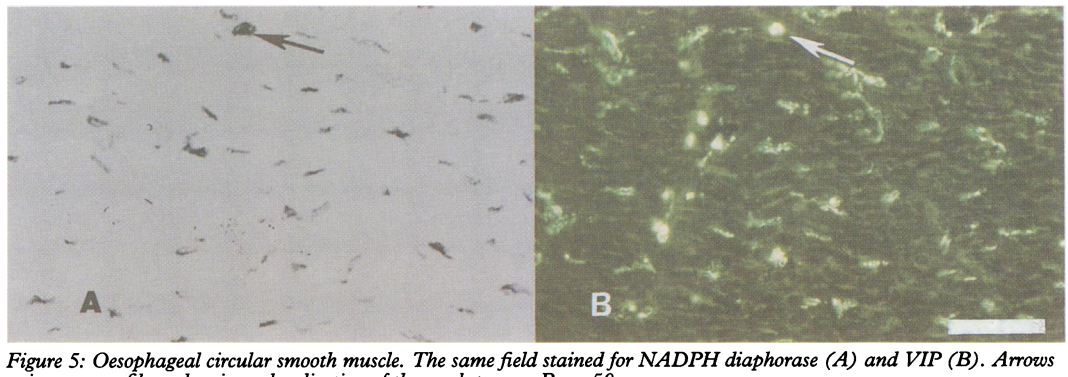

Figure 5: Oesophageal circular smooth muscle. The same field stained for NADPH diaphorase (A) and VIP (B). Arrows point to some fibres showing colocalisation of these substances. Bar=50 μm.

than myenteric neurons, and nitrinergic fibres, though unquestionably present, seemed to be infrequent.

NITRIC OXIDE SYNTHASE AND NADPH DIAPHORASE STAINING

The antibody to nitric oxide synthase stained several of the myenteric neurons in the human oesophagus. All these neurons were also stained by the NADPH diaphorase reaction. Also all the neurons that stained positively for NADPH diaphorase were positively immunostained by the anti-nitric oxide synthase (result not shown). This close association was verified in at least 100 neurons from three subjects.

STAINING IN THE MUSCLE

No specific staining was seen in the muscle fibres using NADPH diaphorase histochemical reaction or using the antiserum against nitric oxide synthase under our experimental conditions. All the cells, however, including the muscles showed non-specific blue staining of varying intensity on prolonged incubation with NADPH and nitro blue tetrazolium salt.

Discussion

This study used neuron specific enolase staining as a marker for all myenteric neurons, NADPH diaphorase staining as a marker for nitric oxide synthase in colocalisation experiments, and the standard fluorescent antibody technique to localise VIP, CGRP, galanin, and other peptides. Though the precise function of neuron specific enolase remains unclear, it is known to stain neurons and their glial cells, and is accepted as a functional marker for neuronal differentiation.[32]

The NADPH diaphorase staining technique has long been used to stain certain populations of neurons.[33] NADPH is essential for the activity of nitric oxide synthase, which participates in neurotransmission and neuromodulation. It was shown recently that neurons accessible to immunostaining with antibodies against nitric oxide synthase also stain with the NADPH diaphorase reaction.[29 30] Although enzymes other than nitric oxide synthase possess diaphorase activity, studies have shown[31 33] that all neurons in the central and peripheral nervous system that stain for diaphorase activity also show nitric oxide synthase activity. It is thus evident that NADPH diaphorase staining under these conditions provides a reliable marker for nitrinergic (nitric oxide synthase containing) neurons and their fibres. Also our experiments on colocalisation of NADPH diaphorase and anti-nitric oxide synthase staining in the human oesophagus corroborate the close association that we have seen between these two kinds of staining in several other tissue systems.

Certain limitations upon technique are imposed because this is a human study. For instance, pretreatment with colchicine was not possible. It was also not practical to make whole mounts (peels), and thus distribution was studied from sections alone. Despite these limitations, animal studies have shown that the immunohistochemical reactions of peptides correlate very closely with results of in situ hybridisation for mRNAs of certain peptides such as VIP and substance P. Thus in human material, even though a statement regarding the positivity of a neuron for a given peptide can be made with certainty, the absence of a peptide in a particular neuron should be taken with caution.

Most interestingly, VIP (96%) and CGRP (80%) terminations were seen on the nitrinergic neurons. On the other hand only 14% of the NADPH diaphorase neurons also had VIP and only 3% of the neurons had CGRP in them. The abundance of these terminations suggests a regulatory system, where VIP and CGRP may act by controlling the release of nitric oxide from nitrinergic neurons. VIP and CGRP have been presumed to serve such a function. VIP has been shown to release nitric oxide from within the smooth muscle cells, however, but not from the nerve fibres.[24] The site at which CGRP releases nitric oxide is not known. In any case, in the opossum oesophageal body, inhibitors of nitric oxide synthase do not modify the inhibitory action of either VIP or CGRP.[20] Further physiological studies are needed to explain the functional significance of the morphological interconnection that are described in this study.

This study shows that NADPH diaphorase positive neurons are widely distributed in the myenteric ganglia of the human oesophagus. Moreover, abundant NADPH diaphorase positive nerve fibres were seen to supply the oesophageal circular muscle. These findings provide a morphological support for functional studies[13 14] suggesting that a product of the L-arginine-nitric oxide synthase pathway may be an NANC inhibitory neurotransmitter in the oesophagus. These experiments showed that inhibition of nitric oxide synthase by analogues of L-arginine (for example, L-NNA and L-NAME) caused (a) a decrease in the latency and amplitude of the oesophageal 'off' contraction; (b) a decrease in the latencies of contraction during physiological swallowing in opossums, and (c) antagonism of hyperpolarisation of the oesophageal smooth muscle, which follows electrical stimulation of the intramural nerves.

Nitrinergic neurons accounted for somewhat more than half of the total myenteric neurons identifiable by neuron specific enolase staining. The other clearly non-nitrinergic neurons may include cholinergic and peptidergic neurons. The comparative abundance of the total peptidergic neuron population in contrast with the nitrinergic one is difficult to estimate because (a) peptides colocalise with nitrinergic neurons, as shown by this study, and (b) when a neuron is purely peptidergic, it may contain a multiplicity of peptides. This study did not include triple localisation for two peptides and the NADPH diaphorase reactive neurons. This study shows, however, that nitrinergic neurons were five times as

abundant as VIP neurons and six times as abundant as CGRP neurons. On the other hand, the number of nitrinergic nerve fibres innervating the oesophageal circular muscle was similar to that of some of the peptide fibres examined such as VIP and CGRP fibres. It is not clear whether this difference in the comparative proportions of nitrinergic, VIP containing, and CGRP containing neurons and fibres is significant from a physiological point of view.

Colocalisation studies on the myenteric neurons yielded information on the coexistence and interconnection of the peptidergic and nitrinergic systems of the oesophagus. Our findings showed that most of the nitrinergic neurons received fibre terminations from CGRP and VIP neurons. About 50% of these neurons received terminations from galanin neurons, but had no input from substance P, the enkephalins, or the other peptides examined. The nitrinergic terminations on an NADPH diaphorase negative, peptide positive neuron could readily be identified. In neurons with both peptide and NADPH diaphorase positivity, it was difficult to identify the peptide termination, especially if the termination happened to lie directly upon the neuron. In most of the neurons, however, the terminations were extensive and usually could be seen all around the soma. Higher resolution using electron microscopy is needed to confirm these findings. The reverse condition also exists: nitrinergic fibres terminated upon the somata of peptidergic neurons, but they were not as easily studied, and could not be quantitated.

In contrast, about half of the total population of VIP positive neurons contained NADPH diaphorase activity. Similarly, galanin and NADPH diaphorase were colocalised in neuronal somata. There was no colocalisation of NADPH diaphorase activity with CGRP. Interestingly, VIP and nitric oxide were also colocalised in about 50% of the VIP positive fibres. Colocalisation of NADPH diaphorase and VIP has been reported in guinea pig ileum.[27] These findings suggest the possibility of a simultaneous (non-cascade) action by peptides and nitric oxide as cotransmitters. Clearly, further work is needed to fully define the functional significance behind nitrinergic and peptidergic NANC neurons in the oesophagus.

Physiological studies have suggested a significant role for nitric oxide in the longitudinal muscle of the gastrointestinal tract.[15 18 20] Our findings of very few nitrinergic fibres in the longitudinal muscle compared with that of the circular muscle may implicate a limited role of nitrinergic innervation in the longitudinal muscle layer in the human oesophagus.

Neither the nitric oxide synthase antiserum nor the diaphorase reaction stained the muscle cells under the conditions specified here. This cannot be sufficient, however, to exclude the possibility that muscle cells may produce nitric oxide. This could happen by other forms of nitric oxide synthase that are not detectable by the present two methods, or the amount of nitric oxide synthase present here may be below the threshold values needed for either method.

In summary, these findings show that NADPH diaphorase positive neurons are widely distributed in the myenteric plexus and that nitrinergic fibres innervate both circular and longitudinal oesophageal muscle. The nitrinergic neurons constitute more than half of the myenteric neuronal population. VIP occurs in about a seventh of these neurons, galanin in about a tenth, and CGRP in none. These nitrinergic neurons receive input from VIP and CGRP neurons, and peptidergic neurons receive input from nitrinergic neurons. The detailed functional significance of these findings remains to be elucidated.

The kind gift of the nitric oxide synthase antiserum provided by Drs Solomon Snyder and Ted Dawson of Johns Hopkins University is gratefully acknowledged. Part of this study has been presented at the annual meeting of the American Gastroenterological Association in Boston in May, 1993 and appears as an abstract in *Gastroenterology* 1993; **104:** A583.

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