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GABA and nitric oxide synthase immunoreactivities are colocalized in a subset of inhibitory motor neurons of the guinea-pig small intestine

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Abstract. Simultaneous immunofluorescence labelling was used to determine the patterns of colocalization of immunoreactivity for γ-aminobutyric acid (GABA-IR) with immunoreactivity for nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP) and tachykinins (TK) in nerve cells and fibres of the guinea-pig small intestine. GABA-IR nerve cell bodies were located in the myenteric plexus and varicose fibres innervated the circular and longitudinal muscle, but did not form pericellular endings in the myenteric ganglia. GABA-IR nerve cells comprised 4–5% of all nerve cells in the myenteric ganglia. Of GABA-IR myenteric nerve cells, about 85% had NOS-IR and of GABA-IR nerve fibres in both muscle layers, about 75% were NOS-IR. Conversely, 20% of NOS-IR nerve cells were GABA-IR. About 6% of GABA-IR nerve fibres innervating the circular muscle, but none innervating the longitudinal muscle, were TK-IR. Most GABA-IR fibres supplying the circular muscle, but none of those supplying the longitudinal muscle, were VIP-IR. From this study, and previous studies of projections of enteric neurons, it is concluded that most GABA-IR neurons in the guinea-pig small intestine are inhibitory motor neurons that also contain NOS-IR. A small proportion represents anally directed excitatory motor neurons that innervate the circular muscle and are also immunoreactive for TK.

Key words: Enteric nervous system – Small intestine – Inhibitory motor neurons – γ-Aminobutyric acid – Nitric oxide synthase – Guinea-pig

Introduction

The most comprehensive description of the chemical coding, projections and connections of neurons forming intrinsic circuits within the intestine exists for the guinea-pig small intestine. Amongst these neurons are several populations of motor neurons that supply the longitudinal and circular muscle layers, including both inhibitory and excitatory motor neurons that innervate the circular muscle (Bornstein et al. 1986; Smith et al. 1990), and excitatory motor neurons that innervate the longitudinal muscle (Ambache and Freeman 1968; Hirst et al. 1975). The longitudinal muscle also receives a minor inhibitory innervation (Bauer and Kuriyama 1982; Osthaus and Galligan 1992).

Retrograde labelling and electrophysiological analysis both suggest that the majority of circular muscle motor neurons projects for short distances along the intestine before innervating the muscle (Bornstein et al. 1986; Smith et al. 1988; Brookes et al. 1991). Pharmacological analysis of excitatory transmission to the muscle indicates that acetylcholine (ACh) and substance P-related peptides (collectively referred to as tachykinins; TK) contribute to the excitation (Holzer 1989; Maggi et al. 1994). Furthermore, histochemical and electron-microscopic analyses show that most excitatory axons to the circular muscle contain both ACh and TK transmitter systems (Llewellyn-Smith et al. 1988; Brookes et al. 1991). Physiological and retrograde tracing experiments both indicate that the majority of the TK innervation of the circular muscle is from orally projecting axons (Smith et al. 1988; Brookes et al. 1991), but a small proportion of excitatory neurons projects locally and for small distances anally (Brookes et al. 1991). The inhibitory neurons project locally and anally and are divided into two main groups on the basis of their axon lengths. The shorter neurons have immunoreactivity for nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) and the longer ones have immunoreactivity for NOS, VIP and bombesin (BN) (Costa et al. 1992a; Furness and Costa 1992; Furness et al. 1994a; Uemura et al. 1995). GABA-immunoreactive (GABA-IR) neurons also project anally for short distances to innervate the circular muscle, as well as locally to the longitudinal muscle (Furness et al. 1989). Howev-

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er, it is not known whether the GABA-IR neurons belong to populations of motor neurons that have already been described in the guinea pig small intestine.

In the present work, the colocalization of GABA-, NOS-, VIP- and TK-IR was investigated in the guinea-pig small intestine in order to determine which functional classes of neurons contain GABA-IR.

Materials and methods

Tissue preparation for GABA loading

Segments of small intestine were removed from guinea-pigs that had been killed by a blow to the head and exsanguination. The tissue was loaded with GABA as described by Furness et al. (1989). In summary, tissue was placed in culture medium (DME medium, Sigma, St Louis, Mo., USA) which was maintained at 37° C and bubbled with a mixture of filtered room air and carbogen (95% O_2 plus 5% CO_2) for 30 min. The culture medium contained the muscle relaxant, nicardipine (3×10^{-5} M; Sigma), amino-oxyacetic acid (AOAA, 2×10^{-5} M; Sigma), an enzyme inhibitor used to block GABA transaminase activity, and β-alanine (10^{-3} M; Sigma) to prevent uptake of GABA into glial cells. Each preparation was then transferred to culture medium of the same composition containing GABA (5×10^{-9} M; Sigma) and incubated for a further 1 h. Tissue was fixed in Zamboni's fixative (2% formaldehyde, 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) containing 0.05% glutaraldehyde (added fresh) for 4 h at room temperature, treated with sodium borohydride (1% solution in 0.1 M phosphate buffer) and washed well in phosphate-buffered saline (PBS, 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0).

Colchicine treatment

Freshly dissected ileum was collected in culture medium bubbled with carbogen and cut open along the mesenteric border. All subsequent procedures were performed under sterile conditions to minimise bacterial contamination of culture medium. Segments of ileum were pinned mucosal side down onto sterile balsa wood and placed in fresh culture medium, containing an antibiotic mixture (10 mg/ml gentamycin, 5000 Units/ml penicillin, 5 mg/ml streptomycin and 0.25 mg/ml amphotericin B) at 37° C and bubbled as described above. Colchicine was added to the medium to provide a final concentration of 100 µM. The following day the tissue was washed well with PBS, repinned on fresh balsa wood and fixed in Zamboni's fixative overnight at 4° C. Tissue was then cleared in dimethyl sulfoxide and washed in PBS.

Tissue preparation for immunohistochemistry

Whole-mounts. Whole-mount preparations were dissected to separate the external musculature from the submucosa/mucosa. The mucosa was removed from the submucosa with a scalpel and circular muscle strips were removed from the external muscle leaving the longitudinal muscle and the myenteric plexus.

Frozen sections. For tissue to be examined in sections, pieces of ileum were placed in PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant for at least 24 h at 4° C. Areas to be sectioned (longitudinal sections, cutting the circular muscle at right angles and transverse sections perpendicular to the long axis of the ileum) were cut from the fixed tissue and immersed in a cryomould containing OCT compound (Lab Tek products, Nashville, Ill., USA). Sections were cut at 10 µm thickness at –20° C on a cryostat, collected on slides coated with amino propyl triethoxy-silane (APTS, Sigma) and left to dry for 1 h at RT.

Immunohistochemistry

Tissue sections or whole-mounts were incubated in 20% normal serum, of the same species in which the secondary antiserum had been raised, containing 1% Triton X-100 for 30 min at room temperature prior to exposure to primary antibodies. Mixtures of antibodies were directed against the following combinations of antigens: GABA+NOS; GABA+VIP; GABA+TK; VIP+NOS; GABA, NOS+VIP and GABA, NOS+TK (see Table 1). Tissue was washed in PBS and then incubated in a mixture of secondary antibodies comprising one antibody linked with biotin and one or two directly labelled with a fluorophore such as fluorescein isothiocyanate (FITC), 7-amino-4-methyl coumarin-3-acetic acid (AMCA) or indocarbocyanine (Cy3) for 2 h (see Table 2). The tissue was then incubated with streptavidin-Texas Red or streptavidin-FITC conjugate for 90 min (see Table 2). A final wash in PBS was made before tissue was mounted in glycerol buffered with 0.5 M sodium carbonate buffer (pH 8.6).

Experiments were performed to ensure that no false colocalisation occurred through inappropriate binding of secondary antibodies. This was done by omitting one primary antibody at a time from double or triple labelling protocols. No nonspecific binding was observed.

Nerve cell and nerve fibre counts

Fluorescence labelling was examined on a Zeiss Axioplan or Axioskop microscope using appropriate filters. Coexistence of immunoreactive markers in cell bodies and nerve fibres was assessed utilising 1000× magnification. GABA-IR and NOS-IR or VIP-IR and NOS-IR nerve cell bodies were counted in myenteric ganglia from whole-mount preparations of small intestine from three different animals. Cell bodies were systematically counted in cohorts of 50 in all planes of focus from at least two preparations for each

Table 1. Characteristics of primary antisera

Tissue antigen	Host	Dilution	Reference
GABA	Mouse	1:10000	Sithigorngul et al. 1989
Neural NOS	Sheep	1:2000	P.C. Emson, unpublished
Neural NOS	Rabbit	1:200	Anderson et al. 1995
VIP	Rabbit	1:1000	M. Epstein, unpublished
TK (raised against substance P)	Rabbit	1:1000	Morris et al. 1986

Table 2. Secondary antibodies and streptavidin complexes used for immunohistochemistry. Supply companies: Amersham Pty Ltd, Melbourne, Australia; Dako Corporation, Calif., USA; Jackson Immunoresearch Lab., Pa., USA; Vector Lab., Burlingame, Calif., USA

Antibody or streptavidin label	Dilution	Source
Biotinylated donkey anti-rabbit IgG	1:200	Amersham
Horse anti-mouse IgG AMCA	1:50	Vector
Biotinylated donkey anti-sheep IgG	1:100	Jackson
Rabbit anti-mouse IgG FITC	1:40	Dako
Streptavidin-Texas Red	1:50	Amersham
Biotinylated horse anti-mouse IgG	1:200	Vector
Donkey anti-sheep IgG FITC	1:100	Jackson
Streptavidin-FITC	1:50	Amersham
Donkey anti-rabbit IgG Cy3	1:1600	Jackson

animal. Tertiary plexus fibres double labelled for GABA-+NOS-IR and triple labelled for GABA-, NOS-+VIP-IR and GABA-, NOS-+TK-IR in the myenteric plexus were counted from whole-mount preparations from three different animals in a similar systematic way. Nerve fibres in the circular muscle showing GABA- and NOS-, VIP- or TK-IR were counted from croystat sections of ileum cut in the longitudinal axis so that circular muscle and nerve fibres were seen in transverse section. Relative numbers of nerve fibres in all planes of focus were counted in preparations from three different animals.

Data are expressed as means±SEM. For each combination of antibodies, the percentages of double-stained and single-stained nerve cells and fibres were calculated. The calculations were from data derived from cohorts of structures viewed first for the presence of one immunoreactivity and then for a second, and cohorts in which evaluation was conducted in the opposite order of viewing immunoreactivity (see Results).

Results

Colocalization of immunoreactivity in cell bodies of the myenteric plexus

Colocalization of GABA- and NOS-IR was examined in GABA-loaded tissue, and colocalization of NOS- and VIP-IR was examined in tissue that had been pretreated with colchicine to enhance the immunoreactivity of VIP-IR nerve cells. It would have been desirable to determine the colocalization of GABA- and VIP-IR in cell bodies, but it was discovered that GABA loading was incompatible with the colchicine treatment that was required to reveal all VIP-IR nerve cells. Thus colocalization of GABA- and VIP-IR was determined only for nerve fibres.

In whole-mounts of tissue labelled for GABA and NOS immunohistochemistry, nerve cells with GABA-IR only (GABA/-), with NOS-IR only (NOS/-) and with both GABA- and NOS-IR (GABA/NOS) were seen in myenteric ganglia (Fig. 1). Nerve cells with GABA-IR were scattered in the ganglia; they were usually found singly, rather than in clumps, and at least one GABA-IR nerve cell was present in almost every myenteric ganglion. All GABA-IR nerve cells had Dogiel type I morphology, that is, they possessed one axon and several short lamellar dendrites. NOS-IR nerve cells were more numerous (Fig. 1) and also had Dogiel type I morphology. Nerve cells that showed colocalization for GABA- and NOS-IR were usually of small to medium size. Macrophages labelled with GABA-IR were consistently seen

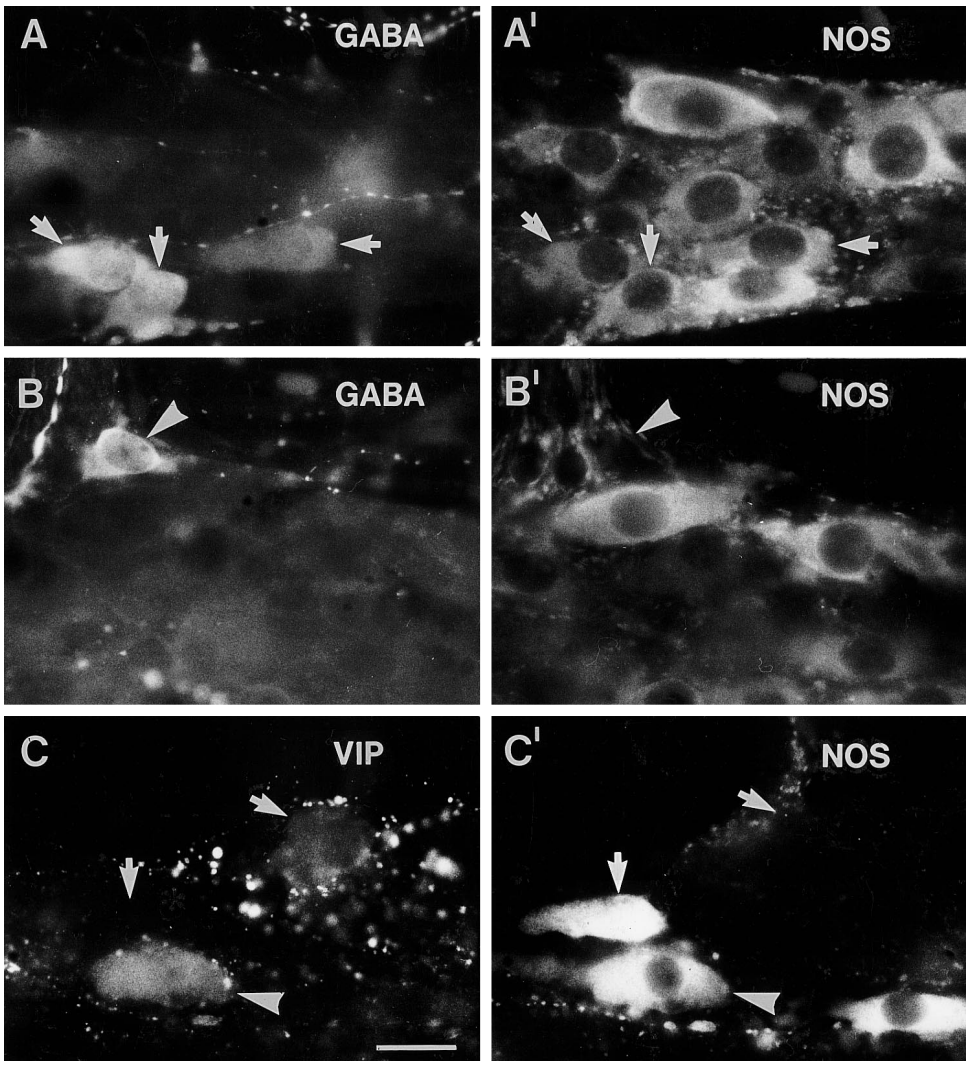

Fig. 1. Colocalization of immunoreactivity in myenteric nerve cells. **A, A′** and **B, B′** Some nerve cells are immunoreactive for both GABA and NOS (*arrows* **A, A′**), many cells are only NOS-IR (compare **A** and **A′**) and few cells have only GABA-IR (*arrowheads*, **B, B′**). **C, C′** Colocalization between VIP-IR and NOS-IR. Most nerve cells that were immunoreactive for one antigen were also immunoreactive for the other (e.g. *arrowheads*), but some cell bodies were immunoreactive for only one of these antigens (*arrows*). *Bar:* 20 μm

Table 3. Analysis of colocalization between GABA-+NOS-IR and NOS-+VIP-IR in myenteric nerve cell bodies of the guinea pig ileum

A

	GABA-IR first (*n*=19)		NOS-IR first (*n*=21)	
	GABA/-	*GABA/NOS*	*NOS/-*	*NOS/GABA*
Total no. of nerve cells	151	799	837	213
%±SEM	16±1	84±1	80±1.2	20±1.2

B

	VIP-IR first (*n*=9)		NOS-IR first (*n*=9)	
	VIP/-	*VIP/NOS*	*NOS/-*	*NOS/VIP*
Total no. of nerve cells	29	421	44	406
%±SEM	6±2	94±2	10±1	90±1

C

Antigen combination	*GABA/-*	*GABA/NOS*	*NOS/-*
% of nerve cell bodies	4%	19%	77%

Antigen combination	*VIP/-*	*VIP/NOS*	*NOS/-*
% of nerve cell bodies	5%	86%	9%

A, B Cells were counted in cohorts of 50 in preparations from 3 animals. The cells were counted by first identifying one immunoreactivity and then determining whether each cell was reactive for the second antigen of the pair; *n*=number of cohorts of 50 cell bodies
C The proportions of nerve cell bodies and their patterns of immunoreactivity derived from data in **A** and **B**, expressed as percentages

Table 4. Colocalization of GABA-+NOS-IR (**A**), GABA-+VIP-IR (**B**) and GABA-+TK-IR (**C**) in nerve fibres in the circular muscle of the guinea-pig ileum

A

	GABA-IR first (*n*=9)		NOS-IR first (*n*=9)	
	GABA/-	*GABA/NOS*	*NOS/-*	*NOS/GABA*
Total no. of nerve fibres	129	371	257	243
%±SEM	26±1	74±1	51±3	49±3

B

	GABA-IR first (*n*=7)		VIP-IR first (*n*=7)	
	GABA/-	*GABA/VIP*	*VIP/-*	*VIP/GABA*
Total no. of nerve fibres	99	251	196	154
%±SEM	28±2	72±2	56±1	44±1

C

	GABA-IR first (*n*=7)		TK-IR first (*n*=7)	
	GABA/-	*GABA/TK*	*TK/-*	*TK/GABA*
Total no. of nerve fibres	328	22	323	27
%±SEM	94±1	6±1	93±1	7±1

D

Antigen combination	*GABA/-*	*GABA/NOS*	*NOS/-*
% of total nerve fibres	15%	42%	43%
Antigen combination	*GABA/-*	*GABA/VIP*	*VIP/-*
% of total nerve fibres	15%	37%	48%
Antigen combination	*GABA/-*	*GABA/TK*	*TK/-*
% of total nerve fibres	52%	4%	44%

A–C Numbers of fibres counted from three different animals. Fibres were counted by first identifying one immunoreactivity and then determining whether immunoreactivity was present for the second antigen in each pair; *n*=numbers of cohorts of 50 fibres
D The proportions of nerve fibres in the circular muscle and their patterns of immunoreactivity derived from data in **A**, **B** and **C**, expressed as percentages

in the same plane as the myenteric ganglia in GABA-loaded preparations.

The numbers of GABA/- and GABA/NOS-IR cells were obtained from analyses in which nerve cells were first identified by their GABA-IR and then viewed with the second filter system to determine whether they were NOS-IR. Similarly, the numbers of NOS/- and NOS/GABA-IR cells were determined for groups of cells first identified by NOS-IR. NOS-IR cells comprised about 85% of the total number of GABA-IR neurons counted and GABA-IR cells represented 20% of the total number of NOS-IR neurons that were counted. The proportions of immunoreactive nerve cells calculated from the two sets of counts were 4% GABA/-, 19% GABA/NOS and 77% NOS/- (Table 3).

GABA-IR nerve fibres were rarely seen in close association with myenteric ganglia. Those that were seen were usually single axons crossing the ganglia and exiting in the primary strands, secondary plexus or tertiary plexus. GABA-IR fibres did not form pericellular baskets around myenteric nerve cells. NOS-IR fibres were common, in comparison, and often formed pericellular varicose baskets around other nonreactive, GABA-IR or NOS-IR neurons.

VIP-IR was present in two groups of nerve cells. The larger group was of cells that had Dogiel type I morphology and had a broad range of sizes; these cells were readily seen after colchicine treatment, but were rare or absent in untreated tissue. Cells of the smaller group with VIP-IR were strongly immunoreactive (even without colchicine treatment) and had fine tapering dendrites and a single axon. These have been identified previously as VIP-IR secretomotor neurons (Song et al. 1991). They were never GABA- or NOS-IR and were excluded from the cell counts. Colocalization of VIP-IR and

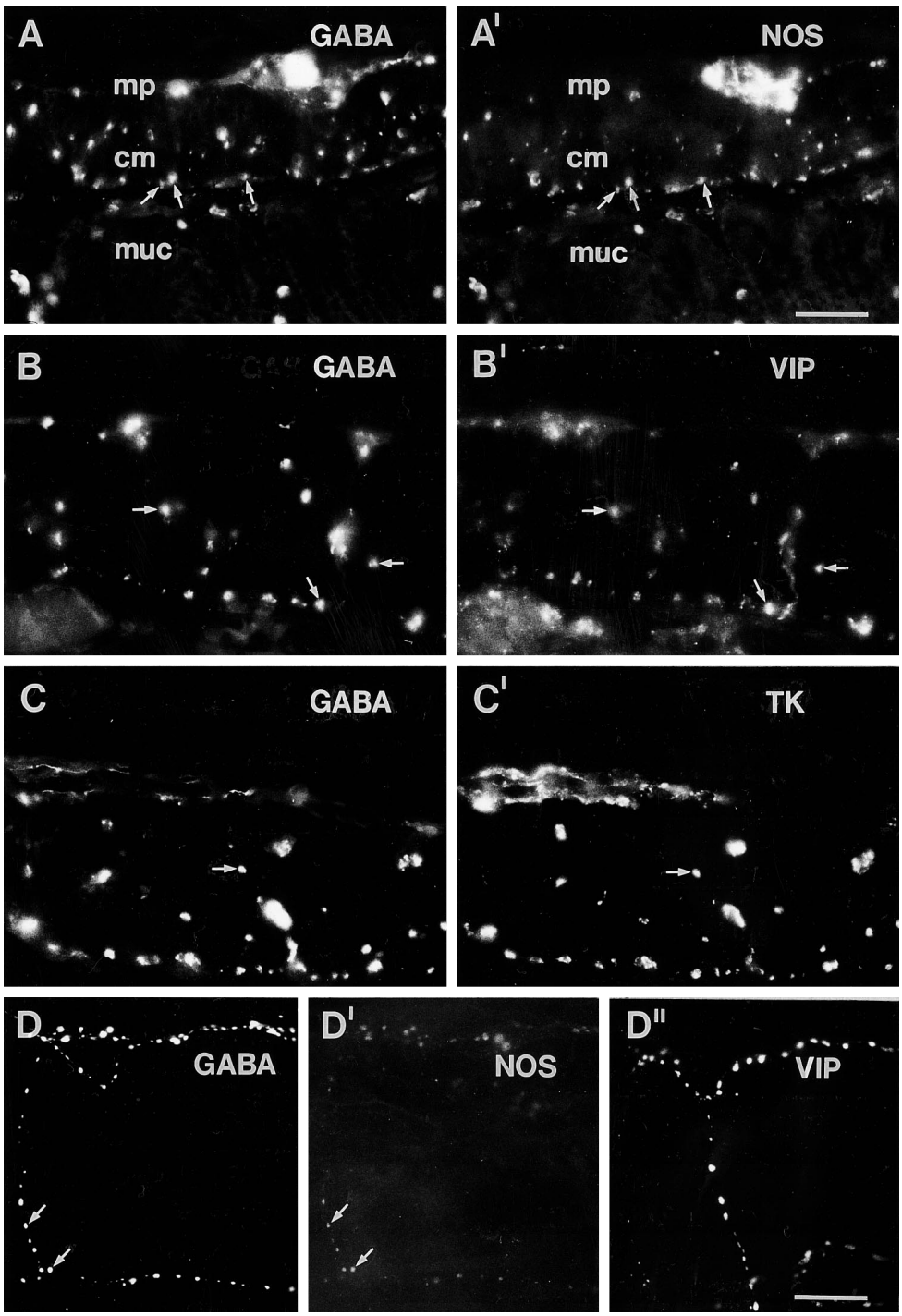

Fig. 2. Patterns of colocalization of immunoreactivity in nerve fibres in the circular muscle (**A–C**), and in the tertiary plexus which innervates the longitudinal muscle (**D**). **A, A′** GABA-IR and NOS-IR are colocalized in about half the NOS-IR nerve fibres that innervate the circular muscle. Fibres in which colocalization occurs are indicated by the *arrows*. **B, B′** Colocalization between GABA-IR and VIP-IR. Fibres in which colocalization occurs are indicated by the *arrows*. **C, C′** Colocalization between GABA-IR and TK-IR. Few fibres show colocalization, one example is indicated by an *arrow*. **D, D′, D″** Simultaneous labelling for GABA-IR, NOS-IR and VIP-IR in the tertiary plexus; GABA- and NOS-IR are colocalized in some fibres (varicosities indicated by *arrows*), but GABA-IR is not colocalized with VIP-IR. *mp* Myenteric plexus; *cm* circular muscle; *muc* mucosa. *Bars:* **A, A′**, 50 µm (on **A**); **B–D″**, 20 µm (on **D″**)

NOS-IR in myenteric neurons was common (Fig. 1), but some Dogiel type I VIP-IR cells were not NOS-IR. Similarly, there were some NOS-IR cells that did not show VIP-IR. NOS-IR neurons comprised about 95% of the total number of VIP-IR neurons counted and VIP-IR neurons represented 90% of the total number of NOS-IR neurons counted. The proportions of immunoreactive nerve cells calculated from the two sets of counts were 5% VIP only (VIP/-), 86% VIP/NOS and 9% NOS/- (Table 3).

Colocalization of GABA-+NOS-IR, VIP-+GABA-IR and TK-+GABA-IR in nerve fibres innervating the circular muscle

Colocalization was examined in groups of 50 fibres by first determining the immunoreactivity of each fibre for one antigen and then for the second. GABA- and NOS-IR nerve fibres were seen throughout the thickness of the circular muscle and in the deep muscular plexus (Fig. 2). Of the total number of GABA-IR nerve fibres counted,

about 75% were also NOS-IR, and GABA-IR was found in about 50% of the NOS-IR nerve fibres. The proportions of immunoreactive nerve fibres calculated from these data were 15% GABA/-, 42% GABA/NOS and 43% NOS/- (Table 4).

Innervation of the circular muscle by VIP-IR nerve fibres was dense throughout the bulk of the muscle and in the deep muscular plexus (Fig. 2). The immunoreactive nerve fibres were counted as described above. VIP-IR nerve fibres comprised about 70% of the total number of GABA-IR nerve fibres and GABA-IR nerve fibres comprised about 45% of the total number of VIP-IR nerve fibres. The proportions of immunoreactive nerve fibres calculated from these data were 15% GABA/-, 37% GABA/VIP and 48% VIP/- (Table 4).

TK-IR nerve fibres comprised approximately 5% of the total number of GABA-IR nerve fibres counted, whereas GABA-IR nerve fibres represented 7% of the total number of TK-IR nerve fibres counted (Table 4). A number of fibres was encountered for which it was difficult to determine whether or not colocalization occurred, possibly because the intracellular distributions of immunoreactivity differed. Generally, the GABA fibres were evenly stained, probably due to the GABA loading causing an even distribution of GABA throughout the cytoplasm of the axon. The TK-IR fibres generally had an irregular labelling that appeared as small granules of immunoreactivity that did not fill the varicosity. This may have been due to a clumping of transmitter vesicles within the nerve fibres. Those fibres in which colocalization was uncertain were not included in the total counts presented in the Tables. If these uncertain colocalizations were counted as true colocalization, TK-IR would comprise about 20% (rather than approximately 5%) of GABA-IR nerve fibres. The proportions of immunoreactive fibres calculated from the total number of fibres immunoreactive for GABA or TK were 52% GABA/-, 4% GABA/TK and 44% TK only (TK/-).

Colocalization of GABA-, NOS-+VIP-IR in nerve fibres of the tertiary plexus

In the tertiary component of the myenteric plexus, nerve fibres were intensely GABA-IR and formed a complex network connecting to secondary strands and internodal strands of myenteric ganglia (Fig. 2). NOS-IR fibres were also present in the tertiary plexus, but the immunoreactivity was much less intense (Fig. 2). Fibres were counted as described above for examination of cell bodies. NOS-IR fibres comprised about 80% of the total number of GABA-IR fibres counted, whereas GABA-IR fibres comprised about 50% of the total number of NOS-IR fibres. The proportions of immunoreactive fibres calculated from these data were 12% GABA/-, 43% GABA/NOS and 45% NOS/- (Table 5).

In addition, tertiary plexus fibres immunoreactive for GABA, NOS+VIP were examined using triple labelling in GABA-loaded ileum. There was substantial overlap of GABA-IR with NOS-IR in nerve fibres, but no GABA-IR nerve fibres were also VIP-IR (Fig. 2, Table 5). Colocalization of NOS-IR with both GABA-IR and VIP-IR in nerve fibres was observed. However, 6% of nerve fibres with GABA-IR were not immunoreactive for either NOS or VIP. In an attempt to account for this 6% of GABA-IR fibres, triple labelling was performed with antibodies to GABA, NOS+TK. Analysis of fibres from two animals showed no colocalization of GABA-IR, or NOS-IR, with TK-IR.

Table 5. Colocalization of GABA-, NOS- and VIP-IR in fibres of the tertiary plexus

A (double label)

	GABA-IR first (*n*=9)		NOS-IR first (*n*=9)	
	GABA/-	*GABA/NOS*	*NOS/-*	*NOS/GABA*
Total no. of nerve fibres	98	352	232	218
%±SEM	22±4	78±4	52±3	48±3

B (double label)

	GABA/-	*GABA/NOS*	*NOS/-*
% of total nerve fibres	12%	43%	45%

C (triple label)

	GABA-IR first (*n*=6)		NOS-IR first (*n*=6)			VIP-IR first (*n*=6)	
	GABA/-	*GABA/NOS*	*NOS/-*	*NOS/GABA*	*NOS/VIP*	*VIP/-*	*VIP/NOS*
Total no. of nerve fibres	77	223	135	60	105	238	62
%±SEM	26±2	74±2	45±2	20±1.5	35±3	79±2	21±2

D (triple label)

Antigen combination	*GABA/-*	*GABA/NOS*	*NOS/-*	*NOS/VIP*	*VIP/-*
% of total nerve fibres	6%	37%	10%	29%	18%

A Counts of nerve fibres double labelled for GABA-IR and NOS-IR
B Proportions of nerve fibres in the tertiary plexus determined from the double-label data
C Counts of nerve fibres triple labelled for GABA-, NOS- and VIP-IR
D Proportions of nerve fibres in the tertiary plexus determined from the triple label data

Discussion

Colocalization of GABA-IR and NOS-IR

In the myenteric plexus of the guinea pig ileum, 19% of total nerve cell bodies are NOS-IR (Furness et al. 1994b). Double staining for NOS- and GABA-IR, in the

present study, indicated that the population of GABA-IR nerve cells was about 25% of the size of the NOS-IR population; GABA-IR can thus be calculated to be in 4.5% of all myenteric nerve cells. Of these, about 85% (which calculates to 3.7% of all nerve cells) were GABA/NOS-IR. GABA is also colocalized with NOS in myenteric nerve cells of human and mouse colon, although the proportional overlaps are quite different; in human, about 25% of myenteric GABA nerve cells were also reactive for NADPH diaphorase (used as a marker for NOS; Nichols et al. 1995) and in mouse colon 35% of GABA-IR cells were NOS-IR (Sang and Young 1995).

GABA-IR in inhibitory motor neurons

Of GABA-IR nerve cells, about 85% were NOS-IR and of GABA-IR nerve fibres, about 80% in the longitudinal muscle innervation and about 75% in the circular muscle innervation were NOS-IR. NOS-IR is present in inhibitory neurons that project anally to the circular muscle, in neurons that project locally to the tertiary plexus innervating the longitudinal muscle and in descending interneurons of the small intestine (Costa et al. 1992b). GABA-IR neurons project slightly anally before entering the circular muscle, but, in contrast to NOS-IR neurons, they do not provide terminals in the myenteric ganglia; separate GABA-IR neurons supply the longitudinal muscle (Furness et al. 1989). Because NO is a transmitter of inhibitory motor neurons, it is deduced that most GABA-IR neurons are inhibitory motor neurons, and that separate GABA/NOS-IR inhibitory neurons supply the longitudinal and circular muscle. The colocalization of GABA- and NOS-IR is an important observation, since the chemical coding of NOS-IR neurons that was previously known did not allow a distinction to be made between motor neurons and interneurons and left open the possibility that the NOS-IR terminals in the myenteric plexus and circular muscle were processes of the same nerve cells (Costa et al. 1992b; Furness et al. 1994b). The present work indicates that one group of inhibitory motor neurons, the GABA/NOS motor neurons, does not have collaterals to other targets.

GABA-IR in excitatory motor neurons

About 5% of GABA-IR fibres that innervate the circular muscle are immunoreactive for tachykinins, which are markers of excitatory neurons innervating the muscle (see Introduction). Brookes et al. (1991) have shown that a small proportion of the choline acetyltransferase/TK-IR neurons project anally for up to 3–4 mm to innervate the circular muscle. Because all GABA neurons innervating the circular muscle project anally (Furness et al. 1989) and TK are excitatory transmitters to the circular muscle (Maggi et al. 1994), it is probable that the GABA/TK-IR neurons are a population of anally projecting excitatory motor neurons, that are likely to be also choline acetyltransferase immunoreactive.

Colocalization of GABA- and NOS-IR in the tertiary plexus

The demonstration that the longitudinal muscle of the guinea-pig ileum has major cholinergic and noncholinergic components of excitatory transmission (Ambache and Freeman 1968; Paton and Zar 1968; Hirst et al. 1975) is supported by immunohistochemistry and retrograde labelling which have shown that most longitudinal muscle motor neurons contain two excitatory transmitters, ACh and TK (Brookes et al. 1992). Tachykinins in fibres innervating the longitudinal muscle appear to be excitatory cotransmitters with ACh (Franco et al. 1979). A minor inhibitory component of longitudinal muscle innervation, which could be mediated by VIP or NO, also appears to exist in the guinea-pig ileum (Bauer and Kuriyama 1982; Osthaus and Galligan 1992). In this study we have shown that about 75% of the GABA-IR tertiary plexus fibres are also NOS-IR, but none are VIP-IR. Additionally, no colocalization was seen between GABA- and TK-IR. Thus, these results distinguish a set of GABA/NOS motor nerve fibres that extend to the longitudinal muscle and are probably inhibitory. The GABA/NOS fibres innervating the longitudinal muscle are not VIP-IR; they probably arise from NOS-IR myenteric nerve cell bodies seen in this work that were not VIP-IR.

Colocalization of NOS-IR and VIP-IR in myenteric cell bodies

There was a high degree of overlap of NOS-IR and VIP-IR in myenteric nerve cell bodies. Nevertheless, 9% of NOS neurons were not immunoreactive for VIP and 5% of VIP neurons, excluding the prominent secretomotor neurons, were not NOS immunoreactive. It is possible that some nerve cells with only VIP-IR that were seen in this study provide innervation of the longitudinal muscle since about 80% of the tertiary plexus nerve fibres were only VIP-IR when labelling for NOS and VIP was performed. A small proportion of neurons with VIP-IR but not NOS-IR could be intestinofugal neurons, since it has been shown that intestinofugal neurons projecting from the small intestine to the coeliac ganglion have immunoreactivity for VIP, but not for NOS (Kuramoto and Furness 1989; Anderson et al. 1995).

Possible roles for GABA

The distribution of GABA nerve cells and nerve fibres differ considerably between species and gut regions. In the guinea-pig small intestine, GABA neurons have their cell bodies in the myenteric ganglia and provide terminals that innervate the longitudinal and circular muscle (Krantis et al. 1986; Hills et al. 1987; Furness et al. 1989). It is notable that GABA fibres do not innervate nerve cells in the myenteric plexus of the guinea-pig small intestine (Krantis et al. 1986; Figs. 6–13; Hills et al. 1987; Fig. 14; Furness et al. 1989). In contrast,

GABA fibres from prominent pericellular networks in myenteric ganglia of the rat small intestine and colon and in the guinea-pig colon (Jessen et al. 1986; Hills et al. 1987). The innervation of the myenteric plexus in the human colon appears to be similar to that in the guinea-pig small intestine (Nichols et al. 1995). There are also differences in muscle innervation; in guinea-pig and human colon the circular muscle is densely innervated, whereas it is sparsely innervated in the rat. The different distributions of GABA nerve fibres imply that GABA has different roles in different species and regions.

In the guinea-pig ileum, exogenously applied GABA has three effects, stimulation of excitatory neurons to the muscle, stimulation of inhibitory neurons to the muscle (both via $GABA_A$ receptors) and inhibition of release of acetylcholine from excitatory muscle motor neurons (via $GABA_B$ receptors), but GABA has no direct effect on the muscle (Krantis et al. 1980; Krantis and Kerr 1981; Giotti et al. 1983; Kleinrok and Kilbinger 1983; Ong and Kerr 1983). It is possible that the stimulatory action of exogenous GABA on the neurons has no physiological significance, since the nerve cells do not receive a GABA innervation and peristalsis in the guinea-pig ileum is not affected by blocking $GABA_A$ receptors (Tonini et al. 1989). In other regions of the gut, GABA may be released at neuro-neuronal synapses and pharmacological studies suggest that it might play a role in peristalsis (Ong and Kerr 1983; Frigo et al. 1987; Grider and Makhlouf 1992). However, a major part of the pharmacological evidence relies on the selective action of bicuculline as an antagonist of $GABA_A$ receptors, whereas Tonini et al. (1989) have shown bicuculline to have non-specific actions, at least in the guinea-pig small intestine. Although it has no direct action on the muscle of the guinea-pig small intestine (Krantis et al. 1980; Krantis and Kerr 1981), GABA released from the endings of motor neurons could possibly reduce the output of acetylcholine from the excitatory motor neurons (Kleinrok and Kilbinger 1983; Ong and Kerr 1983) and thus indirectly contribute to inhibition of motility.

It is concluded from this work that three major groups of GABA-IR neurons occur in the small intestine, GABA/NOS/VIP inhibitory motor neurons, with short anally directed projections to the circular muscle, GABA/SP/ACh excitatory motor neurons also with short anally (or locally) directed projections to the circular muscle and GABA/NOS inhibitory motor neurons to the longitudinal muscle.

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