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REGULAR ARTICLE

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Intimate relationship between interstitial cells of Cajal and enteric nerves in the guinea-pig small intestine

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Abstract Recent studies have suggested that enteric inhibitory neurotransmission is mediated via interstitial cells of Cajal in some gastrointestinal tissues. This study describes the physical relationships between enteric neurons and interstitial cells of Cajal in the deep muscular plexus (IC-DMP) of the guinea-pig small intestine. c-Kit and vimentin were colocalized in the cell bodies and fine cellular processes of interstitial cells of the deep muscular plexus. Anti-vimentin antibodies were subsequently used to examine the relationships of interstitial cells with inhibitory motor neurons (as identified by nitric oxide synthase-like immunoreactivity) and excitatory motor neurons (using substance P-like immunoreactivity). Neurons with nitric oxide synthase- and substance P-like immunoreactivities were closely associated with the cell bodies of interstitial cells and ramified along their processes for distances greater than 300 μm. With transmission electron microscopy, we noted close relationships between interstitial cells and the nitric oxide synthase- and substance P-like immunoreactive axonal varicosities. Varicosities of nitric oxide synthase and substance P neurons were found as close as 20 and 25 nm from interstitial cells, respectively. Specialized junctions with increased electron density of pre- and postsynaptic membranes were observed at close contact points between nitric oxide synthase- and substance P-like immunoreactive neurons and interstitial cells. Close structural relationships (approximately 25 nm) were also occasionally observed between either nitric oxide synthase- and substance P-like immunoreactive varicosities and smooth muscle cells of the outer circular muscle layer. The data suggest that interstitial cells in the deep muscle plexus are heavily innervated by excitatory and inhibitory enteric motor neurons. Thus, these interstitial cells may provide an important, but probably not exclusive, pathway for nerve-muscle communication in the small intestine.

Key words Interstitial cells of Cajal · Enteric nerves · Nitric oxide synthase · Substance P · Gastrointestinal tract · Deep muscular plexus · Guinea pig

Introduction

Morphological and functional studies have suggested that interstitial cells of Cajal (ICC) act as pacemakers and conductors of electrical events in gastrointestinal muscles (Thuneberg 1982; Christensen 1992; Sanders 1996). These cells may also mediate neurotransmission from enteric neurons (Daniel and Posey-Daniel 1984; Daniel and Berezin 1992). Much of the evidence supporting these hypotheses comes from anatomical studies showing that ICC populate pacemaker regions (Faussone-Pellegrini et al. 1977; Thuneberg 1982), form gap junctions with neighboring smooth muscle cells, and form associations, as close as 20 nm, with varicose nerve terminals (Richardson 1958; Rogers and Burnstock 1966; Faussone-Pellegrini et al. 1987; Thuneberg 1982; Daniel and Posey-Daniel 1984). Recently, morphological studies coupled with physiological experiments have revealed that ICC of the myenteric pacemaker region and electrical slow waves are absent from the small intestine in W/W^V mutant mice (Ward et al. 1994; Huizinga et al. 1995). Physiological studies have also shown that inhibitory innervation of gastrointestinal (GI) muscles is concentrated in regions where ICC are located (Smith et al. 1989; Berezin et al. 1990; Huizinga et al. 1990), and isolated ICC have been shown to be responsive to a variety of enteric transmitters including nitric oxide (NO) and substance P (SP; Publicover et al. 1992, 1993). By the use of antibodies against cGMP (de Vente et al. 1989), ICC have been specifically evaluated as targets for NO by monitoring changes in cellular levels of cGMP in response to nitric oxide donors

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Table 1 Details of antibodies used for immunohistochemistry (*ACK2* anti c-Kit, *SP* substance P, *NOS* nitric oxide synthase, *VIM* vimentin)

Combined antibodies	Ressource	Mono- or polyclonal antibodies	Host	Dilution
ACK2/anti-VIM	Gibco BRL, Gaithersburg, Md. /ICN, Costa Mesa, Calif.	Mono-/mono-	Rat/Mouse	1:200/1:50
Anti-SP/anti-VIM	Incstar Co., Stillwater, Minn./as above	Poly-/mono-	Rabbit/Mouse	1:500/1:50
Anti-NOS/anti-VIM	Transduction Lab., Lexington Ky./as above	Poly-/mono-	Rabbit/Mouse	1:500/1:50

and stimulation of enteric neurons (Shuttleworth et al. 1993; Young et al. 1993). The role of ICC in neurotransmission has been supported recently in studies showing that loss of a specific population of ICC in the murine stomach results in loss of NO-dependent neurotransmission (Burns et al. 1996).

Although ultrastructural studies with transmission electron microscopy have shown close apposition between enteric neurons and ICC, the extent and specifics of this innervation have not been documented. In the present study we have used double immunolabeling to show the relationships between ICC of the deep muscular plexus (IC-DMP) and enteric neurons in the guinea-pig small intestine. We have used antibodies directed against the intermediate filament marker, vimentin, and the receptor tyrosine kinase, c-Kit, to label IC-DMP (Ward et al. 1994, 1995; Huizinga et al. 1995; Torihashi et al. 1995, 1997; Burns et al. 1996, 1997) and antibodies directed against SP and NO synthase (NOS) to label excitatory and inhibitory motor neurons, respectively. Immunocytochemistry was also used to demonstrate innervation of IC-DMP and smooth muscle cells by specific populations of motor neurons.

Materials and methods

Animals

Twenty-five male guinea pigs, weighing 250–350 g were sacrificed by asphyxia with CO_2 in a specially constructed chamber. The abdomens were cut open and the ileum of each animal was removed for dissection. The use and treatment of animals was approved by the Institutional Animal Use and Care Committee at the University of Nevada.

Immunohistochemistry

The small intestinal segments were opened along the mesenteric border and lumenal contents were washed away with Krebs Ringer bicarbonate solution (KRB). The opened segments were pinned onto the base of a Sylgard dish with the mucosal side facing up. The mucosa was removed by dissection and the remaining tunica muscularis was relaxed in KRB containing nifedipine (10^{-6} M, Sigma) for 30 min, and then stretched to 150% of the resting length. For experiments utilizing c-Kit and vimentin immunoreactivity, tissues were fixed in ice-cold acetone for 10 min. For double-labeling experiments employing vimentin and NOS or vimentin and SP antibodies, tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 45 min at 4°C.

Following fixation, preparations were washed for 30 min in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Nonspecific antibody binding was reduced by incubation of the tissues in 10% normal goat serum for 1 h at room temperature before addition of the primary antibodies. For double immunostaining, the first incubation was carried out for 48 h at 4°C with a mixture of two primary antisera raised in different species. The two combinations of antibodies were rat/mouse and rabbit/mouse (see Table 1). For the rat/mouse combination, the mixture of labeled second antibodies was fluorescein isothiocyanate (FITC)-coupled goat anti-rat IgG plus Texas red-congugated goat anti-mouse IgG; for the rabbit/mouse combination, a mixture of FITC-coupled goat anti-rabbit IgG plus Texas red-congugated goat anti-mouse IgG was used. All secondary antibodies were purchased from Vector Laboratories (Burlingame, Calif.), and diluted to 1:100 in PBS. Secondary incubations were performed for 1 h at room temperature. Control tissues were prepared by either omitting primary or secondary antibodies from the incubation solutions. All the antisera were diluted with 0.3% Triton-X 100 in 0.01 M PBS (pH 7.4). Tissues were examined with a Bio-Rad MRC 600 confocal microscope (Hercules, Calif.) with an excitation wavelength appropriate for FITC (494 nm) and Texas red (595 nm). Confocal micrographs are digital composites of Z-series scans of 10–15 optical sections through a depth of 6–40 μm. Final images were constructed with Bio-Rad "Comos" software.

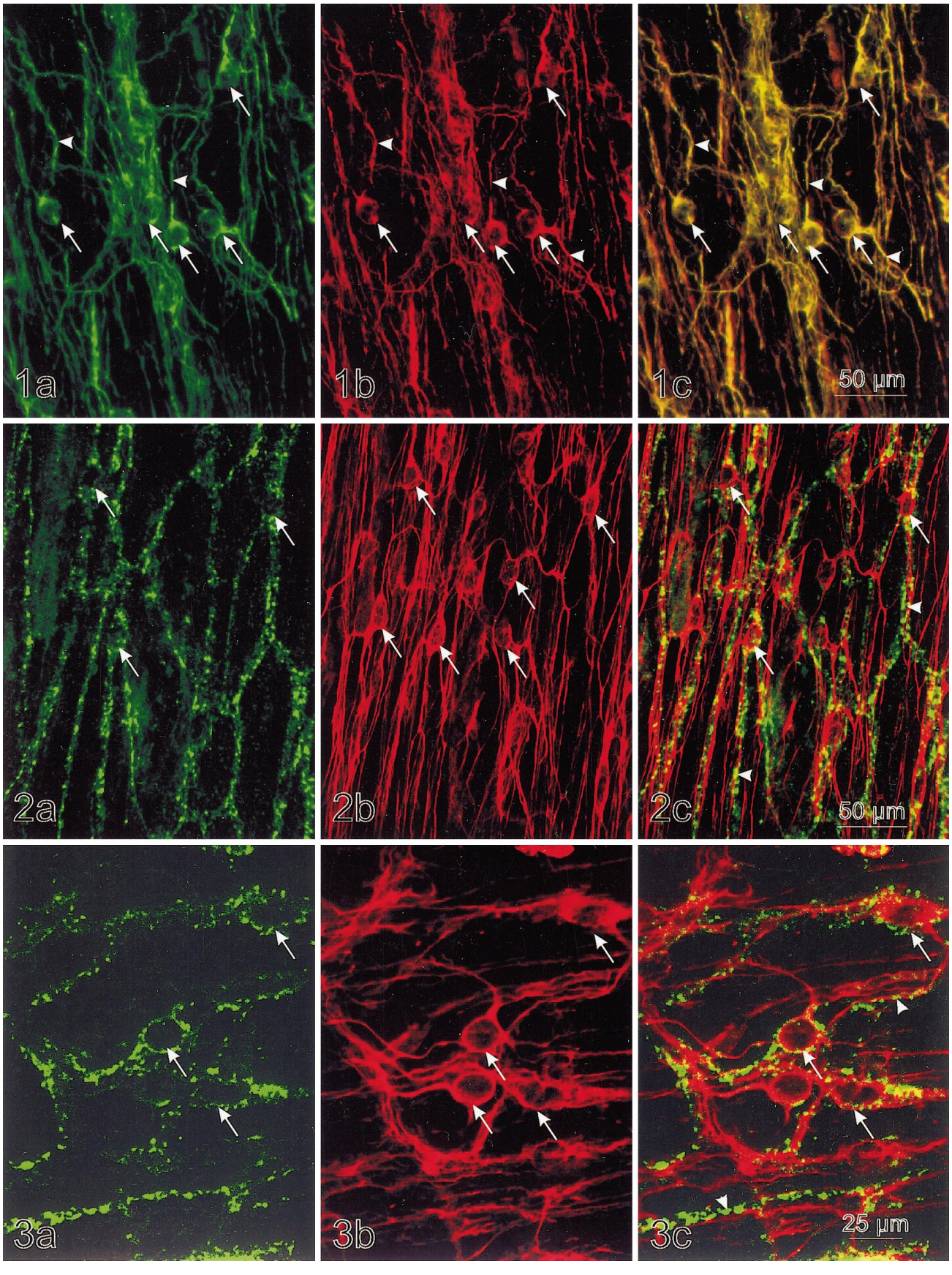
Immunocytochemistry

Tissues were also prepared for immunocytochemistry by incubating at 37^0 C for 60 min in oxygenated KRB solution (pH 7.4). The tissue was then fixed by immersion in 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M PB, pH 7.4, for 45 min at room temperature (Somogyi and Takagi 1982). After a brief rinse in 0.1 M PB, tissue was washed vigorously at room temperature in sev-

Fig. 1a–c Confocal micrographs of whole-mount preparations showing double labeling with c-Kit (FITC) and vimentin (Texas red) antibodies in the deep muscle plexus (DMP) of guinea-pig ileum. **a** c-Kit-like immunoreactivity (*green*), showing that both the cell bodies (*arrows*) and processes (*arrowheads*) of the interstitial cells of Cajal in the DMP (IC-DMP) are immunoreactive for c-Kit; **b** vimentin immunoreactivity (*red*) of the same specimen; **c** colocalization (*yellow*) of c-Kit-LI and vimentin-LI

Fig. 2a–c Confocal micrographs of whole-mount preparations showing double labeling for nitric oxide synthase (NOS; FITC) and vimentin (Texas red) in the DMP of the guinea-pig ileum. **a** Many varicose nerve fibers labeled with NOS-LI (*arrows*); **b** some of the varicose processes surround cell bodies of IC-DMP labeled with vimentin antibodies (*arrows*); **c** the close relationship between nerve fibers with NOS-LI and the cell bodies (*arrows*) and processes (*arrowheads*) of IC-DMP

Fig. 3a–c Confocal micrographs of whole-mount preparations showing double labeling for substance P (SP, FITC) and vimentin (Texas red) in the DMP of guinea-pig ileum. **a** SP-LI of the varicose nerve fibers (*arrows*), some of which surround cell bodies of IC-DMP with vimentin antibodies (**b**, *arrows*); **c** the close relationship between nerve fibers with SP-LI and the cell bodies (*arrows*) and processes (*arrowheads*) of IC-DMP


1a
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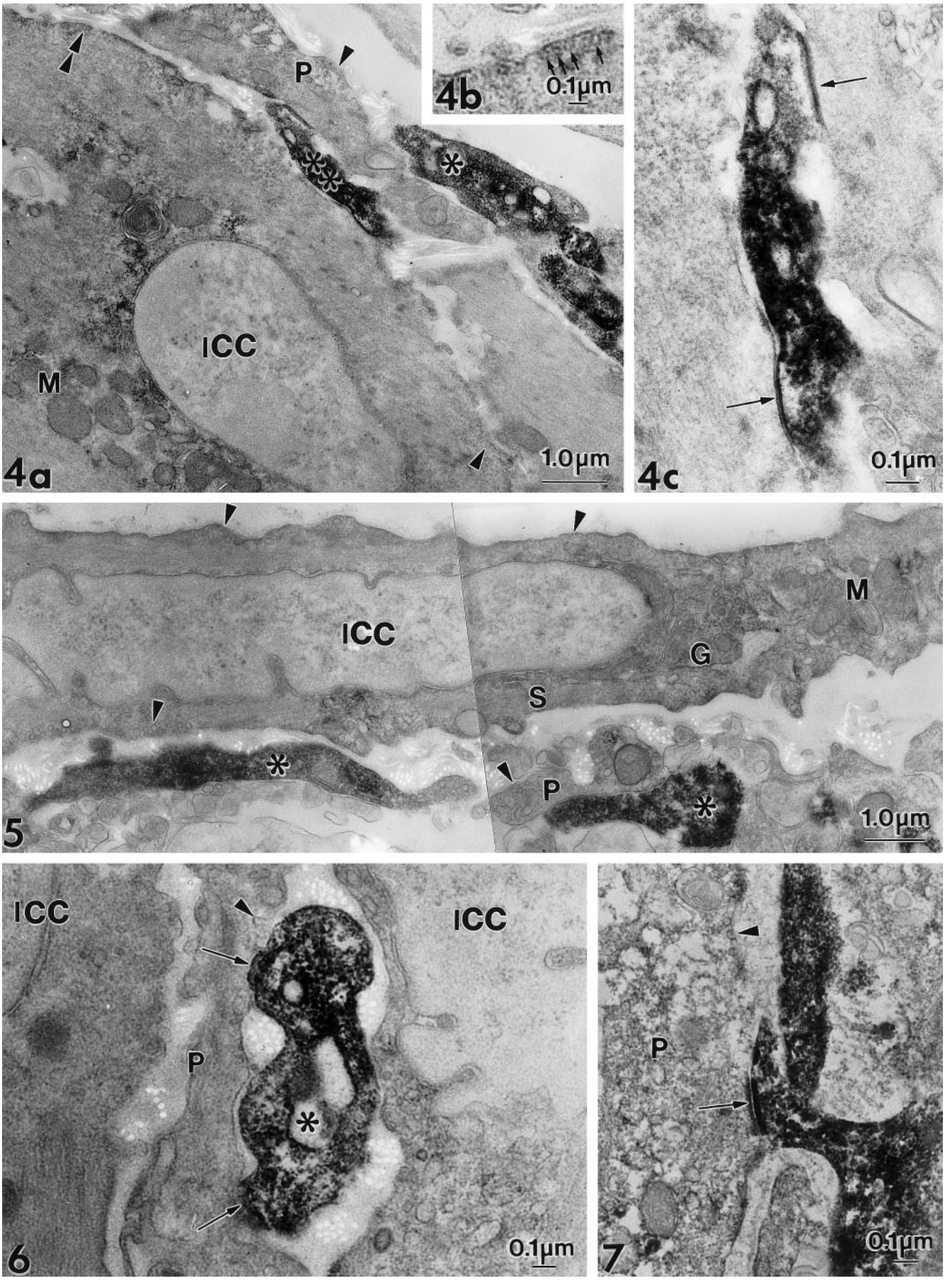
P
ICC
M
1.0μm
4a
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4b
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4c
ICC
M
G
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1.0μm
5
ICC
ICC
P
0.1μm
6
P
0.1μm
7

eral changes of 50% ethanol in distilled water until the picric acid staining of the tissue had disappeared (about 20–30 min). The tissue was then washed in 0.1 M PB and incubated in 0.1% NaCNBH3 (Aldrich Chemical, Milwaukee, Wis.) in 0.1 M PB for 30 min at room temperature. After washing in PB several times, the tunica muscularis was peeled from the remaining gut wall and cut into pieces about 3×3 mm. After nonspecific binding was blocked with goat serum for 1 h at room temperature, the tissues were incubated overnight at room temperature in anti-SP (1:500) and NOS (1:500) primary antisera. On the second day, after washing in PBS several times, secondary immunoreactions were carried out with the Vectastain ABC kit (PK-4001, Vector Lab., Burlingame, Calif.) using 3,3' diaminobenzidine (0.05% DAB plus 0.01% H_2O_2 in 0.05 M TRIS buffer saline, pH 7.6) as a peroxidase substrate. Tissues were continuously checked under the light microscope for suitable reaction, prior to being postfixed in 1% osmium tetroxide in 0.1 M PB (pH 7.4), stained en bloc with 2% aqueous uranyl acetate for 30–40 min, dehydrated, infiltrated and embedded in Medcast resin. Ultrathin sections were cut parallel to the circular muscle layer and stained with lead citrate for 10 min before viewing with a Philips CM10 transmission electron microscope.

Results

Light microscopy

Preliminary results showed that the antibody (ACK2) used to label c-Kit-like immunoreactivity (c-Kit-LI) of ICC in the guinea pig required fixation with acetone. Many of the antibodies available to label specific classes of neurons, however, did not label neurons after acetone fixation. Therefore, another marker for ICC that would give high quality resolution of these cells and not interfere with neural markers was used. Previous studies of the guinea-pig small intestine have shown that ICC in this species are strongly vimentin immunopositive (Komuro and Zhou 1996). Therefore, we employed a secondary immunolocalization step to demonstrate that c-Kit-LI and vimentin-LI colocalized within cell bodies and the processes of IC-DMP (see Fig. 1a–c). After colocalization of c-Kit-LI and vimentin-LI was shown, anti-vimentin antibodies were used for the remainder of the study to label IC-DMP.

IC-DMP labeled with either c-Kit or vimentin antibodies possessed prominent oval nuclei and numerous processes, many of which were oriented parallel to the long axis of the adjacent circular muscle fibers. Major processes of IC-DMP were interconnected to one another by short lateral extensions, forming an integrated network of cells (Fig. 1a–c).

Double staining for vimentin-LI and NOS-LI showed that IC-DMP were closely apposed with varicose nerve fibers containing NOS-LI. The neural processes surrounded the cell bodies and processes of IC-DMP (Fig. 2a–c) for long distances (at least 300 μm), from one IC-DMP to the next.

Double staining with anti-vimentin and anti-SP antibodies revealed numerous IC-DMP that were surrounded by varicose nerve fibers with SP-LI. Neural processes with SP-LI formed basket-like patterns around the cell bodies of IC-DMP (Fig. 3a–c) and could be seen to accompany the processes of IC-DMP for at least 300 μm.

Although confocal microscopy revealed a correlation between IC-DMP and varicose neural fibers with NOS-LI and SP-LI, the precise distances between these cells could not be established by light microscopy. Therefore, we conducted a series of ultrastructural studies.

Electron microscopy

At an ultrastructural level IC-DMP had round or oval nuclei (Figs. 4a, 5, 8a, 9a) and perinuclear cytoplasm containing conspicuous mitochondria, smooth endoplasmic reticulum and Golgi apparatus, relatively sparse rough endoplasmic reticulum, and myofilaments (Figs. 4a, 5, 8a, 9a, 10a). Many caveolae were observed lining the plasma membrane (Figs. 4a,b, 5, 6, 7, 8a, 8b, 9a, 10a,b) and an incomplete basal lamina surrounded the cell bodies (Fig. 8a). These structures are consistent with previously described characteristics of IC-DMP (Faussone-Pellegrini 1987, 1992; Komuro et al. 1994, 1996; Komuro and Zhou 1996).

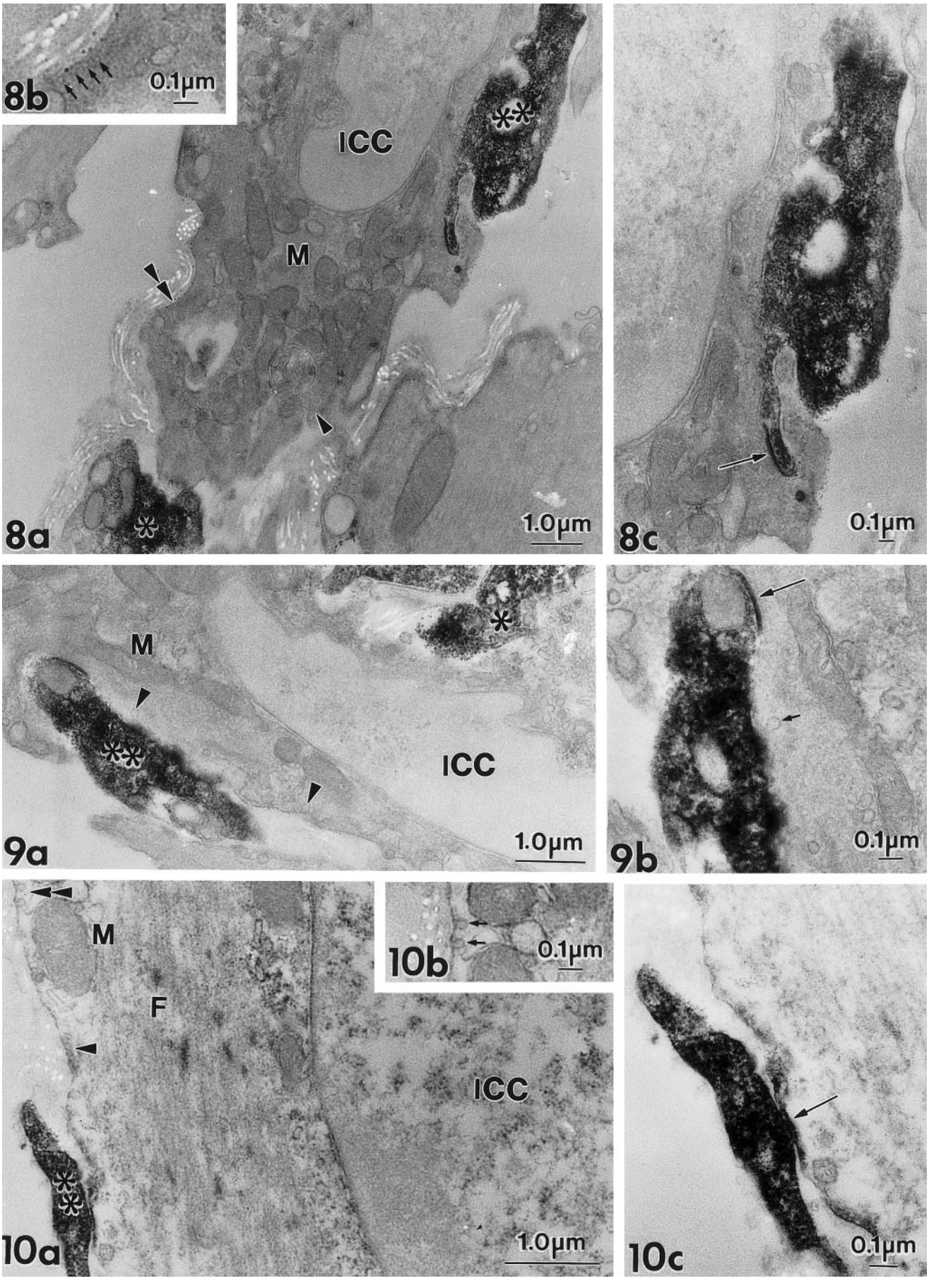
Enteric nerve varicosities in the area of the DMP were immunoreactive for NOS or SP. The immunoreactivity was associated with either the membranes of vesicles within the varicosities or within the lumen of vesicles (Figs. 4a,c, 6, 7, 8a,c, 9a,b, 10a,c). Varicosities with NOS-LI or SP-LI were found as close as 20–25 nm to IC-DMP. Numerous specialized junctions were observed between the cell bodies (Fig. 4c) or processes of IC-DMP and nerve varicosities containing NOS-LI (Figs. 5, 6, 7). Specialized junctions could also be found between varicosities containing SP-LI and the cell bodies of IC-DMP (Figs. 8c, 9b, 10c), but specialized junctions were seldom observed between fibers with SP-LI and the processes of IC-DMP. In some cases double junctions could be found where one nerve terminal with NOS-LI formed junctions with both the cell body and a process of a single IC-DMP (Figs. 4a,c), and in other cases, two or more nerve terminals could be found closely apposed to the cell body of the same IC-DMP (Fig. 8a).

◀ **Fig. 4a–c** Electron micrographs showing immunocytochemistry for NOS in the DMP. **a** An IC-DMP and neural processes labeled with NOS-LI (*asterisks*). IC-DMP possess many caveolae (*double arrowheads*; *small arrows* in enlargement **b**) along the surface membrane, mitochondria (*M*) and filaments. A varicosity with NOS-LI (**) is closely associated with the cell body and the process (*P*) of another IC-DMP. **c** Higher power image of the nerve varicosity identified with ** in **a**. Specialized junctions (with areas of membrane densification) can be observed between the varicosity and the IC-DMP (*arrows*)

Fig. 5 Electron micrograph showing immunocytochemistry for NOS in the DMP. An IC-DMP (*ICC*) with abundant caveolae (*arrowheads*), mitochondria (*M*), Golgi apparatus (*G*) and smooth endoplasmic reticulum (*S*) is shown in close proximity to varicosities with NOS-LI (*asterisks*). One of the nerve varicosities is closely associated with a process (*P*) of an IC-DMP

Fig. 6 Electron micrograph showing immunocytochemistry for NOS in the DMP. Two cell bodies (*ICC*) and one process (*P*) of an IC-DMP are shown to be in close proximity with a varicosity with NOS-LI (*asterisk*). *Arrows* indicate areas of close apposition between the NOS-LI nerve fiber and a process of an IC-DMP; *arrowhead* shows caveolae in the membrane of the IC-DMP process

Fig. 7 Electron micrograph showing immunocytochemistry for NOS in the DMP. A synapselike junction (*arrow*) is located between a varicosity with NOS-LI and an IC-DMP process (*P*). *Arrowhead* shows the caveolae in the membrane of the IC-DMP process


8b
0.1μm
ICC
M
8a
1.0μm
8c
0.1μm
M
ICC
9a
1.0μm
9b
0.1μm
M
F
10b
0.1μm
ICC
10a
1.0μm
10c
0.1μm

The specialized junctions between IC-DMP and nerve fibers contained synapses with electron-dense areas of pre- and postjunctional specialization (Figs. 4c, 7, 8c, 9b, 10c). Occasionally, the nerve terminals protruded into the IC-DMP cell bodies forming a region of interdigitation between the two cell types (Fig. 8a,c).

There were also close associations between varicose nerve terminals containing SP-LI and smooth muscle cells (Fig. 12a), and, less occasionally, junctions between nerve terminals with NOS-LI and smooth muscle cells were observed (Fig. 11a). The distances between terminals with SP-LI or NOS-LI and smooth muscle cells were as little as 25 nm. Specialized synapses with pre- and postjunctional membrane specialization were observed at these points of close apposition (Figs. 11b, 12b).

Discussion

IC-DMP of several animal species and humans have been shown to be closely apposed to varicose terminals of enteric neurons (Richardson 1958; Rogers and Burnstock 1966; Gabella 1972, 1974; Faussone-Pellegrini and Cortesini 1983; Rumessen and Thuneberg 1991; Rumessen et al. 1992). These observations have led to the suggestion that IC-DMP may be innervated and possibly functionally intercalated between enteric nerves and the smooth muscle syncytium. Faussone-Pellegrini (1992), in studies of the ultrastructure of ICC in the circular muscle of rat intestine, found that several types of synaptic vesicles are contained in varicosities close to ICC. This suggested that ICC might be innervated and involved in responses to a variety of enteric neurotransmitters. Our study with immunocytochemical techniques has directly demonstrated that IC-DMP are closely associated and form apparent synapselike contacts with enteric neurons containing SP-LI and NOS-LI. Nerve fibers within the circular muscle layer containing SP-LI and NOS-LI are considered signatures for excitatory and inhibitory motor neurons, respectively (McConalogue and Furness 1994). Thus, our data suggest that IC-DMP may mediate or modulate responses to both excitatory and inhibitory neural signals in the small intestine.

There was an extensive and intimate association between excitatory and inhibitory nerve fibers and IC-DMP in the guinea-pig small intestine. IC-DMP were identified at the light level with immunohistochemistry for c-Kit, which has been shown to label all classes of ICC in the GI tract of this species (Komuro et al. 1996; Burns et al. 1997). Because of difficulties encountered with double labeling with c-Kit (which required acetone fixation) and antibodies to neurochemical elements (which gave poor labeling in acetone-fixed tissues), we sought to find an alternate means of immunolabeling IC-DMP. Others have labeled ICC with antibodies to the intermediate filament vimentin (Komuro 1987; Komuro et al. 1996; Ward and Torihashi 1995). We tested anti-vimentin antibodies and confirmed the high level of colocalization of c-Kit-LI and vimentin-LI in IC-DMP. Double labeling with vimentin and NOS or SP antibodies showed that inhibitory and excitatory inhibitory nerve fibers, which densely innervate the DMP, lie in close proximity with IC-DMP.

In previous studies Komuro et al. (1996) demonstrated that IC-DMP of the guinea-pig small intestine had long branching processes but appeared to be independent from the nerve plexus. These authors did not specifically characterize the relationships between IC-DMP and enteric neurons because of the difficulty of simultaneously visualizing vimentin-positive cells and the nerve plexus (Komuro et al. 1996). Use of double-labeling techniques in the present study clearly shows that IC-DMP were intimately associated with SP-LI and NOS-LI nerve fibers. But since the density of nerve fibers in the DMP is high, precise resolution of the proximity of nerve terminals to IC-DMP was difficult at the level of light microscopy.

Ultrastructural analysis was used to study the fine structures of IC-DMP and to determine the degree of proximity of these cells to enteric nerve terminals. Cells identified as IC-DMP were rich in intermediate filaments, and caveolae were found along the plasma membranes. There were definite similarities between the ultrastructure of IC-DMP and smooth muscle cells, and previous investigations have also noted that IC-DMP are similar in many respects to smooth muscle cells (Faussone-Pellegrini 1987; Rumesson et al. 1992; Torihashi et al. 1993; Komuro et al. 1996). IC-DMP also may correspond to the glycogen-rich cells found within the circular muscle of guinea-pig small intestine (Cook and Burnstock 1976), but in the present study, using immunocytochemistry, we did not find glycogen-rich cells in DMP of the guinea-pig ileum. A possible reason for this difference could be the different processing used for electron microscopy. Ultrastructural features of cells depend upon the fixative employed (Llewellyn-Smith et al. 1983). It is also possible that the glycogen-rich cells represent a different type of cell or IC-DMP under different metabolic conditions.

◀ **Fig. 8a–c** Electron micrographs showing immunocytochemistry for SP in the DMP of guinea-pig ileum. **a** An IC-DMP (*ICC*) is shown in close association with two nerve varicosities with SP-LI (*asterisks*). One nerve terminal protrudes into the cytoplasm of the IC-DMP. *Arrowheads* identify caveolae in the membrane of the IC-DMP. The area of membrane identified with *double arrowheads* is shown in higher power in **b** (*M* mitochondria). **c** Enlarged view of the SP-LI nerve identified with ** in **a**. *Arrow* indicates the specialized junction between the plasma membranes of the nerve terminal and IC-DMP

Fig. 9a,b Electron micrographs showing immunocytochemistry for SP in the DMP. **a** An IC-DMP (*ICC*) and the nerve terminals with SP-LI (*asterisks*). One of the terminals is closely associated with the ICC cell body. *Arrowheads* indicate caveolae in the plasma membrane of the IC-DMP (*M* mitochondria). **b** Enlarged view of the SP-LI nerve identified with ** in **a**. The *long arrow* shows the area of membrane specialization between the nerve terminal and the IC-DMP, and the *short arrow* reveals caveolae in the IC-DMP plasma membrane

Fig. 10a–c Electron micrographs showing immunocytochemistry for SP in the DMP. **a** Shows an IC-DMP (*ICC*) and a nerve terminal with SP-LI (**). *Arrowheads* show caveolae in the membrane of the IC-DMP (*M* mitochondrion, *F* filaments). **b** Enlarged view of the caveolae identified with *double arrowheads* in **a**. **c** Specialized junction between the nerve varicosity and the IC-DMP (*arrow*)

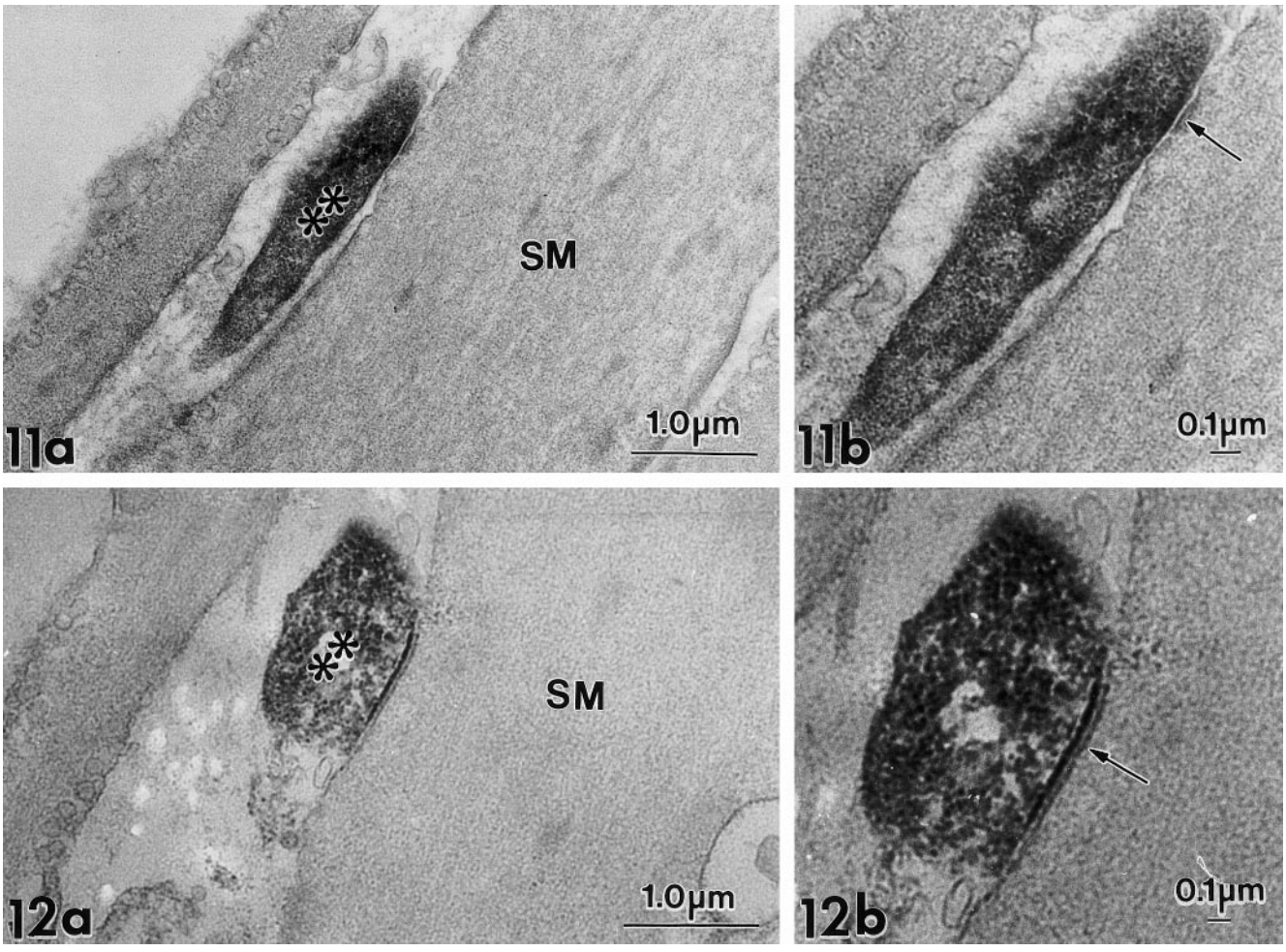


Fig. 11a,b Electron micrographs showing immunocytochemistry for NOS in the DMP. **a** The relationship between a nerve terminal with NOS-LI (*asterisks*) and a smooth muscle cell (*SM*) near the DMP. **b** Enlarged view of the nerve varicosity in **a**. *Arrow* shows a region of membrane specialization at the point of close apposition between the nerve terminal and SM

Fig. 12a,b Electron micrographs showing immunocytochemistry for SP in the DMP. **a** The relationship between a nerve terminal with SP-LI (**) and a smooth muscle cell (*SM*) near the DMP. **b** Enlarged view of the nerve varicosity in **a**. *Arrow* shows a region of membrane specialization at the point of close apposition between the nerve terminal and SM

The immunocytochemical data suggest that IC-DMP are innervated by excitatory and inhibitory motor neurons. In addition to the associations between IC-DMP and enteric nerves observed at the level of light microscopy, we found pre- and postjunctional regions of specialization at points of synaptic contact. Both excitatory and inhibitory neurons formed synapses with IC-DMP. The precise nature of the cytoplasmic structures within the regions of specialization has not yet been investigated.

Some reports suggest a possible role of ICC in mediating nonadrenergic, noncholinergic (NANC) inhibitory inputs in the GI tracts of various species (Huizinga et al. 1990; Burns et al. 1996). The manner in which ICC are involved in inhibitory neurotransmission is not yet clear, however. Inhibitory innervation could occur exclusively through ICC, or there may be parallel innervation of ICC and smooth muscle cells (Sanders 1996). Morphological studies on the opossum esophagus suggest that parallel transmission is likely since enteric neurons are found in close proximity with ICC and smooth muscle cells (Daniel and Posey-Daniel 1984). Studies on the guinea-pig small intestine in which the three-dimensional structure of the nerve terminals was determined by serial sections are consistent with parallel transmission since the specialized structures were found between nerve terminals and ICC as well as between nerve terminals and smooth muscle cells (Klemm, M. and Hirst G.D.S., personal communication). Immunocytochemical experiments in the present study also suggest parallel innervation of the circular muscle layer (Fig. 13), because specialized junctions between enteric nerve terminals and smooth muscle were observed in addition to the synapselike structures between nerve fibers and IC-DMP. At the present time we do not know the relative functional significance of these two possible routes of innervation, but preliminary studies in which IC-DMP were lesioned in the murine small intestine by blocking c-Kit receptors suggest that these cells are critical for NO-dependent neurotransmission (McLaren et al. 1997).

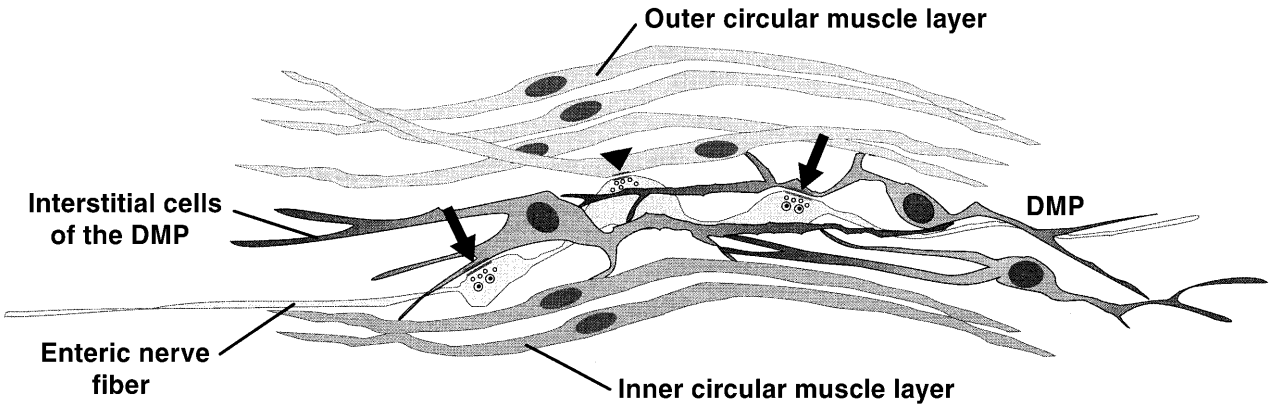


Fig. 13 Model for innervation of the circular muscle layer at the deep muscular plexus (*DMP*). The DMP lies between the inner and outer circular muscle layers in the small intestine and is richly innervated by enteric motor neurons originating in the myenteric plexus. The DMP also contains an extensive network of ICC known as IC-DMP. These cells are closely associated with varicose nerve fibers that form synapselike structures at points of close apposition (*arrows*). Similar structures, however, can also be found between enteric neurons and smooth muscle fibers near the DMP (*arrowhead*), suggesting parallel innervation of IC-DMP and smooth muscle cells

The role of IC-DMP in mediating or modulating nonadrenergic, noncholinergic excitatory responses has also been considered since neurokinin receptors (specifically NK1) have been localized to the cells in the guinea-pig small intestine (Portbury et al. 1996a). Smooth muscle cells in the same preparation lack immunoreactivity to NK1 antibodies, suggesting that tachykinin-dependent regulation of the circular muscle may be partially mediated via IC-DMP. The parallel innervation of IC-DMP and smooth muscle cells by excitatory motor neurons that was observed in the present study may be mediated by different classes of neurokinin receptors. A recent study has reported that NK2 receptors are preferentially expressed by smooth muscle cells in the small intestine (Portbury et al. 1996b).

In conclusion, we have shown that ICC are closely apposed to excitatory and inhibitory enteric motor neurons in the region of the deep muscular plexus. Immunocytochemical evidence strongly suggests frequent points of innervation of IC-DMP since pre- and postsynaptic regions of specialization were observed. Sites of apparent innervation were also detected between enteric motor neurons and smooth muscle cells. These data suggest an important role for IC-DMP in the neural regulation of the small intestine; however, IC-DMP may not be the exclusive route for neural information to reach the smooth muscle.

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