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Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine

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Summary. Immunoreactivity for calretinin, a calcium-binding protein, was studied in neurones in the guinea-pig small intestine. 26±1% of myenteric neurones and 12±3% of submucous neurones were immunoreactive for calretinin. All calretinin-immunoreactive neurones were also immunoreactive for choline acetyltransferase and hence are likely to be cholinergic. In the myenteric plexus, two subtypes of Dogiel type-I calretinin-immunoreactive neurones could be distinguished from their projections and neurochemical coding. Some calretinin-immunoreactive myenteric neurones had short projections to the tertiary plexus, and hence are likely to be cholinergic motor neurones to the longitudinal muscle. Some of these cells were also immunoreactive for substance P. The remaining myenteric neurones, immunoreactive for calretinin, enkephalin, neurofilament protein triplet and substance P, are likely to be orad-projecting, cholinergic interneurones. Calretinin immunoreactivity was also found in cholinergic neurones in the submucosa, which project to the submucosal vasculature and mucosal glands, and which are likely to mediate vasodilation. Thus, calretinin immunoreactivity in the guinea-pig small intestine is confined to three functional classes of cholinergic neurones. It is possible, for the first time, to distinguish these classes of cells from other enteric neurones.

Key words: Calretinin – Enteric nervous system – Calcium binding protein – Small intestine – Cholinergic neurons – Myenteric plexus – Guinea-pig

Immunohistochemical studies of the distribution of a number of peptides and proteins have been valuable in identifying the classes of neurones that contribute to the control of gastrointestinal function (Ekblad et al. 1987; Furness and Costa 1987). By comparing the overlapping distributions of these chemical markers in enteric neurones, it has become apparent that classes of neurones with specific projections can be distinguished according to the combination of markers that they contain. This can be considered as a chemical code that can be used to distinguish different classes of cells (Costa et al. 1986).

Calretinin is a 29 kD calcium-binding protein first identified in chick retina (Rogers 1987), which shares a 58% sequence homology with calbindin. Another highly homologous calcium-binding protein, Protein 10, has been purified from guinea-pig brain (Winsky et al. 1989) and may represent a mammalian form of calretinin. Like calbindin, calretinin immunoreactivity is not found in all neurones, but is restricted to specific populations of neurones in chick sensory ganglia and nuclei (Rogers 1989a), and in chick and rat cerebellum (Rogers 1989b). Calretinin is largely found in neurones that are not immunoreactive for calbindin (Pochet et al. 1989; Rogers 1989a) although coexistence of the two proteins was reported in neurones in the chick dorsal root ganglia, in the inner ear and in the retina (Rogers 1989a).

In the present study the distribution and projections of calretinin-immunoreactive neurones in the guinea-pig small intestine was studied in order to extend our understanding of enteric neuronal circuitry. By combining double and triple labelling immunohistochemistry with lesion studies, the projection and chemical coding of calretinin-immunoreactive neurones was determined.

Materials and methods

Tissue was taken from 38 male and female guinea-pigs (200–350 g), which were killed by a blow to the back of the head followed by severing the carotid arteries. Specimens of small intestine were placed in phosphate-buffered saline (PBS: 0.15 M sodium chloride in 0.01 M sodium phosphate pH 7.2), flushed free of intestinal contents and prepared as whole-mounts for histochemical examination. The intestinal segment was opened along the mesenteric border, stretched and pinned, and fixed for 18 h at 4° C in modified Zamboni's fixative (2% formaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.0) (Stefanini et al. 1967). Tissue was then cleared with dimethylsulphoxide (DMSO), washed

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Table 1. Details of primary antisera used for immunohistochemistry

Antiserum	Code	Species	Dilution	Reference
Calbindin	DEML-RI	Rabbit	1:1600	Furness et al. 1988
Calretinin	Ab-6B	Rabbit	1:1000	Rogers (1989b)
Calretinin	Ab-4B	Rat	1:100	Rogers (1989a, b)
Choline acetyltransferase	Eck2	Rat	1:400	Eckenstein and Thoenen (1982)
Enkephalin	MAS083	Mouse	1:400	Cuello et al. (1984)
Nerve cell body	NK3K	Rabbit	1:200	This paper
Neurofilament protein triplet	IC8	Mouse	1:2000	Vitadello et al. (1986)
Neuropeptide Y	F1/V1	Rat	1:200	Morris et al. (1986)
Protein 10	B10-3	Rabbit	1:800	Winsky et al. (1989)
Substance P	NC1/34MC	Rat	1:200	Cuello et al. (1979)
Tyrosine hydroxylase	2–40-15	Mouse	1:40	Boehringer
Vasoactive intestinal peptide	F1/111	Rat	1:400	Morris et al. (1985)

in PBS and stored in PBS with 0.1% sodium azide. For choline acetyltransferase (ChAT) immunohistochemistry, tissue was collected in phosphate buffer (PB: 0.2 M sodium phosphate pH 7.2) and fixed for 1 h at room temperature in PB with 4% paraformaldehyde and 15% saturated picric acid. It was then cleared with DMSO, washed with PB and stored in PB with 0.1% sodium azide. In order to enhance immunoreactivity for peptides in nerve cell bodies, tissue was maintained in organotypic culture for up to 72 h at 37° C in sterile DME/F12 medium (Sigma). During the final 24 h it was exposed to 250 μM colchicine, then fixed and treated as normal tissue (Steele and Costa 1990b).

Indirect immunofluorescence techniques using primary antisera combined with fluorescent-labelled secondary antibodies or biotin-labelled secondary antibodies with streptavidin-labelled fluorophores, were used for single, double and triple labelling of whole mount preparations. Antisera were diluted with hypertonic PBS to reduce non-specific binding of immunoglobulins to tissue components (Grube 1980). The ChAT antiserum was diluted with 150 mM NaCl, 100 mM TRIS buffer (pH 7.4), containing 2% bovine serum albumin, 5% goat serum, 0.5% Triton X-100 and 0.1% sodium azide. As the actual molecular form of the neurochemicals cannot be established by these techniques, the term 'neurochemical-like immunoreactivity' abbreviated to 'neurochemical immunoreactivity', will be used.

The primary antisera excluding calretinin, protein 10 and nerve cell body (Table 1), have previously been characterized in the guinea-pig small intestine. Calretinin antisera were raised in a rabbit and rat against the 29 kD calretinin protein, and were generously donated by Dr. J. Rogers of Cambridge University, UK. Protein 10 antiserum was raised in a rabbit, and kindly donated by D.M. Jacobowitz (NIH, Bethesda, USA). An antiserum raised by C. Bickford Kandiah (Deakin University, Victoria, Australia) was found to label all nerve cell bodies in the guinea-pig small intestine without labelling axons and varicosities. It was used to establish the proportions of total enteric neurones immunoreactive for various markers and is referred to as nerve cell body (NCB) antiserum. In all double-labelling studies approximately 400 cells in each animal were examined and classified according to their combination of immunoreactivities. Where stated, *n* refers to the number of animals studied.

The second antisera used were as follows. For rabbit primary antisera, fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG (Wellcome Diagnostics, Beckenham, UK, Code 890510) was used at 1:160. For rat primary antisera, one of the following antisera was used: Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, Pa., USA; Code 22172) at 1:80; FITC-conjugated sheep anti-rat IgG (Wellcome Code 6759) at 1:80; biotinylated goat anti-rat IgG (Jackson ImmunoResearch Labs Inc., West Grove, Pa., USA; Code 10956) at 1:50. For mouse primary antisera, either a biotinylated horse anti-mouse IgG (Vector Laboratories Inc, Burlingame, Calif., USA) was used at 1:50, followed by Texas Red-Streptavidin (Amersham, UK; code RPN 1233) diluted 1:50, or 7-amino 4-methyl coumarin (AMCA)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, Code 12671) also used at 1:50.

Tissues were incubated overnight at room temperature with combinations of primary antisera raised in different species for single, double or triple labelling (Wessendorf and Elde 1985; Costa et al. 1986; Staines et al. 1988). Tissues were then rinsed, incubated with combinations of secondary antibodies for 90 min and, where biotinylated secondary antibodies had been used, were further incubated for 90 min with Texas Red-Streptavidin. Following a final rinse, tissues were mounted in bicarbonate-buffered glycerol (pH 8.6) and viewed under fluorescence epi-illumination on a Leitz Laborlux D microscope. Filter block N2 was used to view TRITC and Texas Red fluorescence; filter block L3 to view FITC fluores-

Figs. 1–12. Distribution of calretinin immunoreactivity in whole-mount preparations of the guinea-pig small intestine. Abbreviations: *CALRET* calretinin; *SP* substance P. *Calibration bar* (*bottom right*) – **Fig. 1**: 125 μm; **Figs. 2, 3, 12**: 50 μm; **Figs. 4–11**: 25 μm

Figs. 1, 2. Low and high magnification of myenteric ganglia showing *CALRET*-immunoreactive neurones in myenteric ganglia and many immunoreactive fibres in internodal strands and tertiary plexus

Fig. 3. Myenteric ganglion with a cluster of small simple Dogiel type-I cells near an internodal strand (*arrows*), three faint Dogiel type-II neurones (*arrowheads*), and a single lamellar Dogiel type-I neurone (*open arrow*)

Fig. 4. Cluster of three small simple Dogiel type-I cells in myenteric ganglion near base of an internodal strand (*arrows*)

Fig. 5. Cluster of three lamellar Dogiel type-I cells in centre of myenteric ganglion (*open arrows*)

Fig. 6. Three *CALRET*-immunoreactive neurones in a submucous plexus ganglion (*arrows*)

Fig. 7. *CALRET*-immunoreactive nerve fibres and varicosities in myenteric ganglion (single varicosity, *arrow*)

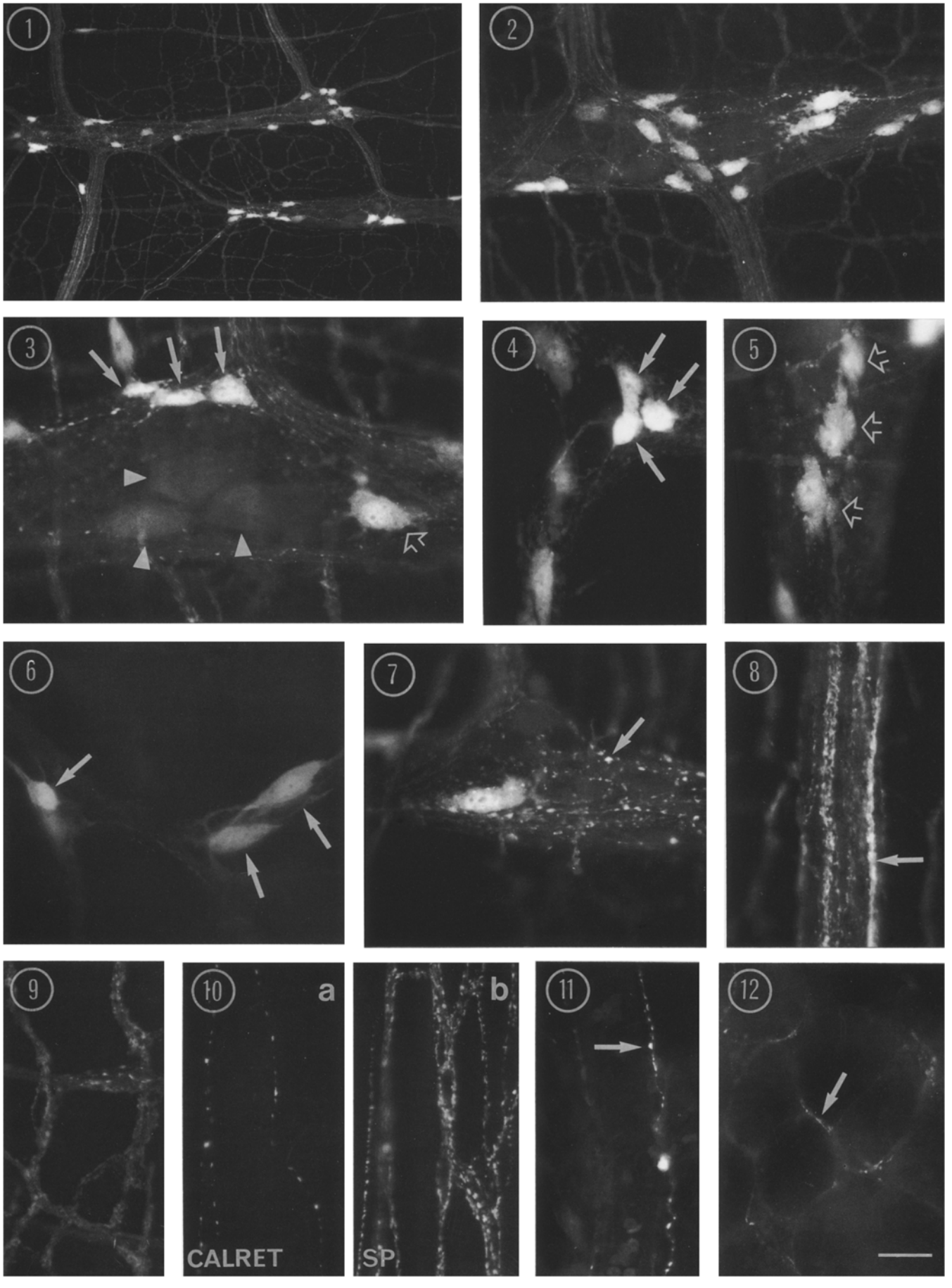
Fig. 8. Immunoreactive nerve fibres in an internodal strand running between rows of myenteric ganglia. Fibres tend to be arranged in bundles with the strand (*arrow*)

Fig. 9. Dense network of *CALRET*-immunoreactive varicose fibres in tertiary plexus

Fig. 10a, b. Sparse innervation of circular muscle by *CALRET*-immunoreactive fibres in deep musular plexus (**a**); for comparison, dense innervation by *SP*-immunoreactive fibres, in the same tissue, is shown in **b**

Fig. 11. Paravascular varicose fibres (*arrow*), immunoreactive for *CALRET*, around a submucous arteriole

Fig. 12. *CALRET*-immunoreactive varicose nerve fibres (*arrow*) around the bases of mucosal glands


1
2
3
4
5
6
7
8
9
10
a
CALRET
b
SP
11
12

cence; and filter block modified from an A2 filter block (with the excitation filter of an H3 filter used as a substitute emission filter together with an additional 430 nm long pass filter) to view AMCA fluorescence. With this combination of filters, AMCA fluorescence was readily distinguishable from FITC fluorescence. Kodak TMAX 400 film, rated at 800 ASA, was used for black and white photography. Controls for double and triple labelling were performed by omitting one or more primary antibodies from the procedure, and by ensuring that all combinations of primary and secondary antisera were free of cross-reactivity.

The three types of in vivo microsurgical lesions have been described in detail elsewhere (Keast et al. 1984). Briefly, animals were anaesthetised with Nembutal (pentobarbitone sodium 60 mg/ml: 0.25 ml/kg) and Hypnorm/Vet (fentanyl citrate 0.315 mg/ml; fluanisone 10 mg/ml: 0.125 ml/kg). To perform a myectomy, a sleeve of longitudinal muscle and attached myenteric plexus was peeled from the full circumference of the gut, leaving a length of intestine without myenteric plexus. Extrinsic denervation was performed by using blunt forceps to crush the nerves running through the mesentery to a loop of intestine. Myotomies consisted of a cut around the full circumference of the gut, to the depth of the submucosa, thus severing all connections in the myenteric plexus. Animals were killed 7 days after the operations to determine the extent of accumulation of immunoreactivity and degeneration of fibres.

For in vitro lesions, tissue was maintained in organotypic culture for up to 3 days. Mucosal and submucosal layers were removed from strips of guinea-pig small intestine in sterile Krebs' solution (composition in mM: NaCl;117, KCl;5, $MgCl_2$;1.2, $CaCl_2$;2.5, NaH_2PO_4;1.2, glucose;10, bubbled with 95% O_2/5% CO_2, pH 7.4) and the remaining muscle layers, with the intervening myenteric plexus, were pinned flat in sterile culture medium as described elsewhere (Brookes and Costa 1990). In lesioned preparations, elements of the myenteric plexus were crushed across the entire circumference of the preparation before the culture period. Over the succeeding 3 days, severed axons degenerated in a similar manner to that seen following in vivo operations, and an accumulation of immunoreactivity developed in the proximal ends of severed axons as described previously (Costa et al. 1980).

Results

Morphology and distribution of nerve cell bodies

Nerve cell bodies displaying calretinin immunoreactivity were abundant in the ganglia of the myenteric plexus, and labelled varicose fibres were clearly visible in the primary, secondary and tertiary branches of the myenteric plexus (Figs. 1, 2). Three subtypes of calretinin-immunoreactive nerve cell bodies could be distinguished. The majority of immunoreactive nerve cell bodies was small (15–25 μm diameter) Dogiel type-I cells (Dogiel 1899; Furness and Costa 1987), with relatively simple shapes, a single axon and few lamellar or filamentous dendrites. These cells were generally located near the edges of the ganglia and were often seen in clusters of 2–4 cells near the internodal strands (Figs. 3, 4). A minority of Dogiel type-I calretinin-immunoreactive neurones were larger (20–50 μm diameter) and had pronounced lamellar dendrites. These cells were usually located on either the mucosal or serosal surface of the ganglia, away from the periphery, and were often clustered in groups of 2–8 cells (Fig. 5). Additionally, very faint calretinin immunoreactivity was occasionally detectable in small numbers of larger (25–60 μm), smooth cells, which displayed Dogiel type-II morphological features.

The proportion of neurones in the myenteric plexus with immunoreactivity for calretinin was established by counting calretinin-immunoreactive neurones in preparations double labelled with nerve cell body (NCB) antiserum. Calretinin immunoreactivity was present in 26 ± 1% (mean ± SD, n = 4) of all myenteric neurones. In submucous ganglia there were relatively few immunoreactive neurones of variable intensity with short, irregular dendrites and a long irregular axon that could be followed, in some cases, to nearby blood vessels, to the non-ganglionated plexus adjacent to the muscularis mucosae and to the base of mucosal glands (Fig. 6). Calretinin-immunoreactive neurones accounted for 12 ± 3% (mean ± SD; $n = 4$) of all submucous neurones.

Nerve fibre distribution

Calretinin-immunoreactive nerve fibres and varicosities formed a moderately dense network within myenteric ganglia of the guinea-pig small intestine (Fig. 7). Numerous varicose nerve fibres were also present in the internodal strands that run between myenteric ganglia and often

Figs. 13–19. Effects of lesions on *CALRET* immunoreactivity; **Figs. 20–22** coexistence of *CALRET* and *ChAT* immunoreactivity. *Abbreviations*: *CONT* control tissue; *CALRET*, calretinin; *ChAT* choline acetyltransferase. *Calibration bar* (*bottom right*) – **Figs. 13–18, 20–22**: 25 μm; **Fig. 19**: 50 μm

Fig. 13. *CONT* after 3 days in organotypic culture with normal density of *CALRET*-immunoreactive varicosities in a myenteric ganglion

Fig. 14. Myenteric ganglion from same tissue as Fig. 13, 200 μm aboral to circumferential nerve crush, with normal density of *CALRET*-immunoreactive varicosities

Fig. 15. Myenteric ganglion from same tissue as Figs. 13 and 14, 200 μm oral to circumferential nerve crush showing complete degeneration of immunoreactive varicose fibres; only axons arising from nerve cell bodies within the ganglion are visible (*arrows*)

Fig. 16. Tertiary plexus in *CONT* after 3 days in organotypic culture with normal density of *CALRET*-immunoreactive nerve fibres

Fig. 17. Tertiary plexus from same tissue as Fig. 16, approximately 400 μm aboral to circumferential nerve crush, with normal density of varicose nerve fibres

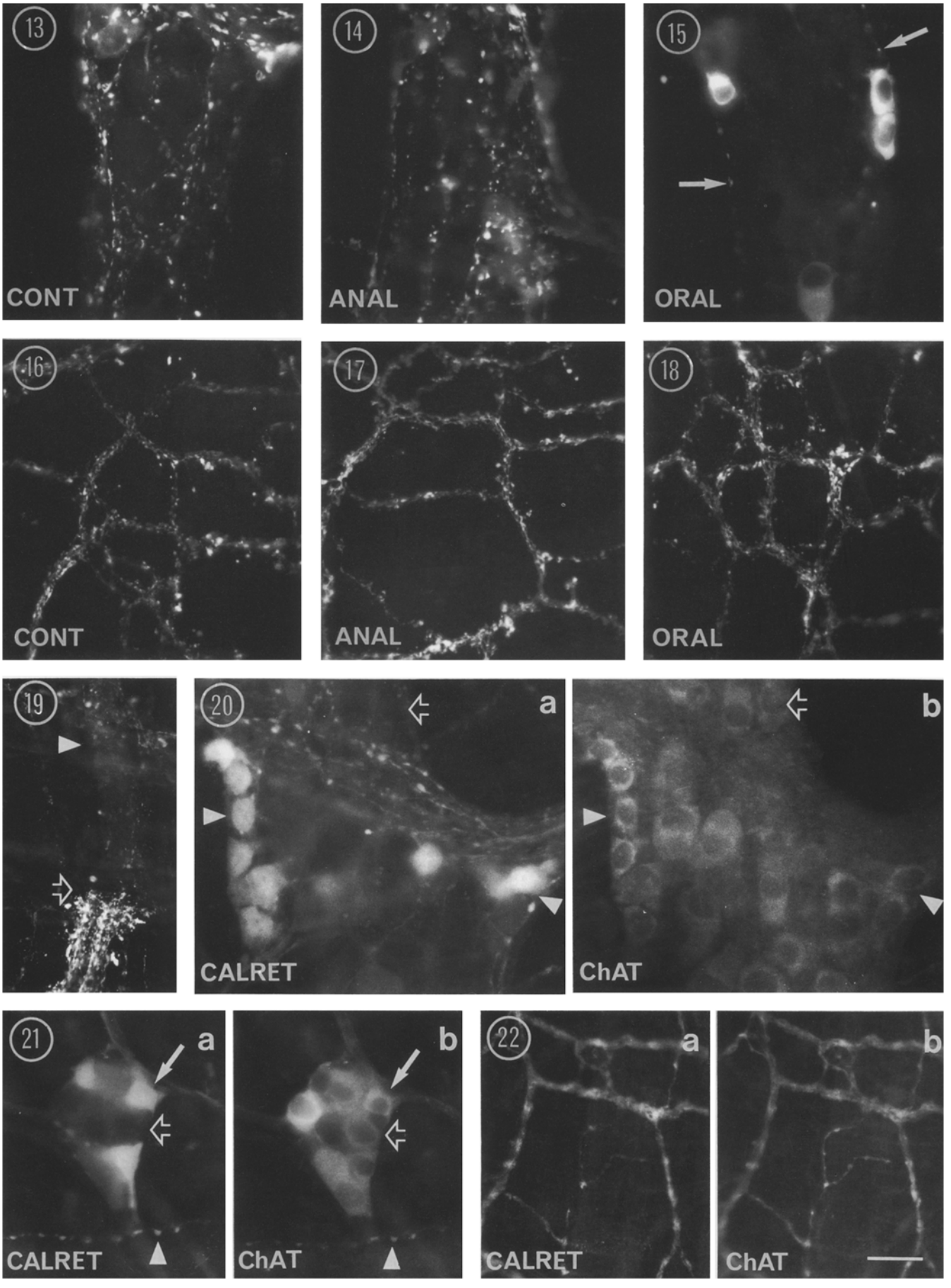
Fig. 18. Tertiary plexus from same tissue as Fig. 16, approximately 400 μm oral to circumferential nerve crush, with normal density of calretinin-immunoreactive varicose nerve fibres

Fig. 19. Accumulation of *CALRET* immunoreactivity (*open arrow*) in internodal strand on aboral side of circumferential nerve crush, after 3 days in organotypic culture. Note the depletion of immunoreactivity oral to the lesion (*arrowhead*)

Fig. 20a, b. Myenteric neurones (*arrowheads*) immunoreactive for both *CALRET* (**a**) and *ChAT* (**b**). All *CALRET*-immunoreactive cells were immunoreactive for *ChAT*, whereas some *ChAT*-immunoreactive neurones were not immunoreactive for *CALRET* (*open arrow*)

Fig. 21a, b. Submucous plexus nerve cell bodies (*arrows*) immunoreactive for *CALRET* (**a**) and *ChAT* (**b**). Some *ChAT*-immunoreactive cell bodies (*open arrow*) are not immunoreactive for *CALRET*. Varicose fibres in paravascular bundles (*arrowhead*) are immunoreactive for both *CALRET* and *ChAT*

Fig. 22a, b. Varicose fibres in the tertiary plexus showing a high degree of coexistence of immunoreactivity for *CALRET* (**a**) and *ChAT* (**b**)


13
CONT
14
ANAL
15
ORAL
16
CONT
17
ANAL
18
ORAL
19
20
a
CALRET
b
ChAT
21
a
CALRET
b
ChAT
22
a
CALRET
b
ChAT

appeared to be organised as discrete bundles within the internodal strands (Fig. 8). Varicose fibres were present in secondary branches of the myenteric plexus. There was a high density of calretinin-immunoreactive varicose fibres in the tertiary plexus (Fig. 9), which lies between internodal strands and myenteric ganglia, and which is believed to innervate the longitudinal muscle layer (Furness and Costa 1987). In contrast, there were very few calretinin-immunoreactive fibres in the deep muscular plexus (Fig. 10), which innervates the circular muscle layer (Llewellyn Smith et al. 1988). In the submucosa and mucosa, calretinin-immunoreactive varicose fibres were seen around submucous arterioles (Fig. 11), in fibres to the muscularis mucosae, and around the base of the mucosal glands (Fig. 12). There were no calretinin-immunoreactive fibres or varicosities in submucous ganglia (Figs. 6, 21a) or in mesenteric nerves.

Projections of myenteric neurones

Projections of calretinin-immunoreactive neurones were established by lesioning experiments carried out in vitro, or in vivo, in 4 animals. After 3 days in organotypic culture a normal density of calretinin-immunoreactive varicosities was present in control myenteric ganglia (Fig. 13). Similarly, in ganglia on the aboral side of a circumferential nerve crush, there was no detectable loss of varicosities although some of the axons showed slight swelling, which could be traced to the site of the crush (Fig. 14). Just oral to such a lesion, however, there was a loss of calretinin-immunoreactive varicosities which was apparent for some distance orally (Fig. 15). Myenteric ganglia are organised into rows, approximately 400–500 μm apart; the loss of varicosities orally, was apparent for up to 6 rows of ganglia (2.5–3 mm). In contrast, no changes in the density of calretinin-immunoreactive varicosities was apparent in the tertiary plexus within a single row of myenteric ganglia from the site of the lesion (Figs. 16–18). There was an accumulation of calretinin immunoreactivity in axons in internodal strands aboral to the lesion but a depletion of immunoreactivity in axons oral to the lesion (Fig. 19). Similar results were obtained with double myotomy lesions performed in vivo, in which the myenteric plexus was severed in two rings around the entire circumference of the gut. These observations are consistent with the existence of two populations of calretinin-immunoreactive neurones in the myenteric plexus; one which projects orally to other myenteric ganglia, and a second population with short projections to the tertiary plexus.

Several days after a myectomy performed in vivo, in which a collar of longitudinal muscle and myenteric plexus was removed from the full circumference of the intestine, there was no detectable change in the distribution of calretinin-immunoreactive fibres to submucous arterioles, muscularis mucosae and around the base of the mucosal glands. This suggests that these fibres do not originate from neurones with cell bodies in the myenteric plexus, but probably arise from cell bodies in submucous ganglia. Extrinsic denervation of a segment of small intestine by crushing the mesenteric nerve trunks did not detectably alter the distribution of calretinin-immunoreactive cells and fibres in the myenteric plexus, submucosa or mucosa.

Coexistence with other neuronal markers

Using double and triple labelling immunohistochemistry, we examined the coexistence of calretinin with previously studied peptide and protein markers in order to distinguish calretinin-immunoreactive neurones that project to the tertiary plexus from those that project orally to myenteric ganglia. Previous studies have shown that there are populations of enteric neurones immunoreactive for substance P (Costa et al. 1981), enkephalin (Furness et al. 1983c), choline acetyltransferase (ChAT; Furness et al. 1983a), vasoactive intestinal polypeptide (Costa and Furness 1983), neuropeptide Y (Furness et al. 1983b; Furness et al. 1985), calbindin (Furness et al. 1988a) and neurofilament protein triplet (Costa et al. 1989) in the guinea-pig small intestine. Double labelling studies were carried out with antiserum to each of these peptides or proteins, together with calretinin antiserum. Two calretinin antisera (both supplied by J. Rogers, Cambridge, UK), raised in rabbit or rat, were used in double-labelling studies after ascertaining that they revealed identical populations of cells in double-labelled preparations.

Initial studies indicated that many enteric neurones were immunoreactive for choline acetyltransferase (ChAT) and hence were likely to be cholinergic (Furness et al. 1983a). Recent developments in ChAT immunohistochemistry (Steele and Costa 1990a) have permitted double labelling with calretinin antisera, which showed that all calretinin-immunoreactive nerve cell bodies in

Figs. 23–27. Coexistence of *CALRET*, *ENK*, *NF* and *SP* in nerve cell bodies and fibres in the guinea-pig small intestine. *Abbreviations*: *CALRET* calretinin; *ENK* enkephalin; *NF* neurofilament protein triplet; *SP* substance P. *Calibration bar* (*bottom right*); all figures: 25 μm

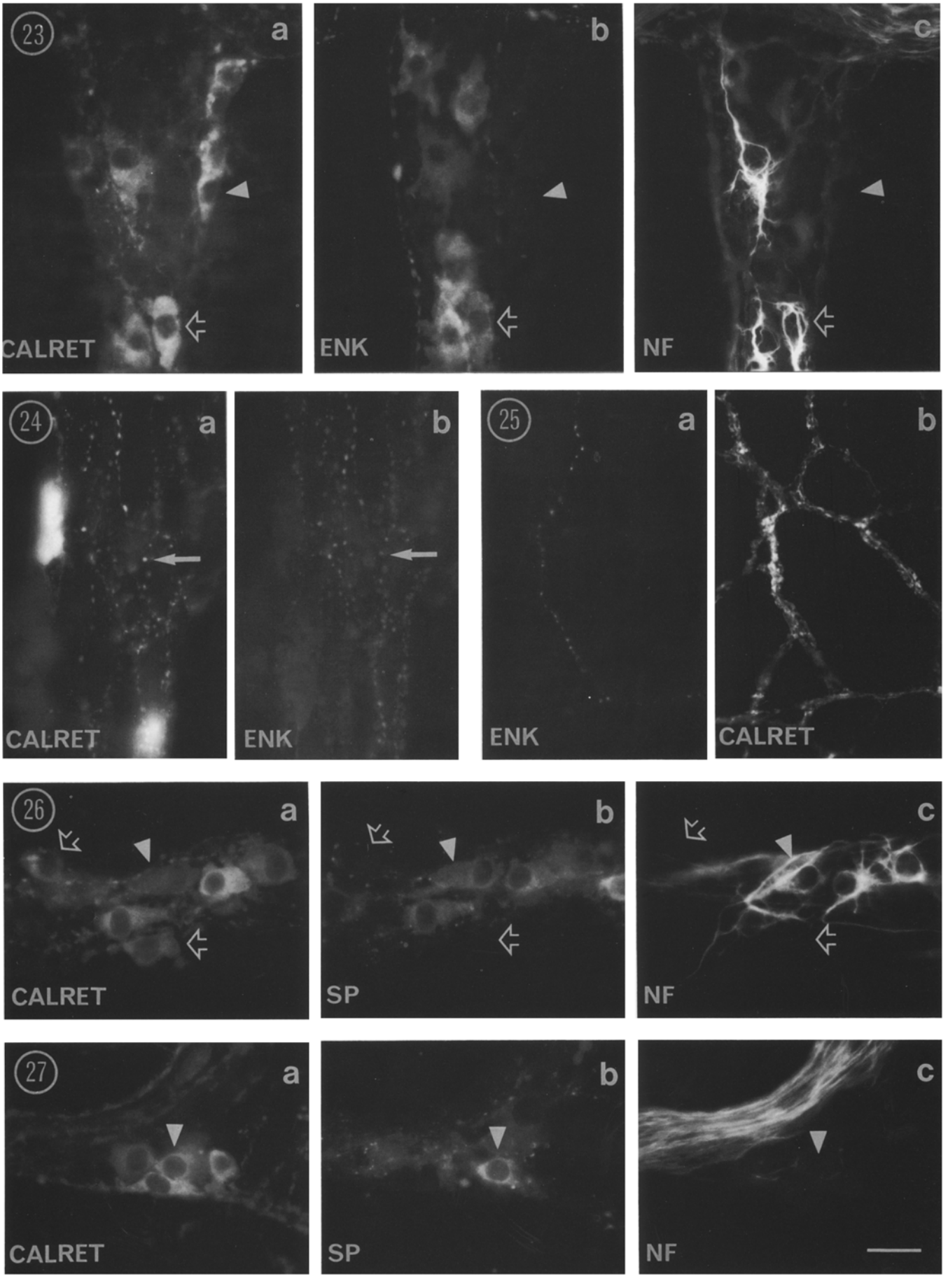
Fig.23a–c. Myenteric neurones immunoreactive for *CALRET* (**a**), *ENK* (**b**) and *NF* (**c**). *CALRET*-immunoreactive cells were either immunoreactive for both *ENK* and *NF* (*open arrow*) or for neither (*arrowhead*)

Fig. 24a, b. Varicose fibres (*arrow*) in myenteric ganglion immunoreactive for *CALRET* (**a**), and also immunoreactive for *ENK* (**b**). Likewise, *ENK*-immunoreactive fibres were also immunoreactive for *CALRET*

Fig. 25a, b. *ENK*-immunoreactive varicose nerve fibres were sparse in the tertiary plexus (**a**) compared to *CALRET*-immunoreactive fibres (**b**). There was no detectable coexistence of *CALRET* and *ENK* immunoreactivities in nerve fibres in the tertiary plexus

Fig. 26a–c. Lamellar neurones (*arrowhead*) immunoreactive for *CALRET* (**a**) were also immunoreactive for *SP* (**b**) and *NF* (**c**). Also present, two cells immunoreactive for *CALRET* (*open arrows*), which were not immunoreactive for either *SP* or *NF*

Fig. 27a–c. Cluster of four small, simple *CALRET*-immunoreactive neurones near the base of internodal strand (**a**). Only one of these cells (*arrowhead*) was immunoreactive for *SP* (**b**), none was immunoreactive for *NF* (**c**)


23
a
CALRET
b
ENK
c
NF
24
a
CALRET
b
ENK
25
a
ENK
b
CALRET
26
a
CALRET
b
SP
c
NF
27
a
CALRET
b
SP
c
NF

the myenteric (Fig. 20) and submucous ganglia (Fig. 21) were also ChAT immunoreactive. Calretinin-immunoreactive varicosities in tertiary plexus (Fig. 22), myenteric ganglia, around submucous arterioles (Fig. 21) and in the muscularis mucosae were also ChAT immunoreactive. There were, however, many ChAT-immunoreactive neurones in myenteric and submucous ganglia that were not immunoreactive for calretinin. Preliminary studies of the chemical coding of ChAT-immunoreactive neurones in the myenteric plexus have been published elsewhere (Steele and Costa 1990a).

Previous studies have demonstrated the existence of a population of enkephalin-immunoreactive myenteric neurones that project orally, giving rise to immunoreactive varicosities in myenteric ganglia (Furness et al. 1983c). The same studies also showed a relatively sparse innervation of the tertiary plexus by enkephalin-immunoreactive varicose fibres. The coexistence of enkephalin with calretinin in myenteric neurones was studied to determine whether it could distinguish calretinin-immunoreactive neurones that project orally to myenteric ganglia from those that project locally to the tertiary plexus. In colchicine-treated, double-labelled preparations, $17 \pm 7\%$ (mean $\pm$ SD; $n=4$) of calretinin-immunoreactive neurons were also immunoreactive for enkephalin.

Previous studies have revealed a population of myenteric neurones with orally directed axons, immunoreactive for neurofilament protein triplet and enkephalin (Costa et al. 1989). In double-labelled preparations, $21 \pm 5\%$ (mean $\pm$ SD) of calretinin-immunoreactive neurones were also immunoreactive for neurofilament protein triplet. In order to test the possibility that the same population of calretinin-immunoreactive neurones might be immunoreactive for both enkephalin and neurofilament protein triplet, triple-labelling immunohistochemical preparations were studied. In preparations labelled for calretinin, enkephalin and neurofilament protein triplet, there was complete coexistence of enkephalin and neurofilament protein triplet immunoreactivities in calretinin-immunoreactive neurones (Fig. 23).

Within myenteric ganglia there was complete coexistence of calretinin and enkephalin immunoreactivity in varicose fibres (Fig. 24); however, there was no detectable coexistence of calretinin and enkephalin in fibres and varicosities in the tertiary plexus (Fig. 25). This confirmed that neurones immunoreactive for calretinin, enkephalin and neurofilament protein triplet project orally to myenteric ganglia, whereas calretinin-immunoreactive motor neurones that lack enkephalin or neurofilament protein triplet immunoreactivity project locally to the tertiary plexus.

In submucous ganglia there was no coexistence of calretinin, enkephalin or neurofilament protein triplet immunoreactivities.

Costa et al. (1989) reported that orally projecting neurones immunoreactive for enkephalin and neurofilament protein triplet are also immunoreactive for substance P. In double-labelled preparations in the present study, a substantial proportion ($35 \pm 8\%$, mean $\pm$ SD; $n=4$) of calretinin-immunoreactive neurones in myenteric ganglia were also immunoreactive for substance P (SP). To determine whether the calretinin/enkephalin/neurofilament protein triplet-immunoreactive neurones that project orally to myenteric ganglia were also SP-immunoreactive, triple-labelled immunohistochemical preparations were studied. In preparations labelled with antisera to calretinin, neurofilament protein triplet and SP, all calretinin/neurofilament protein triplet-immunoreactive cells were intensely immunoreactive for SP (Fig. 26). In contrast, SP immunoreactivity was present in only a small proportion of calretinin-immunoreactive nerve cell bodies that were not neurofilament protein triplet immunoreactive, (Fig. 27), and in most of these cells SP immunoreactivity was relatively faint.

In double-labelled preparations, there was no coexistence of calbindin immunoreactivity in Dogiel type-I calretinin-immunoreactive myenteric neurones. As mentioned previously, Dogiel type-II cells with very faint calretinin immunoreactivity were occasionally seen in the myenteric plexus; these cells were immunoreactive for calbindin in double-labelled preparations but were not studied further, due to the unreliability of labelling. There was no coexistence of calretinin and calbindin immunoreactivities in the submucous plexus.

Double labelling with antisera to calretinin and protein 10 revealed identical distributions of immunoreactive nerve cell bodies and fibres in myenteric and submucous plexuses.

Double labelling with antisera to calretinin and either vasoactive intestinal polypeptide or neuropeptide Y showed that there was no detectable coexistence of immunoreactivity in nerve cell bodies or varicosities in the myenteric or submucous plexuses.

Discussion

All calretinin-immunoreactive neurones in the guinea-pig small intestine were also immunoreactive for choline acetyltransferase (ChAT), and comprise a proportion of enteric cholinergic neurones. On the basis of distribution, morphology, projections and neurochemical coding, calretinin-immunoreactive cells could be divided into two classes of myenteric, and one class of submucous neurones.

Myenteric interneurones

The present study has described a class of myenteric neurones with orally directed axons and lamellar dendrites, located in clusters on either surface of the ganglia, which are immunoreactive for calretinin, ChAT, enkephalin, neurofilament protein triplet and substance P. From total cell counts, this class of neurones comprises approximately 5% of all cells in myenteric ganglia. Neurones with this coding are likely to give rise to all of the enkephalin-immunoreactive varicosities that have been previously described in the myenteric ganglia (Furness et al. 1983c) and which the present study has shown to be immunoreactive for calretinin.

The presence of calretinin/enkephalin-immunoreactive varicosities within myenteric ganglia, which may be

presumed to be transmitter release sites, suggests that nerve cells of this class are either interneurones or sensory neurones. Because no nerve fibres with this coding are present in longitudinal or circular muscle layers, submucosa, mucosa or mesenteric nerves, they are unlikely to be motor neurones. Calretinin-, ChAT-, enkephalin-, neurofilament protein triplet- and substance P-immunoreactive neurones, which have Dogiel type-I morphological features, are unlikely to be sensory neurones, for three reasons. Firstly, the morphology of Dogiel type-I neurones, which have a single long nerve process, is less suited to a sensory role than the multipolar morphology of Dogiel type-II cells (Furness et al. 1988b). Secondly, the electrophysiological characteristics of Dogiel type-I cells are less compatible with a sensory function than the electrophysiology of Dogiel type-II cells. It is well established that Dogiel type-I neurones have S-cell electrophysiology, and that Dogiel type-II neurones have AH-cell electrophysiological characteristics, in the guinea-pig small intestine (Bornstein et al. 1984; Erde et al. 1985; Katayama et al. 1986; Iyer et al. 1988). Fast excitatory synaptic inputs, a characteristics feature of S cells, are largely absent from AH cells, an observation that led to the initial suggestion that AH cells (and hence Dogiel type-II cells) were likely to play a sensory role (Hirst et al. 1974). The presence of strong excitatory synaptic inputs in Dogiel type-I cells is fully compatible with calretinin-, ChAT-, enkephalin-, neurofilament protein triplet-substance P-immunoreactive nerve cells being interneurones. Thirdly, calretinin-immunoreactive neurones cannot be sensory neurones that respond to mucosal stimulation (Smith and Furness 1988) since calretinin-immunoreactive myenteric neurones do not project to the mucosa. Thus, the chemical coding of the calretinin-, ChAT-, enkephalin-, neurofilament protein triplet- and substance P-immunoreactive cells makes it possible to distinguish this class of orally directed cholinergic interneurones from other types of neurones in the myenteric plexus of the guinea-pig small intestine.

Physiological studies are consistent with the existence of a population of cholinergic ascending interneurones in the guinea-pig small intestine since ascending reflexes, sensitive to hexamethonium, can propagate over long distances in the gut (Costa and Furness 1976; Tonini and Costa 1990). It is interesting that there is a hexamethonium-resistant component of intestinal reflexes that may be mediated by substance P (Bartho et al. 1989; Holzer 1989), which the present study has also localised immunohistochemically in this class of orally directed interneurones.

Myenteric motor neurones to the longitudinal muscle

The population of calretinin-immunoreactive neurones that are also immunoreactive for ChAT, but not for enkephalin or neurofilament protein triplet, are found in the tertiary plexus and thus supply the longitudinal muscle. Since fibres with this coding are present only in the tertiary plexus, they must be cholinergic motor neurones to the longitudinal muscle. There was no detectable degeneration of calretinin-immunoreactive nerve fibres in the tertiary plexus beyond 400 μm of a circumferential lesion. This indicates that calretinin-immunoreactive motor neurones to the longitudinal muscle have short local projections without distinct oral or anal polarity. From total cell counts, calretinin-immunoreactive longitudinal muscle motor neurones account for approximately 21% of all myenteric neurones. Since calretinin-immunoreactive varicose nerve fibers are scarce in the circular muscle layer but very dense in the tertiary plexus, motor neurones to the longitudinal muscle and circular muscle layer must be largely separate populations.

Acetylcholine is the major excitatory transmitter to the longitudinal muscle layer although a substantial atropine-resistant excitatory input also exists (Kosterlitz and Lees 1964; Ambache and Freeman 1968). The non-cholinergic excitatory transmitter to the longitudinal muscle layer is likely to be substance P since the atropine-resistant contraction is reduced by substance P desensitisation (Franco et al. 1979). The presence of substance P immunoreactivity in some calretinin-immunoreactive longitudinal muscle motor neurones suggests that substance P is released from some cholinergic motor neurones. However, many substance P-immunoreactive varicosities in the tertiary plexus were not immunoreactive for calretinin; so the possibility of an additional population of non cholinergic excitatory motor neurones, containing substance P, remains open.

Thus, calretinin immunoreactivity, combined with a lack of enkephalin or neurofilament protein triplet immunoreactivity, distinguishes a significant proportion of motor neurones to the longitudinal muscle from other cell types in the myenteric plexus.

Submucous vasomotor neurones

Calretinin-immunoreactive nerve cell bodies in submucous ganglia, and immunoreactive varicose fibres in the muscularis mucosa, around the base of glands and submucous arterioles, were all ChAT immunoreactive. Cholinergic neurones in the submucous plexus have recently been demonstrated to cause vasodilation in submucosal arterioles (Neild et al. 1990), and it is likely that this represents a role for some of the calretinin-immunoreactive neurones in the submucous plexus. However, calretinin-immunoreactive varicose fibres, which do not disappear following surgical removal of the underlying myenteric plexus, are also found in other targets in the mucosa and submucosa. It is possible that the same calretinin-immunoreactive neurones in the submucous plexus supply varicose fibres to submucous blood vessels, to the non-ganglionated plexus adjacent to the muscularis mucosa and to the base of the mucosal glands.

Significance of calretinin in enteric neurones

Calretinin and calbindin immunoreactivities have been found in largely separate populations of neurones in the

guinea-pig small intestine as has previously been reported in rat and chick cerebellum (Rogers 1989b), in chick sensory nuclei and ganglia (Rogers 1989a), and in regions of mammalian brain (Pochet et al. 1989) although coexistence of the two calcium-binding proteins was seen in neurones in the dorsal root ganglia, inner ear and retina (Rogers 1989a). It remains uncertain whether the very faint calretinin immunoreactivity observed in occasional calbindin immunoreactive Dogiel type-II cells in the guinea-pig myenteric plexus reflects the presence of low levels of true calretinin, or cross reactivity of calretinin antisera against calbindin. Complete coexistence of calretinin and protein 10 immunoreactivity in enteric neurones supports the suggestion that protein 10 is a mammalian form of calretinin (Winsky et al. 1989).

The role of calcium-binding proteins in enteric nerve cells remains uncertain. It has been suggested that calbindin in AH neurones in the myenteric plexus (Iyer et al. 1988) might be involved in the buffering of calcium that enters the cell body during the soma action potential (Hirst and Spence 1973; North 1973; Furness et al. 1988a). In this study it has been demonstrated that calretinin immunoreactivity is localised in the myenteric plexus, exclusively in Dogiel type-I cells, which have been previously demonstrated to have S-cell electrophysiological characteristics (Bornstein et al. 1984; Erde et al. 1985; Katayama et al. 1986; Iyer et al. 1988) and which do not have a significant calcium component to their soma action potentials (Hirst et al. 1974). However, S cells do receive fast excitatory nicotinic synpatic inputs (Nishi and North 1973; Hirst et al. 1974); activation of nicotinic receptors in these cells by acetylcholine can evoke a calcium influx (Tokimasa et al. 1983). Calretinin might be involved in buffering intracellular calcium from this or other sources. It should be noted that a considerable proportion of Dogiel type-I cells (and hence S cells, physiologically) are immunoreactive for neither calbindin nor calretinin but may contain other, as yet unspecified, calcium-binding proteins.

Calretinin immunoreactivity in the guinea-pig small intestine is confined to three classes of cholinergic neurones for which functional roles have been proposed. For the first time it is possible to distinguish these classes of cells from the many other neurones in the enteric nervous system of the guinea-pig small intestine.

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