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Galanin-like immunoreactivity in enteric neurons of the human colon

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INTRODUCTION

Galanin, a peptide of twenty-nine amino acid residues which was originally isolated from porcine intestine (Tatemoto *et al.* 1983), has pharmacological actions which suggest physiological importance in several gastrointestinal systems. For example, it causes contraction in rat jejunum by a direct action, stimulates release of acetylcholine and substance P from neurons in guinea-pig taenia coli, and has an inhibitory modulatory action on excitatory neuromuscular transmission in the guinea-pig taenia coli (Ekblad, Håkanson, Sundler & Wahlestedt, 1985*a*). In the guinea-pig ileum, galanin inhibits release of acetylcholine, whether evoked by electrical stimulation, vasoactive intestinal polypeptide or substance P (Yau, Porsett & Youther, 1986). Further, galanin has been shown to inhibit insulin secretion and induce hyperglycaemia in dogs (McDonald *et al.* 1985).

The peptide appears to be located throughout the gastrointestinal tract of many species, with cell bodies that contain galanin-like immunoreactivity (galanin-LI) in the myenteric plexus and submucous plexus, and with fibres projecting to all layers of the gut wall of several species (Melander *et al.* 1985; Ekblad, Rökåeus, Håkanson & Sundler, 1985*b*; Furness *et al.* 1987; Fehér & Burnstock, 1988) including man (Bauer *et al.* 1988). In the rat, most of the galanin in the gastrointestinal tract belongs to intrinsic neurons with little or none contained in the vagus or sympathetic neurons (Ekblad *et al.* 1985*b*); however, a primary sensory distribution has also been observed in the rat (Ch'ng *et al.* 1985). In addition to being located in the spinal cord, galanin is widely distributed in the brain (Ch'ng *et al.* 1985; Rökåeus *et al.* 1984; Skofitsch & Jakobowitz, 1985, 1986) and galanin-binding sites have been identified therein (Melander, Hökfelt, Nilsson & Brodin, 1986).

The object of the current study was to identify the sublocalisation of galanin-LI in human distal sigmoid colon using immunocytochemical techniques. In particular, its distribution within the submucous plexus was examined.

MATERIALS AND METHODS

Samples of human distal sigmoid colon were obtained from patients who had undergone a resection for rectal carcinoma or villous adenoma (one patient). The samples were taken from uninvolved regions remote from the tumour or adenoma. Tissues were obtained from six females aged 38–71 years (mean = 52 years) and six males aged 26–90 years (mean = 59 years). Samples the full thickness of the gut wall, approximately 1 cm square, were fixed in 4% (w/v) paraformaldehyde in 0·1 M phosphate-buffered saline (PBS) (pH 7·3) for 90–120 minutes. After washing in PBS

(3 × 10 minutes), tissues for cryostat section (5–10 μm) were placed in 7% sucrose in PBS overnight at 4 °C. Wholemounts of divisions of the submucous plexus, i.e. Henle's plexus, intermediate plexus and Meissner's plexus, were prepared as described previously (Hoyle & Burnstock, 1989). Wholemount preparations were dehydrated and rehydrated through graded alcohols and washed with 0·1% Triton in PBS (3 × 10 minutes). Sections and wholemounts were incubated with primary antibodies (1:250 and 1:200 dilution respectively) at room temperature (19–23 °C) for 15 hours; the preparations were then washed with PBS and incubated with goat-anti-rabbit fluorescein isothiocyanate- (FITC, Nordic) conjugated secondary antibodies (1:50 dilution) for 1 hour at room temperature. Preparations were then washed in PBS. In order to reduce background fluorescence, sections were incubated with pontamine sky-blue (PBS/0·05% pontamine sky-blue/1% dimethylsulphoxide) for 10 minutes (Cowen, Haven & Burnstock, 1985) before rinsing in PBS and mounting in Citifluor (City University, London). Owing to the high content of connective tissue in the wholemount preparations, there was a very high background fluorescence. Neither pontamine sky-blue nor pre-adsorption of the secondary antibodies with either normal goat serum (1:50–1:10 dilution) or normal human serum (1:50–1:10 dilution) improved the background; however, pre-incubation of the secondary antibodies with submucosal tissue (1:1, v/v) for 30–60 minutes at room temperature appreciably reduced the background non-specific fluorescence. Some sections and wholemounts were stained for NADH-diaphorase (Hoyle & Burnstock, 1989) before being dehydrated and before the primary antibodies were applied. However, this raised the level of autofluorescence in the tissues and also suppressed the specific fluorescence.

Galanin-antibody specificity

The antigalanin antibody was generously supplied by Dr J. Wharton of The Royal Postgraduate Medical School, Hammersmith Hospital. Specificity in the human distal colon was tested by pre-adsorbing the antibody (diluted 1:250) with either galanin (6×10^{-11}–6×10^{-4} M) or an inappropriate peptide for 1 hour at room temperature before applying it to the sections. At 6×10^{-9} M, galanin noticeably decreased the specific fluorescence in sections, and at 6×10^{-7} M all specific fluorescence was abolished. Bombesin (3×10^{-6}–3×10^{-4} M), vasoactive intestinal polypeptide (3×10^{-7}–3×10^{-5} M), neuropeptide Y (10^{-6}–10^{-4} M), methionine–enkephalin (10^{-6}–10^{-4} M), leucine–enkephalin (10^{-6}–10^{-4} M), substance P (10^{-6}–10^{-4} M) and neurotensin (10^{-6}–10^{-4} M) had no effect on the galanin-LI.

Statistical analyses

Means of groups were compared using analysis of variance, and distributions were compared using chi-squared tests. A probability level of $P < 0.05$ was accepted as being significant.

OBSERVATIONS

Nerve fibres containing galanin-LI were present in all the muscle layers of the distal sigmoid colon. The innervation appeared denser in the circular muscle than in the longitudinal muscle, whether taenia coli or inter-taenial, and denser than that of the muscularis mucosae.

Within the mucosa, fine branching fibres were seen in close apposition to epithelial cells at the base of the crypts and, within the lamina propria, extending to the bases of the epithelial cells lining the lumen of colon (Fig. 1*a*,*b*). There was no galanin-LI in epithelial cells.

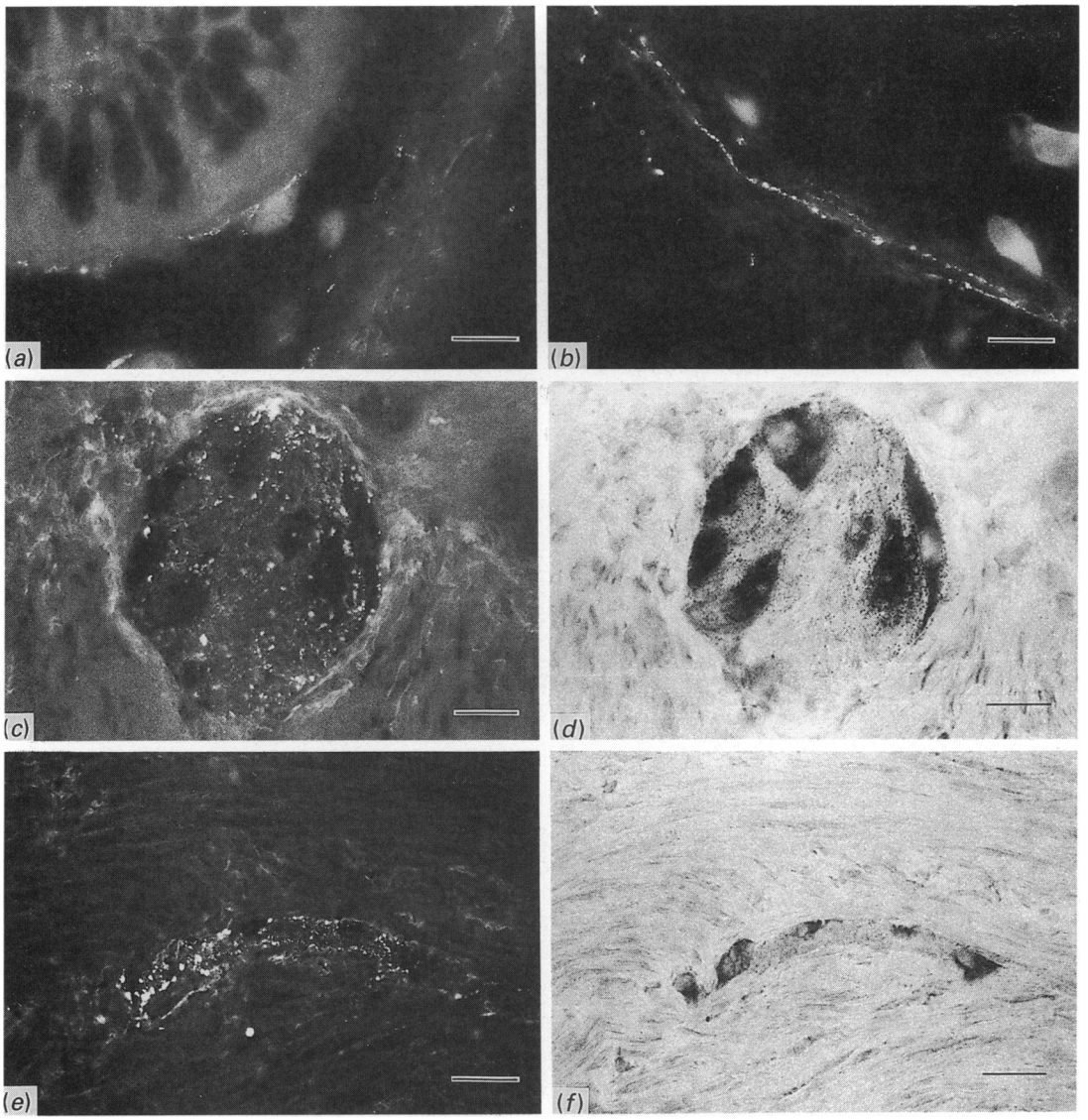

Fig. 1 (*a–f*). Galanin-like immunoreactivity in the wall of the human distal colon. (*a*) Fine varicose fibres with galanin-LI lie around the base of a crypt (top left) and (*b*) within the lamina propria (the bright epithelial cell did not contain galanin-LI). In ganglia of the myenteric plexus, (*c*) and (*e*), galanin-LI was dispersed. (*d*) and (*f*) are the same fields as in (*c*) and (*e*) respectively; these sections were stained for NADH-diaphorase activity prior to immunostaining for galanin-LI. The ganglion shown in (*c*) and (*d*) lies between the longitudinal muscle layer (above it) and the circular muscle layer (below it). The ganglion in (*f*) was embedded within the longitudinal muscle. Scale bars represent 25 μm in (*a–d*) and 40 μm in (*e*) and (*f*).

In the myenteric plexus, nearly every ganglion contained galanin-LI (Fig. 1*c–f*). However, nerve cell bodies positive for galanin-LI were seen only rarely in the plexus.

In the submucosa, all the galanin-LI was related to the submucous plexus. No association of fibres containing galanin-LI with blood vessels was seen. Many ganglia from all divisions of the submucous plexus (Henle's plexus, the intermediate plexus and Meissner's plexus, as defined by Hoyle & Burnstock, 1989) contained varicose

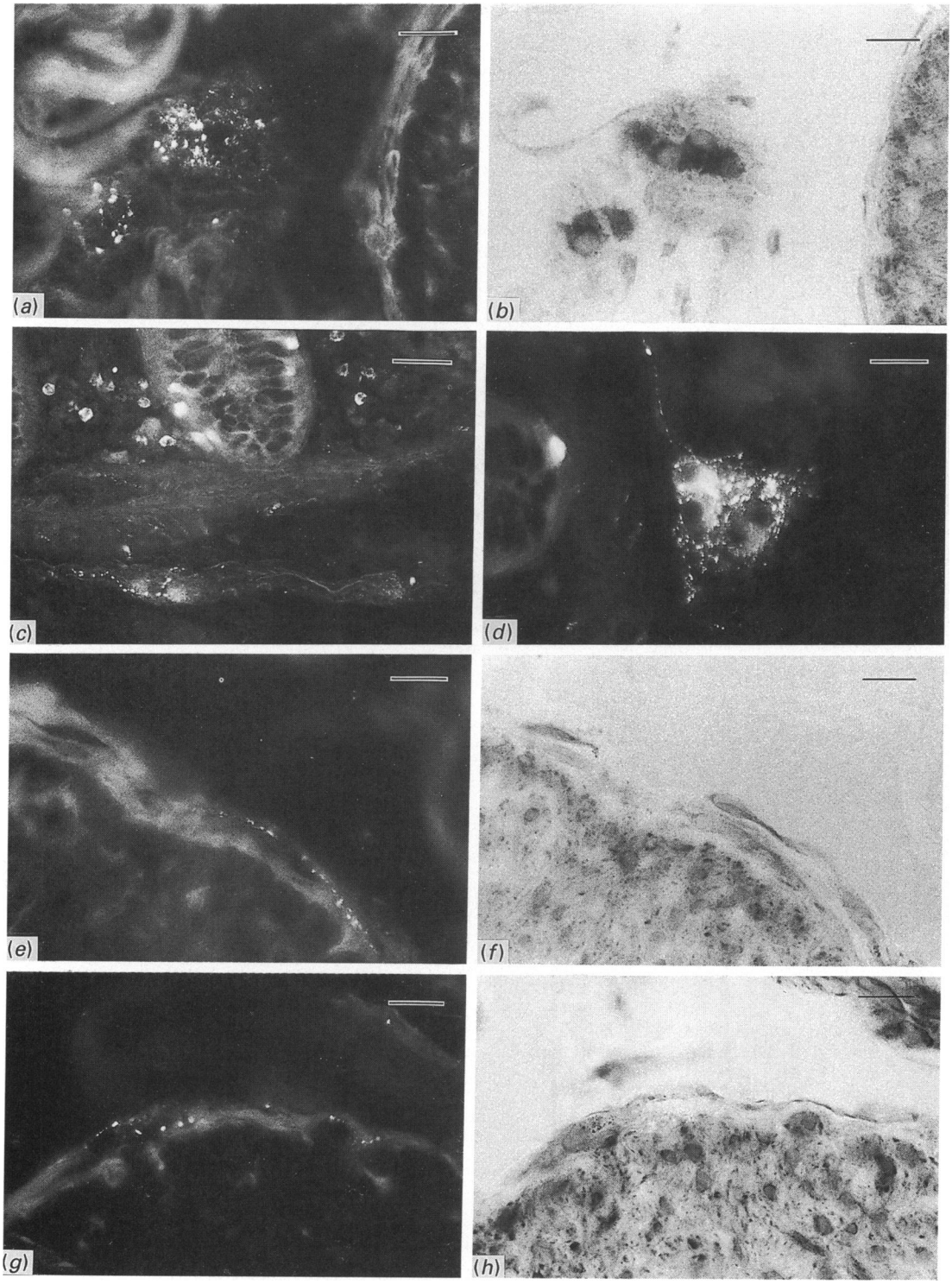

Fig. 2(*a–h*). Galanin-like immunoreactivity in the submucous plexus of the human colon. A ganglion from Henle's plexus (*a*) contains galanin-LI; the same field is shown in (*b*) with NADH-diaphorase staining. Ganglia in Meissner's plexus (*c*,*d*) often appeared to send varicose processes which ran close to the muscularis mucosae (running left to right in (*c*), below the base of a crypt, and running vertically in (*d*) with the base of a crypt on the left). Varicose fibres of the *plexus entericus* (*submucosus*) *extremus* containing galanin-LI were associated with the fibroblast-like cells adjacent to the circular muscle (*e*,*g*). The same fields stained for NADH-diaphorase activity are shown in (*f*) and (*h*). Scale bars represent 16 μm in (*a*,*b*,*f–h*) and 25 μm in (*c*) and (*d*).

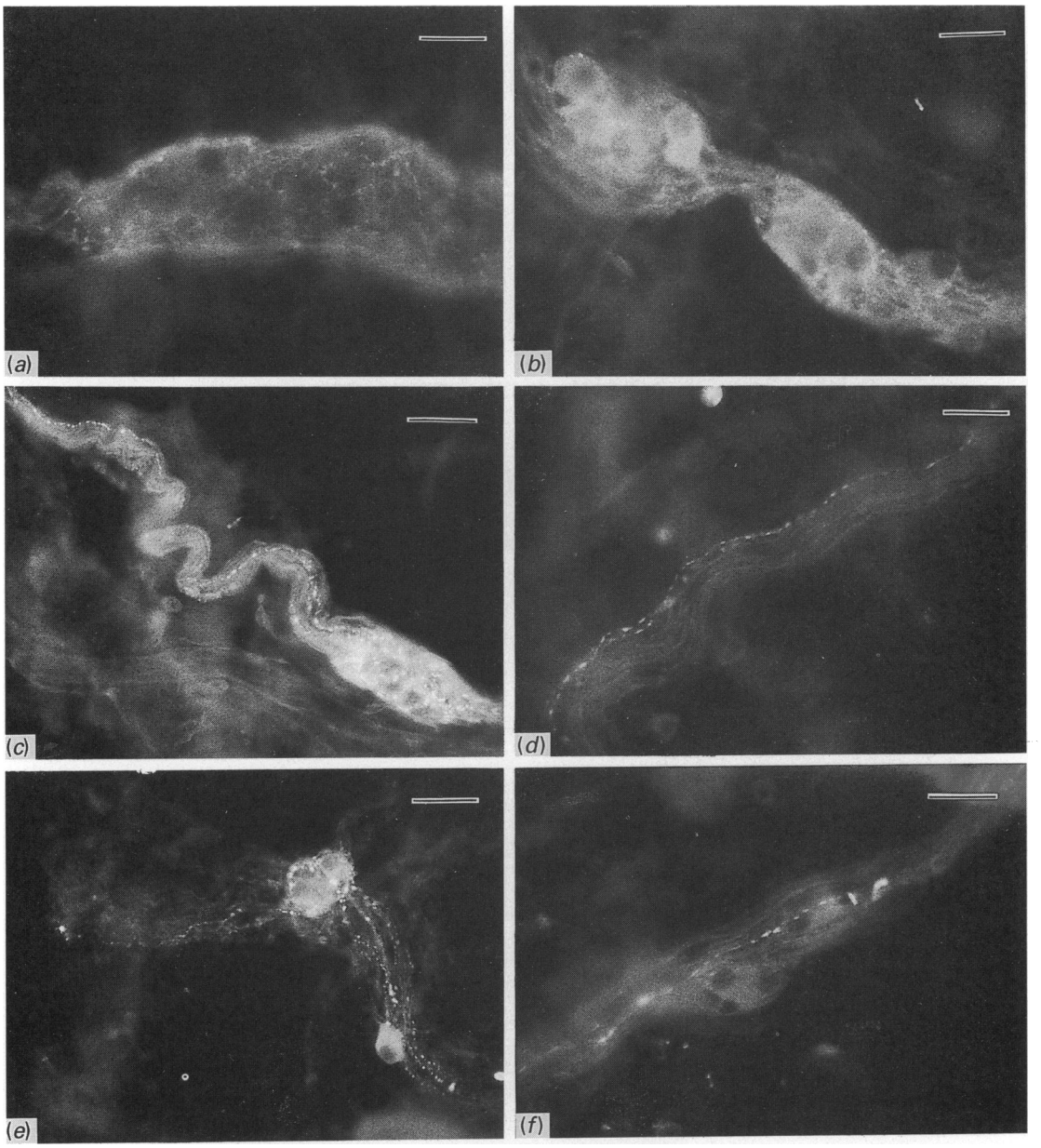

Fig. 3(*a–f*). Galanin-like immunoreactivity in wholemount preparations of divisions of the submucous plexus of the human distal colon. Ganglia with immunoreactive cell bodies and varicose networks were seen in all divisions. (*a*) and (*b*) were from Henle's plexus, (*c*) and (*e*) were from the intermediate plexus and (*f*) was from Meissner's plexus. Varicose fibres were seen in tracts as in (*d*), from the intermediate plexus. Scale bars represent 25 μm in (*a,b,d*) and (*f*), and 40 μm in (*c*) and (*e*).

nerve fibres and/or cell bodies with galanin-LI (Fig. 2*a–d*). Neurons in Meissner's plexus appeared to project to the muscularis mucosae and structures of the mucosa.

Very fine nerve fibres containing galanin-LI were seen at the margin of the circular muscle. At the junction of the submucosa and the circular muscle, a layer of non-neuronal, non-muscular fibroblast-like cells was often seen which stained for NADH-diaphorase (Fig. 2*b,f,h*). This layer was up to two cells thick but not always clearly

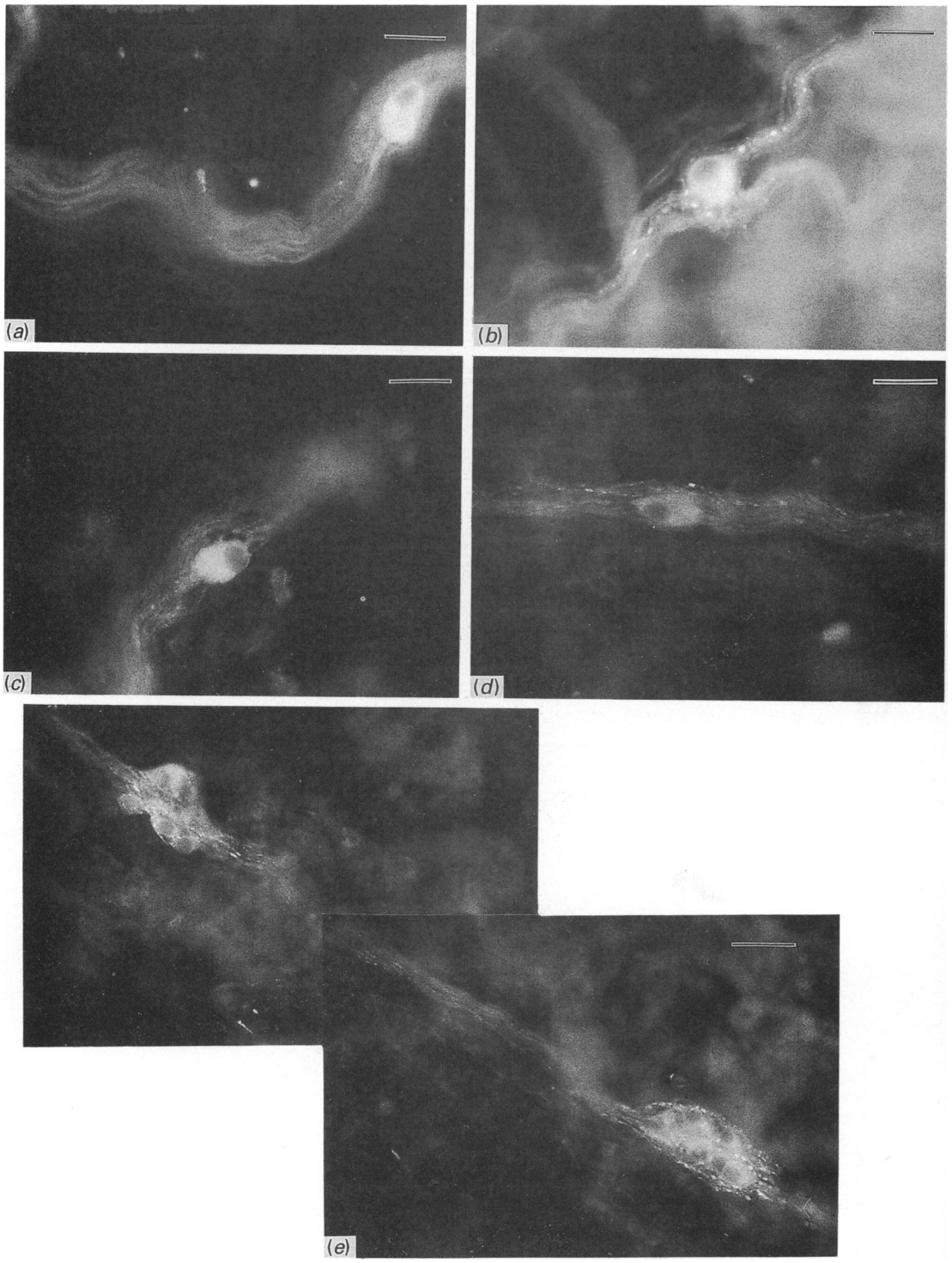

Fig. 4(*a–e*). Galanin-like immunoreactivity in wholemount preparations of divisions of the submucous plexus of the human distal colon. Single neurons contained galanin-LI; (*a*) and (*b*) were from Henle's plexus, (*c*) and (*d*) were from the intermediate plexus. In (*e*) two ganglia from the intermediate plexus are connected by a fibre tract containing galanin-LI in varicose fibres. Scale bars represent 25 μm for (*a–d*) and 40 μm for (*e*).

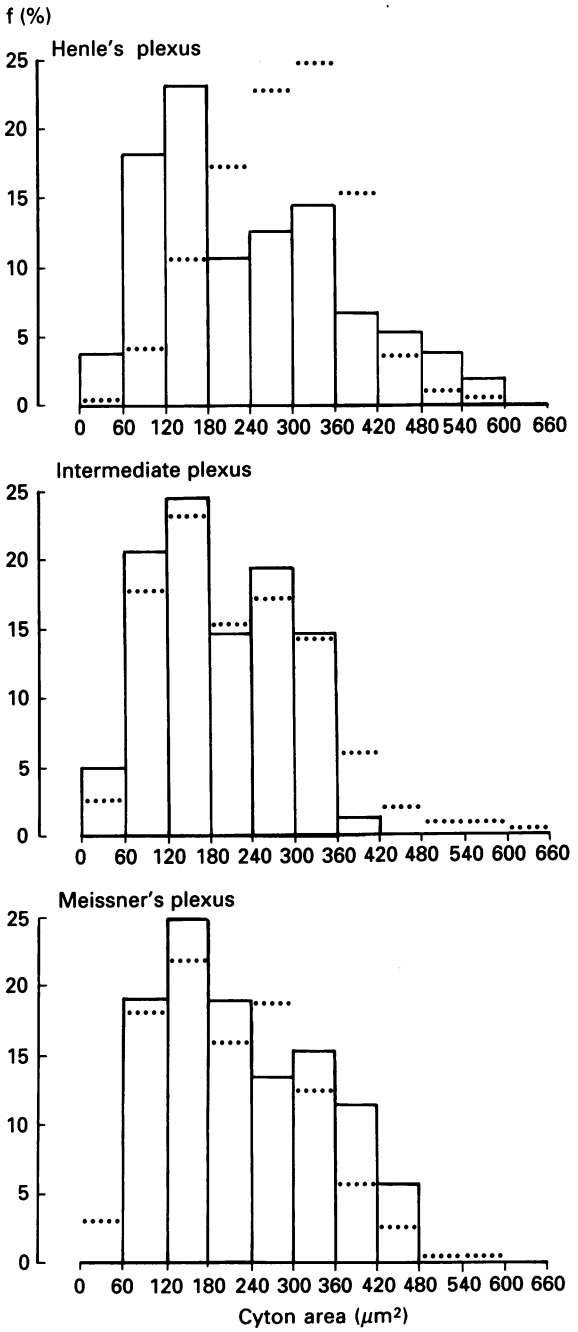

Fig. 5. Frequency-distribution histograms of areas of nerve cell bodies (cyton area) containing galanin-like immunoreactivity in subdivisions of the submucous plexus. Areas were put into bins with a width 60 μm^2 and a rolling average with a width of 2 bins was taken to provide the final histogram (this process was performed in order to smooth errors in estimating the cell body area). The dotted bars are the frequency-distribution of neuron cell body areas stained with NADH-diaphorase from Hoyle & Burnstock (1989) and are shown here for convenience. The area of 28 cells was measured in Henle's plexus, 41 in the intermediate plexus and 26 in Meissner's plexus. The distribution in Meissner's plexus significantly differed from that in the intermediate plexus ($P < 0.05$). For all the divisions, the distribution of the galanin-like immunoreactivity was significantly different from that of the NADH-diaphorase activity ($P < 0{\cdot}001$).

Table 1. *Area of cell bodies with galanin-like immunoreactivity in divisions of the submucous plexus of human distal sigmoid colon*

Division	Galanin-LI μm^2	NADH-diaphorase* μm^2
Henle's plexus	265 ± 24·3 (28)	326 ± 9·4 (90)**
Intermediate plexus	220 ± 12·7 (41)	266 ± 11·7 (84)***
Meissner's plexus	264 ± 22·4 (26)	244 ± 10·2 (85)

* Cell bodies visualised with NADH-diaphorase, data repeated here for convenience from Hoyle & Burnstock (1989).

,* Significant difference versus galanin-LI, $P < 0.025$ and $P < 0.001$ respectively.

Values given are mean ± S.E. (*n*).

visible. Fibres with galanin-LI seemed to cross this layer from the submucosa (Fig. 2*e*,*g*).

In wholemount preparations of separate layers of the submucous plexus, ganglia containing immunoreactive nerve cell bodies and ganglia containing basketworks of varicose fibres were observed (Figs. 3, 4). Immunoreactive bundles of fibres could be followed for long distances from ganglion to ganglion. It was not possible to count the percentage of ganglia containing cell bodies with galanin-LI because the NADH-diaphorase counterstain prevented immunoreactive cell bodies from being seen. In ganglia which contained immunoreactive cell bodies, not all the nerve cell bodies necessarily contained immunoreactivity. There was no difference in the pattern of distribution of cell bodies or fibres with galanin-LI in the three divisions of the submucous plexus: Henle's plexus, the intermediate layer and Meissner's plexus.

The area of cell bodies in the submucous plexus containing galanin-LI was estimated, using a graticule fitted in the eyepiece of the microscope (Table 1), and the frequency-distribution of cell body areas was compared (Fig. 5). The mean cell body area was similar for each division of the submucous plexus, and within each subdivision of the plexus there was a peak in the frequency-distribution of cell body area at 121–180 μm^2. In Henle's and Meissner's plexuses 82–83 % of nerve cell bodies were up to 360 μm^2, but in the intermediate plexus nearly all (99·8 %) were up to 360 μm^2.

DISCUSSION

The results presented here show that galanin-LI is distributed in neural elements throughout the wall of the human distal sigmoid colon. Although there is less galanin immunoreactivity in the human colon than in the stomach or small intestine, there are still substantial amounts present (Bauer *et al.* 1988). In the human jejunum, galanin is localised mainly in the muscularis externa and the submucosa with none in epithelial cells (Bauer *et al.* 1988). That situation would appear to be true for the colon as well, with the large submucosal quantity of galanin being present in neurons of the submucous plexus. Likewise, no galanin-LI was seen in the colonic epithelium.

The distribution of galanin-LI in the human colon was similar to that of the guinea-pig or rat small intestine (Furness *et al.* 1987; Fehér & Burnstock, 1988). However, unlike the guinea-pig and rat small intestine, no galanin-immunoreactive nerve fibres were seen which were closely related to blood vessels in the submucosa. As pointed out by Bauer *et al.* (1988), because of molecular variations in galanin molecules between species, extrapolation of experimental results between species should be treated with caution.

Galanin-LI appeared to be contained in certain subpopulations of neurons with cell bodies in Henle's plexus. In this plexus, although galanin-LI appeared in larger nerve cell bodies, above 420 μm^2, it was more likely to be seen in nerve cell bodies up to 180 μm^2, and less likely to be seen in nerve cell bodies from 181–420 μm^2 than was NADH-diaphorase activity. It is also interesting to note that, while the frequency-distribution of the cell body area of galanin-containing neurons was very similar between Henle's plexus and Meissner's plexus, there were significant differences between the frequency-distribution of the total population of neurons of these plexuses (Hoyle & Burnstock, 1989). Further, while the intermediate plexus and Meissner's plexus had similar frequency-distribution of cell sizes which stained for NADH-diaphorase (Hoyle & Burnstock, 1989), the frequency-distribution of cell sizes containing galanin-LI markedly differed. This implies that the neurons of the intermediate plexus and Meissner's plexus which contain galanin-LI are not from an homogeneous population, and supports the argument that an intermediate plexus can be identified, separate from Meissner's plexus.

The *plexus entericus extremus*, or *plexus entericus* (*submucosus*) *extremus* has been described as an aganglionated plexus, lying subjacent to the circular muscle and closely associated with interstitial cells of Cajal or fibroblasts (Stach, 1972, 1975; Faussone Pellegrini & Cortesini, 1984; Faussone Pellegrini, 1985). The cell bodies of the nerve fibres of this plexus lie in Henle's plexus (Stach, 1972, 1975; Christensen & Rick, 1987), which itself is closely apposed to the circular muscle coat. The localisation of galanin-LI in this region is the first description of the presence of a neuropeptide in this part of the submucous plexus.

Whether or not the fibroblast-like cells are in fact fibroblasts is beyond the scope of this communication. Interstitial cells of Cajal have been described in this region in many species (Stach, 1972, 1975; Faussone Pellegrini, 1985; Christensen & Rick, 1987). It has been suggested that there is a correlation between the presence of interstitial cells at the conjunction of the submucous plexus with the colonic circular muscle layer and the generation of rhythmic activity (Christensen & Rick, 1987). This is unlikely to be the case in the human distal sigmoid colon because, at the electron microscope level, these fibroblast-like cells do not have features of interstitial cells of Cajal (Faussone Pellegrini & Cortesini, 1984), nor is there regular rhythmic slow-wave activity in normal human distal sigmoid colon (Couturier, Roze, Couturier-Turpin & Debray, 1969; Taylor *et al.* 1974; Narducci, Basotti, Gaburri & Morelli, 1987); furthermore, slow-wave activity in the human colon is believed to be generated in the longitudinal muscle layer (Vanasin, Ustach & Schuster, 1977).

In the guinea-pig, the co-localisation of galanin with other neuro-substances has led to the suggestion that it may participate in control of gastrointestinal motility, blood flow, and water and electrolyte transport (Furness *et al.* 1987). The extensive presence of varicose nerve fibres in ganglia of the enteric plexuses suggests that neuronally-released galanin might act on ganglionic neurons. Also, in the guinea-pig, galanin has been shown to have an inhibitory action on neurons in the myenteric plexus of the ileum (Palmer, Schemann, Tamura & Wood, 1986) and to stimulate release of acetylcholine and substance P from myenteric neurons of the guinea-pig taenia coli (Ekblad *et al.* 1985*a*). As yet, actions of galanin in the human gut are unknown.

The distribution of galanin-LI in the muscularis mucosae and the mucosa was strikingly similar to that of substance P and vasoactive intestinal polypeptide in these regions in human colon (Ferri *et al.* 1982, 1983) and it is possible that in man, as in the guinea-pig, there is a close relationship between galanin and other neuropeptides.

Nerve cell bodies containing galanin-LI were found at all levels within the

submucous plexus and these neurons supplied the fibres innervating the muscularis mucosa, lamina propria and mucosal epithelial cells as well as fibres of the *plexus entericus* (*submucosus*) *extremus*. The difference in the frequency-distribution of cell body sizes of galanin-like immunoreactive neurons between the intermediate plexus and Meissner's plexus further supports the argument that these are two separate plexuses and that in the human colon the submucous plexus contains three ganglionated sub-plexuses, namely: Henle's plexus, which lies in close apposition to the circular muscle layer, the intermediate plexus, which lies in the submucosa and towards the muscularis mucosae, and Meissner's plexus, which lies in close apposition to the muscularis mucosae (Hoyle & Burnstock, 1989). The physiological significance of the structural organisation of the neurons in the submucous plexus is largely unknown and we await the time when a unique function can be attributed to the various subdivisions which will complement our current knowledge of sensory, secretory, integrative and motor functions (Furness & Costa, 1987).

SUMMARY

The distribution of galanin was investigated at the light microscopic level in the human distal colon using immunocytochemical techniques. Galanin-like immunoreactivity was seen in nerve cell bodies in ganglia of the myenteric and submucous plexuses and in nerve fibres innervating all the muscle layers of the colon, the lamina propria, and epithelial cells at the base of crypts or lining the colonic lumen. Immunoreactivity was more intense in the circular muscle than in the longitudinal muscle or the muscularis mucosae. Immunoreactive nerve cell bodies were much scarcer in the myenteric than in the submucous plexus. Within subdivisions of the submucous plexus, galanin-like immunoreactivity was heterogeneously distributed. In Henle's plexus and Meissner's plexus 82–83% of galanin-positive cell bodies were up to 360 μm^2 in profile-area, but in the intermediate plexus nearly all (99·8%) were below 360 μm^2. The frequency-distribution of cell body area of galanin-containing nerve cell bodies was similar for Henle's plexus and Meissner's plexus but these two plexuses contain different size-populations of neurons when stained for NADH-diaphorase activity. Galanin-like immunoreactive nerve fibres were found in the *plexus entericus* (*submucosus*) *extremus*, and this is the first report of neuropeptide in this location.

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