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Secretoneurin: a new peptide in the human enteric nervous system

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Abstract Secretoneurin is a functional neuropeptide derived from secretogranin II (chromogranin C). This proprotein is processed to varying degrees in neuroendocrine tissues. In the present study we established by gel filtration high performance liquid chromatography that in human intestinal wall and mucosa an antiserum against secretoneurin detects as the major immunoreactive moiety the free peptide secretoneurin. In the mucosa some larger immunoreactive peptides were also present, however, a significant amount of the intact proprotein secretogranin II could not be detected. By immunohistochemistry we studied the distribution of secretoneurin within the gut. Antibodies to protein gene product 9.5 and chromogranin A were used to identify all neurons and endocrine cells, respectively, whilst those to the peptides substance P, CGRP and somatostatin were used for the further characterization of individual secretoneurin-positive structures. Secretoneurin immunoreactivity was found in nerve fibres in all layers of the gut wall. In both myenteric and submucous plexuses, nerve fibres and the majority of ganglion cells were secretoneurin-immunoreactive. In the mucosa, some secretoneurin-positive nerve processes ran parallel to the basal membrane of epithelial cells, occasionally invading the epithelial layer. Secretoneurin immunoreactivity was found in endocrine cells, mostly D cells, in the following regions in descending order of density: stomach/duodenum; rectum; colon; ileum. Thus, secretoneurin is a new major peptide within the human enteric neuroendocrine system. Its presence in abundant myenteric ganglion cells may imply a role in the modulation of gastrointestinal motility. The chemotactic properties of secretoneurin and its possible localization in sensory fibres suggest that this peptide may be involved in the genesis of intestinal inflammation.

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Introduction

The chromogranins are a family of secretory proteins comprising the peptides chromogranin A and B, secretogranin II and 7B2 (Blaschko et al. 1967; Schneider et al. 1967; Fischer-Colbrie and Frischenschlager 1985; Marcinkiewicz et al. 1985; Rosa et al. 1985). These proteins are widespread constituents of neuroendocrine granules, which are used for the storage and release of peptide hormones and neuropeptides (for review see Wiedenmann and Huttner 1989; Winkler and Fischer-Colbrie 1992). Since the chromogranins have a much wider distribution than any other individual hormone or neuropeptide, they are the most widespread markers known for the matrix of neuroendocrine granules and, as such, are valuable tools for the identification of neuroendocrine cells and neuroendocrine tumours (Weiler et al. 1988; Wiedenmann and Huttner 1989; Schürmann et al. 1991).

Secretoneurin is a peptide derived from secretogranin II (Kirchmair et al. 1993). Secretogranin II (also named chromogranin C) was initially described in anterior pituitary cells (Rosa and Zanini 1981) and secretogranin II immunoreactivity has been shown subsequently to occur in the brain (Rosa et al. 1985; Cozzi et al. 1989; Weiler et al. 1990; Mahata et al. 1991), peripheral nerves (Hagn et al. 1986), axons and nerves of intestinal intramural plexuses (Pelagi et al. 1992), the endocrine pancreas (Lassmann et al. 1986; Yoshie et al. 1987), C-cells of the thyroid (Rosa et al. 1985; Lassmann et al. 1986; Weiler et al. 1989) and in several endocrine cell types of the human intestinal tract (Rindi et al. 1986; Wiedenmann et al. 1988). Apart from various other neuroendocrine tumours, secretogranin II immunoreactivity is present in

gastrinomas of the pancreas, in insulinomas and in intestinal carcinoids (Weiler et al. 1988; Wiedenmann et al. 1988).

The chromogranins become proteolytically processed within the large dense-core vesicles, their subcellular storage site in neuroendocrine tissues (Winkler and Fischer-Colbrie 1992). In order to study the processing of secretogranin II, an antiserum was raised against a synthetic peptide corresponding to amino acids 154–186 of bovine secretogranin II. This peptide is flanked in the secretogranin II molecule by pairs of basic amino acids representing proteolytic cleavage sites (Fischer-Colbrie et al. 1990). The immunoreactive moieties in various tissues were analyzed with this antiserum employing radioimmunoassay and high performance liquid chromatography (Kirchmair et al. 1993). In adrenal medulla and anterior pituitary, processing of secretogranin II is limited; on the other hand, in brain, spinal cord and in neurons of the posterior pituitary it is almost completely processed (Kirchmair et al. 1993; Egger et al. 1994; Marksteiner et al. 1994). Thus, most of the immunoreactivity in neurons is due to the free peptide, secretoneurin. For the gastrointestinal tract it has not yet been established to what extent secretogranin II is proteolytically processed.

Secretoneurin has been localized by immunocytochemical and radioimmunological studies to several areas of human and rat brain, including the lateral septum, the hypothalamus, the brain stem and primary afferent fibres (Marksteiner et al. 1993, 1994). Secretoneurin shows an overlapping distribution with substance P, somatostatin and calcitonin gene-related peptide (CGRP; Marksteiner et al. 1993; 1994). The recent discovery that secretoneurin can release dopamine from rat striatal slices (Saria et al. 1993) and during microdialysis (Agneter et al. 1995) establishes that this peptide belongs to the group of functional neuropeptides. In sensory C-fibres secretoneurin is colocalized with substance P and CGRP and is released from these nerves by capsaicin (Kirchmair et al. 1994). Since secretoneurin is chemotactic for monocytes (Reinisch et al. 1993), it seems likely that this peptide, when released from afferent nerve fibres, may participate in neurogenic inflammation and immune response regulation.

The aim of the present study was to investigate the distribution of secretoneurin immunoreactivity in the normal human gut and to characterize it in molecular terms by high performance liquid chromatography.

Materials and methods

Tissue preparation

Samples of histologically normal human adult (age range 42–76 years) stomach (fundus and antrum), duodenum, ileum, colon and rectum (n=3–6 for each) were taken in the course of routine surgery for cancer. Tissue blocks comprising full thickness of the gut wall were fixed by immersion for 4 h in Zamboni's fluid (0.1 *M* phosphate buffer containing 2% w/v paraformaldehyde and 15% v/v saturated picric acid; Stefanini et al. 1967). Following washing in 0.01 *M* phosphate-buffered 0.15 *M* saline containing 15% w/v sucrose, cryostat blocks were prepared. For distribution studies and co-localization with protein gene product 9.5 (PGP), two serial 6-μm sections were cut at −20° C and collected onto poly-L-lysine coated (Huang et al. 1983) glass slides. For co-localization studies on endocrine cells, serial 3-μm sections were cut from Bouin's-fixed, paraffin-embedded duodenum and rectum, as described previously (Bishop et al. 1984).

High performance liquid chromatography

Extracts of pieces of surgically resected human intestine were produced by sonication (3×5 s) of the tissue in distilled water followed by immediate boiling and centrifugation for 20 min at 14,000×g. Supernatants were subjected to gel filtration chromatography (Bio-Stil TSK 400) followed by radioimunoassay with the secretoneurin antiserum, as described in detail previously (Kirchmair et al. 1993; Egger et al. 1994).

Immunohistochemistry

Tissue sections were immunostained using a range of antibodies (see Table 1) by an indirect immuno-peroxidase method (Hsu et al. 1981) as previously described (Walters et al. 1993). Ganglion cells and nerve fibres were identified by immunostaining for PGP as a conventional neuronal marker (Lundberg et al. 1988; Gulbenkian et al. 1987). Some cases showed secretoneurin-immunoreactive fibres in the mucosal epithelium, a localization associated with sensory fibres (Singaram et al. 1990). In order to characterize these nerves further, the neuropeptides, substance P and CGRP, shown in animal experiments to subserve a sensory function in the gut (Costa et al. 1981; Furness et al. 1982; Su et al. 1987), were immunostained in consecutive sections. Intraepithelial endocrine cells were detected using chromogranin A antisera and further characterized by immunostaining for somatostatin (D cells) and serotonin (enterochromaffin cells) (Table 1). Routine negative control immunostaining included the replacement of the primary antibodies with pre-immune serum or omission of one or several reagents in the immunostaining procedure. Preincubation of the primary antibody with rat secretoneurin peptide abolished specific staining. Immunoreactive sites were photographed using a Polyvar (Leica UK, Milton Keynes, UK) photomicroscope with bright field illumination.

Table 1 Primary antibody characteristics

Antibodies to	Donor species	Titre	Source
Secretoneurin	Rabbit	1:2000	Reiner Fischer-Colbrie
Protein gene product 9.5	Rabbit	1:80 000	Ultraclone, UK
Calcitonin gene-related peptide	Rabbit	1:4000	Hammersmith Hospital
Substance P	Rabbit	1:10 000	Hammersmith Hospital
Chromogranin A	Mouse	1:1000	Boehringer Mannheim, Germany
Somatostatin	Rabbit	1:8000	Hammersmith Hospital
Serotonin	Mouse	1:1000	Dako, Denmark

Evaluation of immunohistochemical results

In three visual fields per section, the density of nerve processes showing immunoreactivity for secretoneurin was estimated as the percentage of total nerve processes demonstrated by PGP immunoreactivity, using a four-point scoring system (see reference to Table 2). The proportion of secretoneurin-positive ganglion cells was estimated from counts of total PGP-positive cell bodies in one tissue sample per gut region.

Results

Characterization of secretoneurin immunoreactivity by HPLC

Extracts of mucosa and of the muscle layers of human intestine were subjected to gel filtration chromatography. For both samples the major immunoreactive peak eluted exactly in the position of the free peptide secretoneurin (Fig. 1). In the mucosa an additional peak was present eluting in the position of secretogranin II-derived intermediate peptides. Significant amounts of the unprocessed proprotein secretogranin II were not detectable.

Immunohistochemical data

Innervation

Secretoneurin immunoreactivity was found in all regions of the gut in numerous nerve cel bodies and fibres. Table 2 indicates the relative density of the immunoreactive fibres at different sites within normal human gut. The majority of PGP-positive nerve fibres co-expressed secretoneurin, in nearly all localizations and layers (Table 2). However, the intensity of immunostaining for secretoneurin was generally slightly

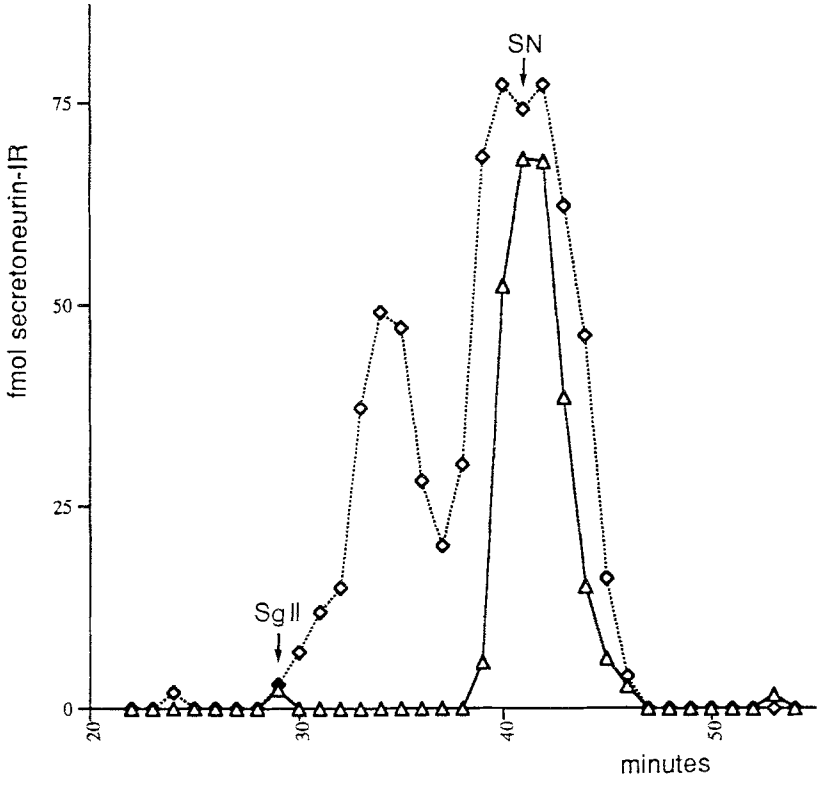

Fig. 1 Gel-filtration high performance liquid chromatography of intestinal extracts. Extracts from human intestinal mucosa (◇) and from the intestinal wall (△) were subjected to gel-filtration chromatography. The individual eluted fractions were analyzed by radioimmunoassay for secretoneurin. The elution position of the secretogranin II (*Sg II*) and of the free peptide secretoneurin (*SN*) are indicated by *arrows*. The total content of secretoneurin was 15.5 fml/mg (wet weight) mucosa and 10.1 fml/mg intestinal wall

Table 2 Density of nerve fibres with secretoneurin immunoreactivity in different regions of normal human gut. Semiquantitative estimation using a four-point scoring system. 0, +, ++, +++ represent increasing scores of secretoneurin-immunoreactive nerve fibres expressed as the percentage of nerve fibres positive for protein gene product 9.5, i.e. +++=widespread positive reaction (>80% of all nerve fibres stained), ++=focally positive (20–80%), +=some nerve fibres positive (<20%)

	Longitudinal muscle	Myenteric plexus	Circular muscle	Submucosa	Submucous plexus	Muscularis mucosae	Mucosa
Stomach	++	+++	++	++	+++	++	++
Duodenum	++	+++	++	+/++	+++	++	++
Jejunum	++	+++	++	++	+++	++	++
Ileum	++	+++	++	++	+++	+/++	+++
Colon	++	+++	++	++	+++	++	++
Rectum	++	+++	++	++	+++	++	++

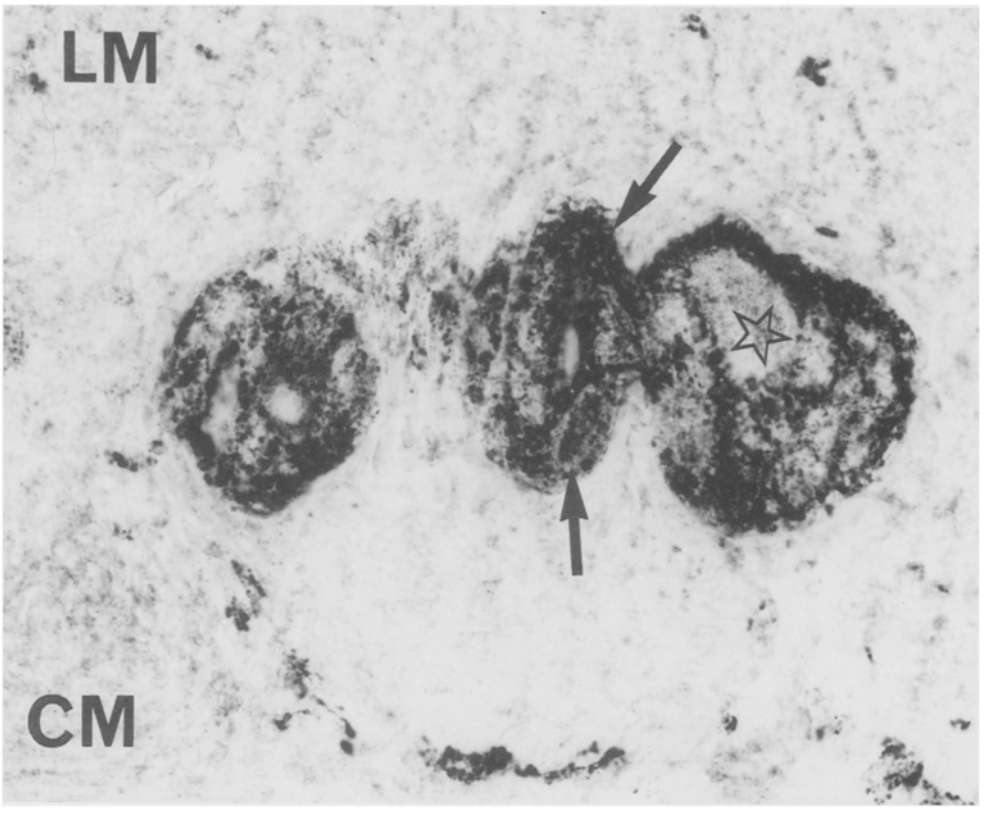

Fig. 2 Secretoneurin immunoreactivity in the muscularis propria of the human duodenum. Intense immunostaining of the myenteric plexus and of nerve fibres in the longitudinal (*LM*) and circular (*CM*) muscle layers. Most ganglion cells show secretoneurin immunoreactivity *(arrows).* However, some ganglion cells *(asterisk)* seem not to contain secretoneurin (magnification ×441)

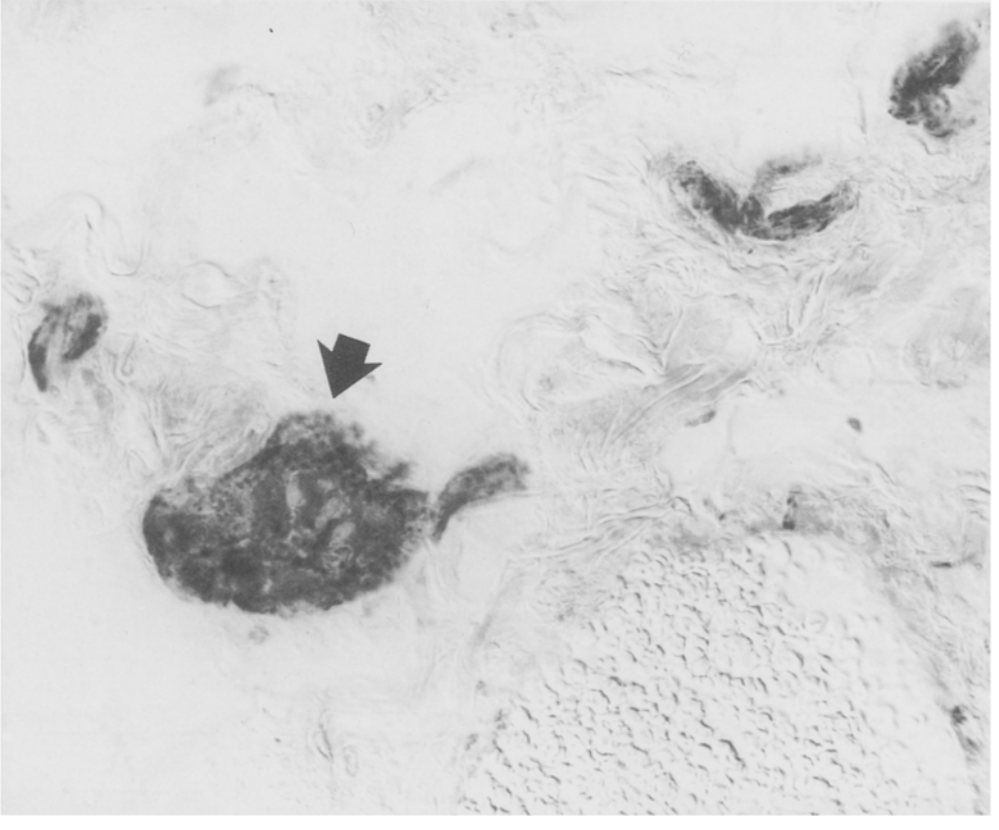

Fig. 3 Secretoneurin immunoreactivity in the ileal submucosa. Intense staining of a submucous ganglion *(arrow),* and of submucous nerve fibres and fascicles (magnification ×694, Nomarski optics)

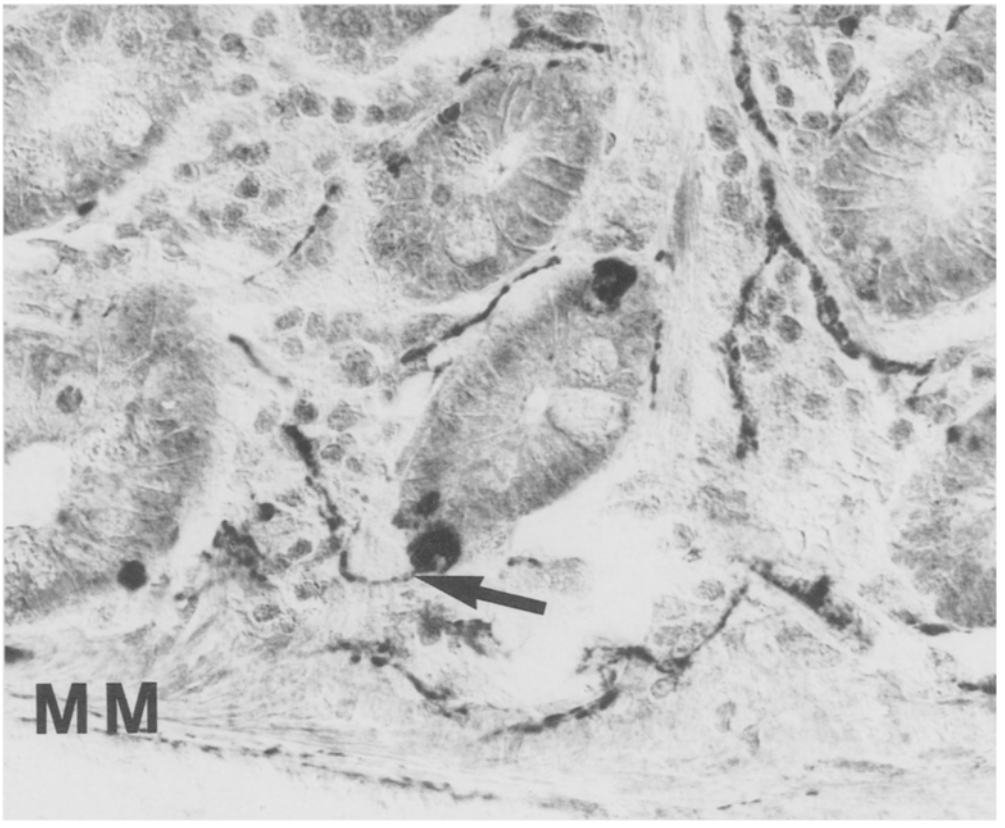

Fig. 4 Secretoneurin immunoreactivity in the duodenal mucosa. Secretoneurin-positive nerve fibres are mostly present towards the muscularis mucosae (*MM*), occasionally in close contact with endocrine cells *(arrow)* (magnification ×694, Nomarski optics)

weaker when compared with that for PGP. We observed a homogeneous staining pattern (with 20–80% of nerve fibres stained) in all muscle layers (Figs. 2, 5). Similar immunoreactivity was obtained in the submucosa (Fig. 3) and mucosa, with a slight reduction of secretoneurin-positive fibres in the submucosa of the duodenum and a slight increase in the ileal mucosa. In the mucosa, immunoreactive nerve fibres were found predominantly towards the lamina muscularis mucosae and around the base of crypts (Fig. 4), but small proportions of fibres also reached the villus tips. Some secretoneurin-immunoreactive fibres ran parallel to the basal membrane of epithelial cells (Fig. 5), occasionally, in three colonic samples, even invading the epithelial layer or surrounding the crypts in a network of fibres. In two of these cases, substance P-immunoreactive fibres could also be identified in between epithelial cells, but in smaller numbers than those containing secretoneurin. No intraepithelial CGRP-immunoreactive neurons were observed. In some sections, secretoneurin-positive fibres were in close contact with intraepithelial endocrine cells (Fig. 4).

In both the myenteric and submucous plexuses, virtually all PGP-positive nerve fibres also expressed secretoneurin immunoreactivity (Table 2). Furthermore, the vast majority of ganglion cells were secretoneurin-positive without apparent differences between the gut regions (Figs. 2, 3). A small proportion of ganglion cells of both plexuses, however, did not show secretoneurin immunoreactivity (Fig. 2).

Endocrine cells

Secretoneurin immunoreactivity was found in endocrine cells (Fig. 6) in the following regions in descending order of density: stomach/duodenum; rectum; colon; ileum. This distribution pattern matched with the general distribution of endocrine cells in the gut identified by the immunoreactivity for chromogranin A in serially sectioned cells. Secretoneurin-immunoreactive cells were far outnumbered by those displaying chromogranin A immunoreactivity.

Both the distribution pattern and morphology of secretoneurin-immunoreactive endocrine cells indicated that they were D cells, an observation confirmed by co-localization studies in serial sections of wax-embedded tissue. In representative sections of different localizations, secretoneurin-positive endocrine cells co-expressed somatostatin (Fig. 7). Some cells, however, were somatostatin-positive but secretoneurin-negative. Co-localization of secretoneurin and serotonin, the only other regulatory factor present in endocrine cells in all areas of the human gut, could not be detected. As expected, despite strong staining of endocrine cells in Bouin's-fixed, wax-embedded tissue, immunoreactivity for secretoneurin in neurons was markedly reduced and sometimes absent. A similar lack of neural somatostatin immunoreactivity was also observed in these samples as this method of tissue processing is not optimal for preservation of peptide immunoreactivity in nerves (Springal et al. 1984).

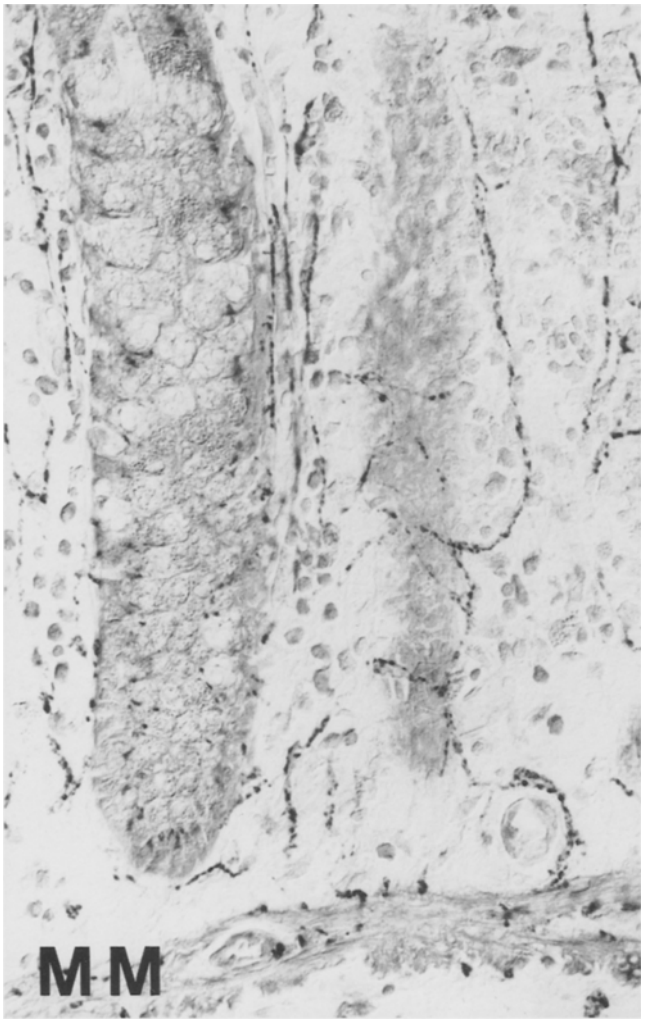

Fig. 5 Secretoneurin immunoreactivity in the colonic mucosa. Some secretoneurin-positive fibres run parallel to the basal membrane of epithelial cells, occasionally invading the epithelium. Note the varicose staining pattern, predominantly present in intraepithelial nerve processes. Nerves within the muscularis mucosae (*MM*) are also secretoneurin-positive (magnification ×360, Nomarski optics)

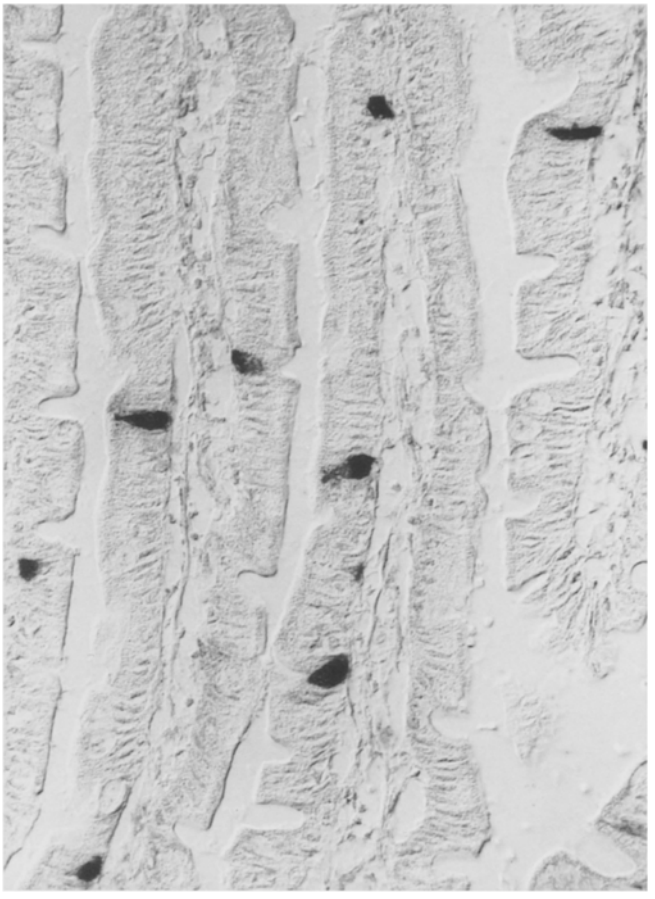

Fig. 6 Secretoneurin immunoreactivity in the duodenal epithelium. Intraepithelial secretoneurin-positive cells show the typical shape and distribution of intestinal endocrine cells (magnification ×280, Nomarski optics). Bouin's fixation usually disclosed secretoneurin staining in mucosal nerve fibres

Discussion

The antiserum used in the present study is directed against secretoneurin representing a 33-amino acid sequence of the proprotein secretogranin II. It does not cross-react with other peptides obtained from the same molecule and has no cross-reactivity with classical neuropeptides (Kirchmair et al. 1993). The antiserum, however, not only detects the free peptide secretoneurin, but also larger secretoneurin-containing peptides formed from secretogranin II and the proprotein secretogranin II itself (Kirchmair et al. 1993). On the other hand, previous studies (see Introduction) on the immunohistochemistry of secretogranin II employed antisera which not only reacted with the proprotein but also with peptides derived from it, since such peptides still carry antigenic epitopes. As stated in the Introduction we have recently established that, depending on the tissue, secretogranin II is proteolytically processed to a considerable degree varying from about 50% in the adrenal and anterior pituitary to more than 90% in brain and nervous tissue (Kirchmair et al. 1993; Egger et al. 1994). Thus in order to reach a conclusion which molecular moiety of secretogranin II is recognized by secretoneurin antisera, the immunoreactive material has to be characterized. For intestinal tissues we have done this for the first time with HPLC gel chromatography. Rather surprisingly a high degree of processing was found. In the intestinal wall the major immunoreactive material co-eluted with the free peptide secretoneurin, whereas in the mucosa some intermediate peptides were also present. It should be emphasized that also in rat intestine (from stomach to colon) the free peptide represents the major immunoreactive material (B. Leitner and H. Winkler, to be published).

We can therefore conclude that in the intestine, secretogranin II is processed to a high degree. This is not surprising as far as secretoneurin-containing intestinal nerves are concerned, since an analogously high process-

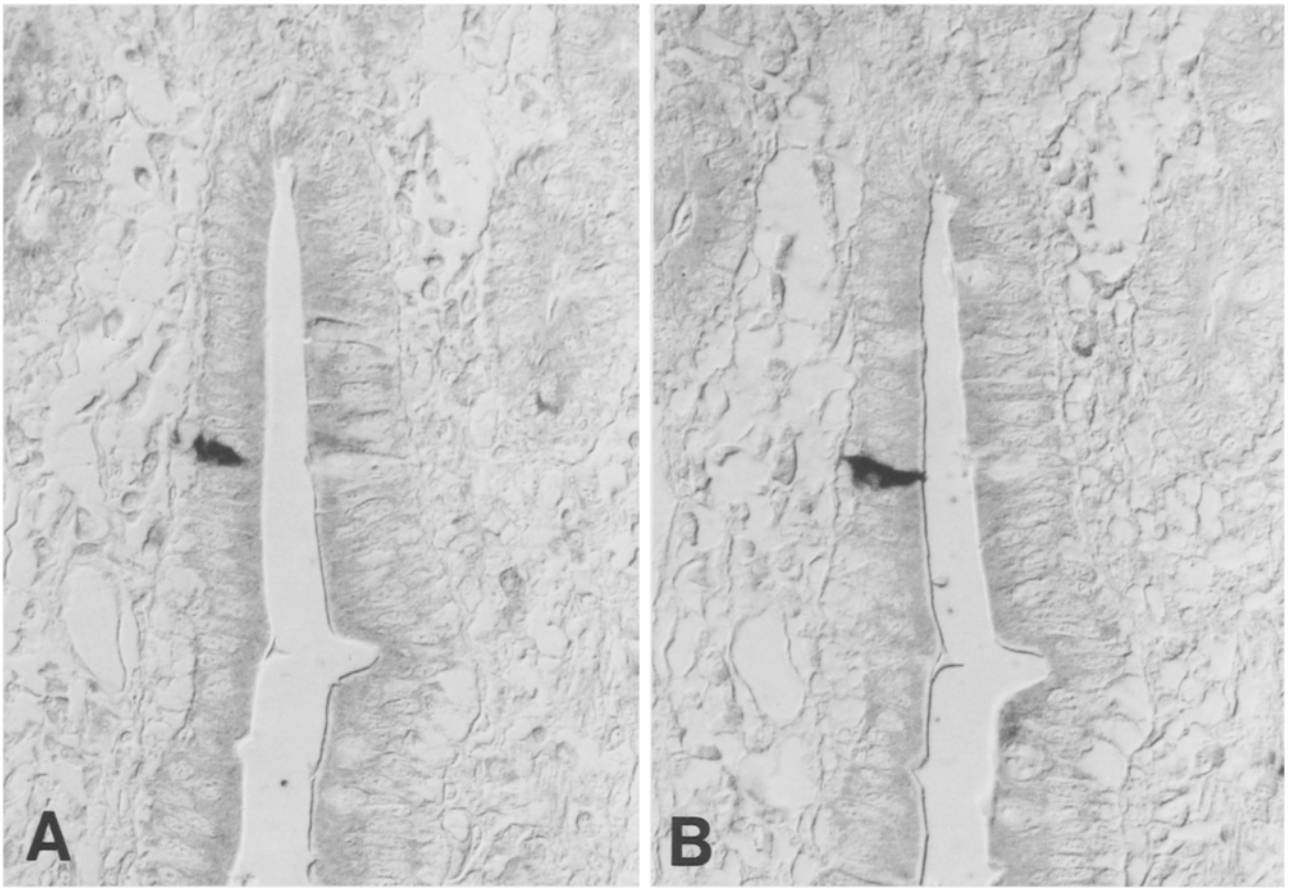

Fig. 7A, B One serially sectioned intraepithelial duodenal endocrine cell immunostained for **A** secretoneurin and **B** somatostatin showing the co-localization of both peptides (magnification ×548, Nomarski optics, Bouin's fixation)

ing was found for brain, spinal cord and posterior pituitary neurones (Kirchmair et al. 1993; Egger et al. 1994). However, it appears now that in endocrine cells also, secretogranin II must be processed to a significant degree, since in mucosa only intermediate peptides and secretoneurin were found. Thus previous studies employing an antiserum against secretogranin II were apparently localizing epitope-carrying peptides derived from the proprotein. For the present study we are justified in assuming that our immunohistochemical data (especially for nerves) actually identify and localize secretoneurin.

The immunohistochemical data firmly establish the presence of secretoneurin immunoreactivity in the human enteric neuroendocrine system. Comprehensive mapping of the immunocytochemical localization of secretoneurin shows that this peptide is present in a significant proportion of human enteric neurons and in a subpopulation of mucosal endocrine cells, mostly D cells.

A considerable proportion of the nerve fibres throughout the intestine were immunostained for secretoneurin. In a previous study (Kirchmair et al. 1994), it was shown that capsaicin treatment of rats led to a significant decline of secretoneurin in bladder and trachea, to a lesser degree, however, than that seen for substance P. This indicated that secretoneurin was not only present in capsaicin-sensitive afferent C-fibres but also in additional nerves. The present study now shows that secretoneurin is apparently present in a wide spectrum of nerves, both afferent and efferent.

Some functional implications can be drawn from our morphological observations. Firstly, the peptide was found in abundant myenteric ganglion cells, implying some role in the modulation or direct control of gastrointestinal motility. It is not possible to characterize capsaicin-sensitive C-fibres in the human gut. However, a striking finding in some samples of colon was an exceptionally close relationship seen between secretoneurin-immunoreactive fibres and the mucosal epithelium, with the fibres apparently penetrating the epithelial layer, a localization known to be associated with sensory fibres (Singaram et al. 1990). It may be that these fine nerve endings in the epithelium directly affect mucosal secretion or, possibly, subserve a sensory function and, thus, represent C-fibres in the gut. The latter possibility is supported by our observations of a few intraepithelial fibres showing immunoreactivity for substance P, a sensory neuropeptide. As secretoneurin attracts human monocytes in vitro and in a model of human skin chambers (Reinisch et al. 1993), a further action is likely to be some contribution to the development of the inflammatory cellular infiltrate, for example by attracting monocytes to the intestinal mucosa in inflammatory bowel disease.

In summary, we have described in the human gastrointestinal system discrete neuroendocrine cell populations that are immunoreactive for the recently described sensory neuropeptide secretoneurin. Its widespread distribution in different regions of the gut and throughout the gut wall suggests several specific functions for secretoneurin that may be involved in or serve as markers for disease states. In particular, its presence in apparent sensory fibres and the observation that secretoneurin is chemotactic for monocytes suggest that it may contribute to inflammatory processes in the human gastrointestinal tract.

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