Gut Hormones in *Salamandra salamandra*

An Immunocytochemical and Electron Microscopic Investigation

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Summary. Histological, cytochemical and immunocytochemical methods were used in light and electron microscopical studies to demonstrate the presence of a neuroendocrine system in the gut of the urodele, *Salamandra salamandra.*

Cytochemical stains capable of detecting peptide-producing endocrine cells demonstrate cells reacting with Masson's silver (argentaffin) method, Grimelius' argyrophil silver method, masked metachromasia method and the lead haematoxylin stain.

Using antisera raised to a variety of mammalian gut peptides, cells containing bombesin-, gastrin-, somatostatin-, substance P- and glucagon-like immunoreactivity were identified; vasoactive intestinal polypeptide- and substance P-like immunoreactivities were found in nerve fibres in the submucous and myenteric plexus. No immunoreactivity was detected for motilin, gastric inhibitory polypeptide, cholecystokinin or secretin.

The ultrastructure of the immunoreactive cells and nerves was revealed by the semithin/thin method. All the cells identified contained numerous electron-dense secretory granules, which varied in their chracteristic morphological structure from one cell type to another.

The evidence collected in this study indicates that a complex neuroendocrine system regulating gut function is present in this amphibian and may have developed prior to the emergence of the phylum.

Key words: Gut hormones – Endocrine cells – Electron microscopy – Immunocytochemistry – Peptidergic innervation.

Salamandra salamandra, a member of the amphibian order Urodela, is one of the most primitive extant land dwelling vertebrates. In the Urodela the skeletal arrangement remains almost unaltered from that of the first fossil Amphibia (Kingsley Noble 1954).

The skin glands of Amphibia, especially the anurans, have been shown to possess a wealth of biologically active peptides (Ersparmer and Melchiorri 1973); these peptides are also found in the mammalian diffuse neuroendocrine system either as identical substances or closely related forms. There are several reports in the literature of the occurrence of polypeptide hormones in the gut of anurans. Larsson and Rehfeldt (1977) have reported in Ranidae the existence of a gastrin/cholecystokinin (CCK)-like peptide in the stomach. Lechago and colleagues (1978), also working with Ranidae, have reported the presence of bombesin-like immunoreactive cells in the gastric mucosa. There is, however, to date no systematic study of the presence of a full complement of gut hormones in any amphibian species.

As the salamander is, comparatively, the most primitive extant amphibian, the investigation of the gastrointestinal tract of this species for the presence of biologically active peptides is of interest in tracing both the evolutionary development and the phylogenetic antiquity of the diffuse neuroendocrine system.

Materials and Methods

The gastrointestinal tract from six specimens of *Salamandra salamandra* was processed for immunocyto-chemistry and electron microscopy. The gastrointestinal tract was divided into upper and lower stomach, duodenum, upper and lower intestine and large intestine.

Fixation

1. Light Microscopy. Small pieces of mucosa (1 cm^2) were immediately quenched in Arcton at $-156°$ C and subsequently freeze-dried overnight. The tissue was vapour-fixed at 60° C for 3 h in p-benzoquinone (Pearse and Polak 1975) and the samples vacuum-embedded in paraffin. 5 μm thick sections were cut for immunostaining.

2. Electron Microscopy. Samples from each area were chopped finely and immersed in the fixative of choice.

a) For *conventional morphology,* the pieces were fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4° C, then postfixed in 1 % osmium tetroxide in Millonig's buffer, pH 7.2, for 1 h at 4° C.

b) For *comparative immunocytochemistry/electron microscopy,* the pieces were fixed in 2.5 % purified glutaraldehyde in 0.05 M phosphate buffer, pH 7.3, for 15 min at 4° C (Buchan et al. 1978).

In both cases the fixed material was rinsed in buffer, dehydrated and embedded in Araldite at 60° C.

60 nm thin sections of the osmicated tissue were cut and mounted on 200 mesh copper grids and counterstained with 15 % uranyl acetate for 3 min and 5 % lead citrate for 2 min.

For the semithin/thin method (Polak et al. 1975) the non-osmicated tissue was used. Serial 800 nm-sections were mounted on glass slides and used for immunostaining. The 80 nm-sections were mounted on 200 mesh copper grids and counterstained as above.

Staining Methods

Prior to immunostaining, the paraffin was removed from the 5 μm-sections by immersion in xylene (2 min), then cleared in petroleum ether (2 min) and allowed to air-dry.

Removal of Araldite from the semithin (800 nm) sections was achieved by immersion in saturated sodium ethoxide for 15 min (Lane and Europa 1965) followed by re-hydration and a short wash in phosphate buffer, pH 7.2).

Table 1. Characterization of antisera

Antiserum	FITC dilution	Specificity	Peptides that absorb staining[a]
Gastrin A	1/400	C-terminal	Gastrin & CCK
Gastrin B	1/200	N-terminal	Gastrin
CCK A	1/600	C-terminal	CCK & Gastrin
CCK B	1/200	Mid (9–20)	CCK
VIP	1/400	C-terminal	VIP
Somatostatin	1/400		Somatostatin
Secretin	1/2,000		
GIP	1/600		
Neurotensin	1/400		None
Glucagon	1/1000	N-terminal	Glucagon
Bombesin	1/400	N-terminal	Bombesin
Substance P	1/2000	C-terminal	Substance P
Enkephalin	1/400		
Motilin	1/200	N-terminal	

[a] The absorptions reported are only those concerning the salamander and do not refer to the results in mammals. In each case the amount of peptide added was 1nmol/ml of the diluted antiserum

Immunostaining

The indirect immunofluorescence method (Coons et al. 1955) was used. A first layer of rabbit antiserum at the optimal dilution was applied for 48 h at 4° C. After washing in 0.01 M phosphate-buffered normal saline, pH 7.1 (PBS), a second layer of fluoresceine-conjugated goat anti-rabbit globulin (Miles) was applied at a dilution of 1:100 for 1 h at room temperature. The sections were washed in PBS and mounted in PBS/glycerine (1:9). The stained sections were examined using a Leitz Orthoplan microscope and the positively reacting areas photographed. After photography the sections were restained by cytochemical methods (see below).

Antisera

Specific antisera to vasoactive intestinal polypeptide (VIP), gastrin (N and C-terminal), cholecystokinin (CCK) (mid and C-terminal), somatostatin, secretin, gastric inhibitory polypeptide (GIP), neurotensin, glucagon, bombesin, substance P, enkephalin and motilin were used in this study. Table 1 gives the specificity and dilution of the individual antisera.

Controls

The following tests were performed to show the specificity of the immunocytochemical staining. Absorption of antisera prior to immunostaining: the diluted antisera were incubated with synthetic or purified peptide antigens. The results of the absorption controls are shown in Table 1. Other controls included the use of normal rabbit serum in place of the antiserum and the fluoresceine conjugate alone.

Cytochemical Stains

Before re-staining, the immunostained semithin sections were rinsed thoroughly in distilled water to remove contaminants. After cytochemical staining, the sections were washed in distilled water, mounted in PBS/glycerine and the positively-reacting areas photographed.

Masson: This technique was performed according to the method of Solcia (Solcia et al. 1969).
Grimelius: The sections were treated with silver nitrate solution in acetate buffer, then transferred into hydroquinone with sodium sulphite (Grimelius 1968).
Masked Metachromasia: The sections were hydrolysed in HCl and then stained with toluidine blue (Solcia et al. 1968).
Lead Haematoxylin: The sections were incubated in Solcia's lead haematoxylin (Solcia 1969b) for 2h at 45°C.

Semithin/Thin Technique

The characterisation of the ultrastructural appearance of the individual peptide-containing cells and nerves was achieved by this method. Photographs of the positively immunostained or cytochemically stained areas in the semithin sections were used to identify the same area in the serial thin sections.

Results

The endocrine cells of the mucosa found scattered amongst the enterocytes and mucin-containing cells were located close to the basal lamina with fine processes reaching the lumen. They contained large nuclei with the secretory granules concentrated around the periphery of the cell. Some cells show long "dendritic" processes, which also contain granules (Fig. 1).

Light Microscopy

Antisera to gastrin, somatostatin, neurotensin, glucagon, bombesin, substance P and VIP successfully stained nerves and endocrine cells of the intestine. Table 2 summarises the full distribution as shown by the immunostaining. Specificity of the immunostaining was shown by the complete quenching of staining after absorption of the antisera with their corresponding peptides and not with any of the other available purified peptides. The exception to this was neurotensin; the antisera to neurotensin immunostained numerous cells both in the mucosa and the lamina propria throughout the intestine. However, the immunostaining could not be removed even with the addition of large quantities of neurotensin, up to 50 nmol/ml of the diluted antiserum. Absorption with the related amphibian peptide xenopsin (Table 3) also failed to remove the staining. The immunostaining produced by the neurotensin antiserum was therefore non-specific.

Gastrin-containing cells were found only in the antral region of the stomach. They were mainly rectangular in profile with large nuclei (Fig. 2). Bombesin-containing cells were found from the stomach to the colon. They appeared mostly oval in shape with a large central nucleus. Glucagon cells, found from the fundus of the stomach to the colon, contained a smaller nucleus and frequently showed an immunofluorescent cellular process lying parallel to the basal lamina (Fig. 3).

Somatostatin was present in endocrine cells from the stomach to the colon. They contained large nuclei, and commonly multiple immunofluorescent-cell processes were observed, which interdigitated with the surrounding cells (Fig. 4). No somatostatin-containing nerves were observed. Substance P-immunoreactive cells
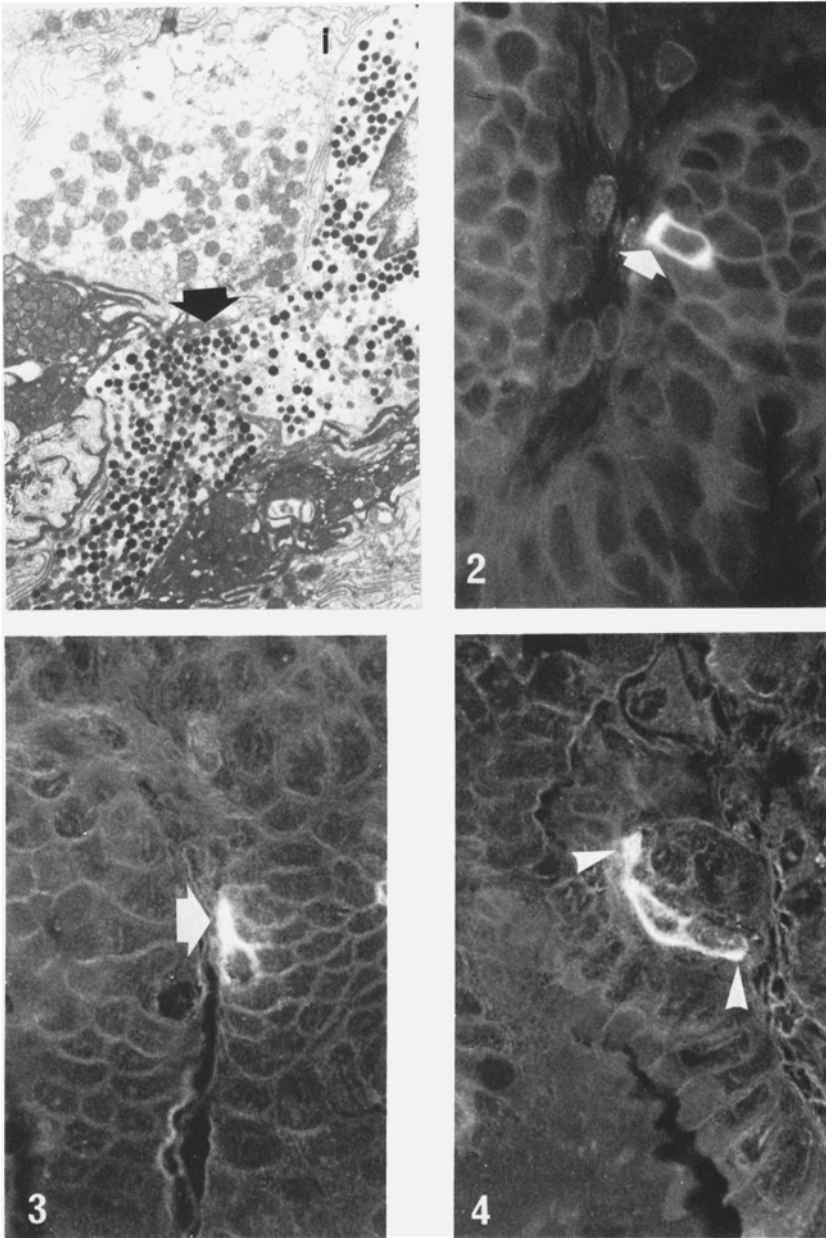
Fig. 1. A granular cell in the duodenal mucosa with a long process (*arrow*) Glutaraldehyde-osmium fixation. × 8000

Fig. 2. Antral mucosa showing a gastrin-immunoreactive cell. × 500

Fig. 3. Fundic mucosa containing a glucagon-immunoreactive cell with a basal process (*arrow*). × 450

Fig. 4. Fundic somatostatin-immunoreactive cell with several processes (*arrows*). × 400

Table 2. Distribution of the gut peptides in the salamander

	Fundus	Antrum	Duodenum	S. I. 1	S. I. 2	Colon
Gastrin A		+				
Gastrin B						
CCK A		+				
CCK B						
VIP	+	+	+	+	+	+
Somatostatin	+	+	+	+	+	+
GIP						
Enkephalin						
Substance P	+	+	+	+	+	+
Bombesin	+	+	+	+	+	+
neurotensin	+	+	+	+	+	+
Glucagon	+	+	+	+	+	+
Secretin						
Motilin						

Table 3. Similarity between neurotensin and xenopsin

C-terminal region

– Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	Neurotensin
– Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu	Xenopsin

and nerve fibres were found throughout the intestine. These cells had large round nuclei central to the immunostaining. The nerve fibres were present in both the submucous and myenteric plexus, being most abundant in the fundus and colon (Fig. 5).

VIP-immunoreactivity was found only in nerve fibres, throughout the length of the intestine. Positively-reacting fibres were seen in both the submucous and myenteric plexus (Fig. 6).

Electron Microscopy

Of the mucosal endocrine cells immunostained, the following ultrastructural profiles have been demonstrated:

Bombesin cells contained secretory granules around the periphery of the cells. The granules were relatively polymorphic in shape with a mean diameter of 220 nm (60 profiles).

Gastrin-immunoreactive cells contained secretory granules with an average diameter of 180 nm (50 profiles). They were predominantly round and electron-dense (Fig. 7). Glucagon cells contained mostly round, electron-dense granules averaging 160 nm in diameter (50 profiles).
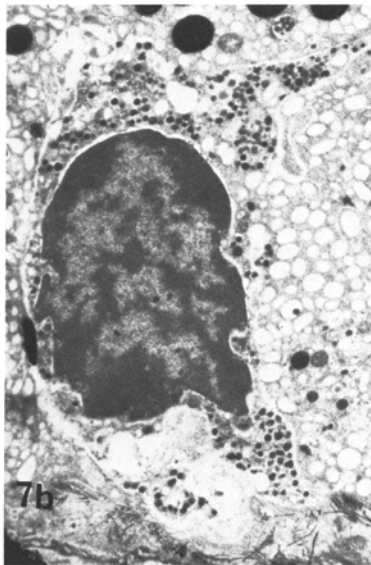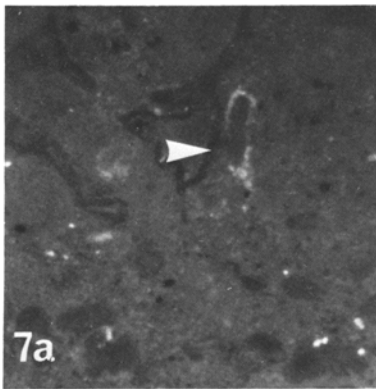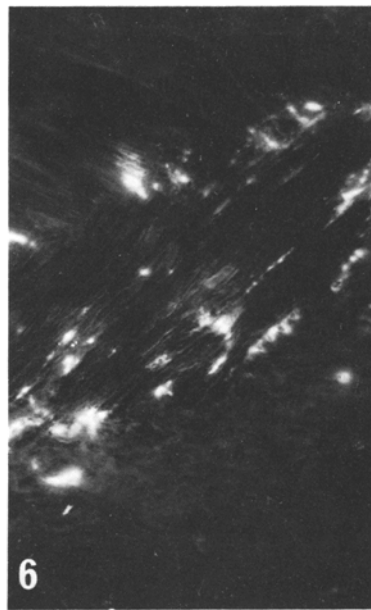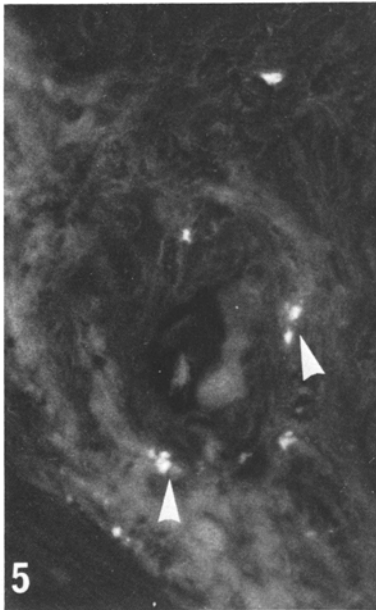
Fig. 5. Substance P-immunostained fibres around a blood vessel in the fundus. × 500

Fig. 6. VIP-immunoreactive fibres in the muscle layers of the duodenum. × 200

Fig. 7. a Gastrin-immunoreactive cell in a 800 nm-semithin section × 500. **b** The same cell in the serial thin (80 nm)-section. × 3,000
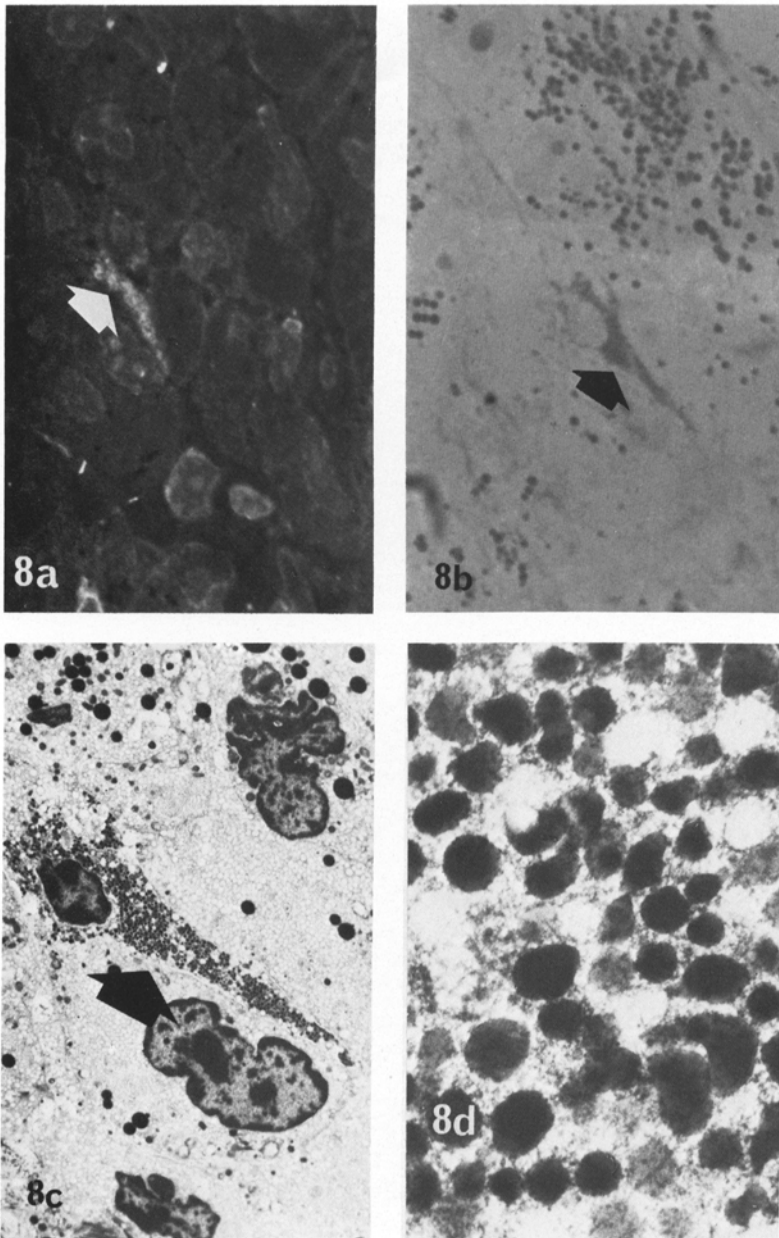
Fig. 8. a Semithin section stained with somatostatin antiserum; *arrow* indicating the positive cell. × 500. **b** Same cell after re-staining with the masked metachromasia method. × 500. **c** Same cell in a serial thin (80 nm)-section. × 2,000. **d** Detail of the secretory granules. × 28,000
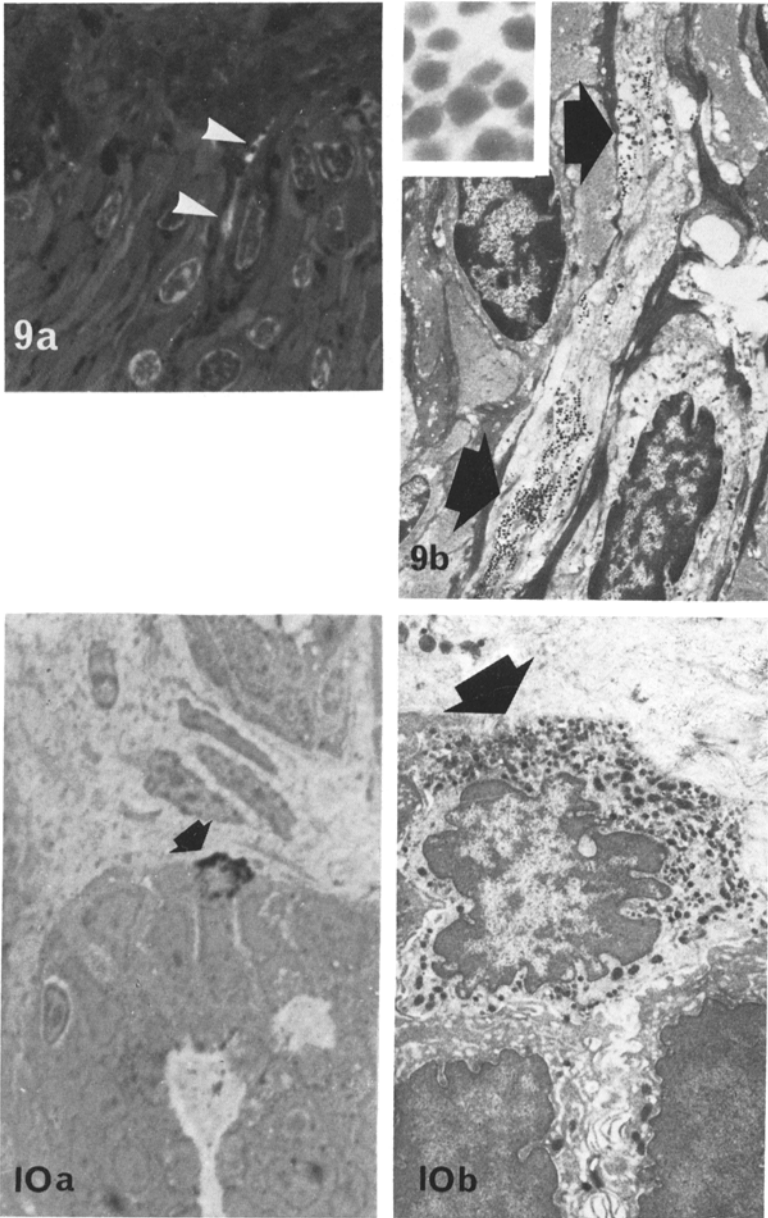
Fig. 9. a Semithin section showing VIP-immunoreactive fibres (*arrows*). × 500. **b** Same area in serial thin section. × 2,000. *Insert:* Secretory granules. × 28,000

Fig. 10. a Semithin section showing argentaffin, Masson-positive cell. × 500. **b** Same cell in the serial 80 nm-section. × 6,000
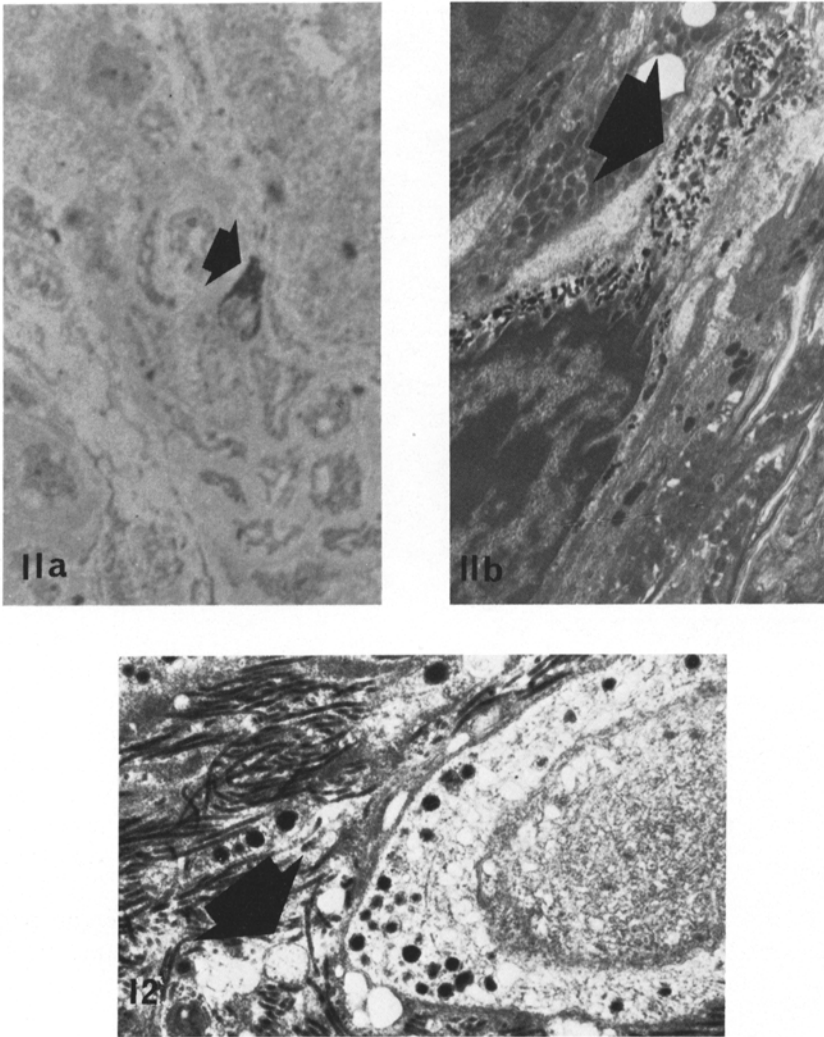
Fig. 11. a Grimelius'-stained semithin section: *arrow* indicates the positive cell. × 500. **b** The same cell in the serial thin section. × 5,000

Fig. 12. Endocrine cell in the lamina propria of the duodenum near nerve fibres (*arrow*). × 14,000

Somatostatin-immunoreactivity was present in cells containing granules that were slightly polymorphic with an average diameter of 300 nm (50 profiles) (Fig. 8). Substance P-immunostaining was found in cells containing polymorphic granules with an average diameter of 300 nm (50 profiles). The nerve fibres containing substance P were not identified in the resin-embedded material, possibly due to the smaller quantities present.

VIP-immunoreactivity was present in fibres containing numerous electron-dense secretory granules averaging 150 nm in diameter (50 profiles) (Fig. 9).

Cytochemically identified cells: Argentaffin cells (amine-containing), as identified by Masson's silver stain, contained granules 200 nm in diameter (Fig. 10). These cells did not react with any of the antisera employed in this study.

Lead haematoxylin-positive cells were numerous throughout the intestine, especially in the stomach. The cells were often found at the periphery of the glands and were filled with large electron-dense granules, 300 nm in diameter (100 profiles). The masked metachromasia-technique revealed numerous cells that were found to contain somatostatin-immunoreactivity (for description, see somatostatin).

Grimelius-positive cells were present throughout the intestine and contained secretory granules ranging from 160–190 nm (50 profiles) (Fig. 11). These cells did not react with any of the antisera used in the study.

Additional cells were identified solely on the basis of their morphology. These consisted of a population of cells resembling endocrine cells in close association with nerve fibres (Fig. 12).

Discussion

The salamander, belonging to the order Urodela, is one of the most primitive land dwelling vertebrates. These animals have remained unspecialised in comparison to the other two extant amphibian orders, the Apoda (caecilians) and the Anura (frogs and toads). Although fully terrestrial, salamanders have a freely permeable skin, which restricts their habitat to areas where water is readily available. The basic structure of the amphibian intestine resembles that of mammals with mucosa, lamina propria, nerve plexuses and muscle layers. Villi and crypts do not develop in these animals, although folding of the gut epithelium gives rise to villus-like structures.

Immunostaining shows that the mucosa and submucosa of salamanders are rich in peptide-containing cells and nerves in a similar dual localisation to that found in mammals.

In mammals, somatostatin is thought to act in a paracrine (local) manner (Polak and Bloom 1979), and it has been suggested that the long processes found on these cells indicate that the individual somatostatin-containing cells influence directly several surrounding cells (Larsson et al. 1979). If the amphibian peptide acts in the same manner, this would explain the occurrence of the "dendritic" processes found in these cells.

The significance of the cells found in the lamina propria in association with nerve fibres is unknown, although they resemble those found in the finch (Katakoa 1974). It has been suggested that they are the "missing link" between peptide-containing submucosal nerve fibres and mucosal endocrine cells (Matsuo et al. 1976).

The cells identified in this study differ markedly in their ultrastructural appearance from those identified in mammals using the same techniques (Buchan and Polak 1980). Therefore, it is essential to identify cell types by product, not morphology, in these non-mammalian species.

In the mammalian intestine, VIP-nerves are implicated in the stimulation of water and electrolyte secretion (Gaginella and O'Dorisio 1979), vasodilation (Said and Mutt 1970) and relaxation of the muscle fibres (Kachelhoffer et al. 1976). In the salamander, VIP-immunoreactivity was confined to the submucous and myenteric plexus, innervating the longitudinal muscle layer (see Fig. 7) and suggesting that the effect of this peptide may be concerned with muscle relaxation.

Substance P is known, in mammals, to have a potent effect on smooth muscle contraction and vasodilation (Bury and Mashford 1977; Von Euler 1977). Substance P-immunoreactive fibres were found in both plexus and around blood vessels (see Fig. 6), but did not infiltrate the muscle layers, thus suggesting a primarily vasodilatory role for this peptide in the salamander.

GIP, secretin, CCK and motilin were not detected in the salamander intestine, raising the possibility that, since the antisera were raised in mammals, they were unable to detect the amphibian form of the peptide. The present study cannot determine whether closely related peptides were present in the gut.

Interesting information can be gathered from the results of immunostaining for CCK and gastrin. One of the antisera to CCK used in this study was specific for the mid-portion (9–20) of the molecule, which has no similarity to gastrin, unlike the C-terminal pentapeptide, which is identical in both hormones (Polak et al. 1977). In addition to this antiserum, a C-terminal specific one was also used. The specific (9–20) CCK-antiserum detects neither gastrin nor the C-terminal octapeptide of CCK. Larsson and Rehfeldt (1977) showed that antisera to the C-terminal region of gastrin immunostained numerous endocrine cells of the antral mucosa of amphibians and that a C-terminal (octapeptide)-specific CCK antiserum detected the same cells. This correlates well with our finding of numerous cells reactive to both the C-terminal gastrin and CCK antiserum in the stomach, and unreactive with the specific CCK and N-terminal gastrin antiserum.

Larsson and Rehfeldt (1977) suggested that CCK and gastrin do not occur as separate entities in Amphibia, but are represented by a single peptide that might be related to an ancestral molecule of the gastrin family. This could be a caerulein-like peptide, since caerulein, which was originally isolated from frog skin, is known to have the same C-terminal pentapeptide sequence as gastrin and CCK. If this is correct, it would explain the positive reaction to the C-terminal gastrin and CCK antisera found in the present study and the lack of immunoreactivity to the CCK specific antiserum and the N-terminal gastrin antiserum.

Thus, we have demonstrated that the majority of the peptides comprising the mammalian neuroendocrine system are recognizable, immunologically, within the gut of a primitive amphibian. Whether the actions of these peptides in Amphibia bear any resemblance to that in Mammalia remains to be elucidated.

References

Buchan AMJ, Polak JM, Solcia E, Capella C, Hudson D, Pearse AGE (1978) Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK. Gut 19:403–407

Buchan AMJ, Polak JM (1980) The classification of the human gastroenteropancreatic endocrine cells. Invest Cell Pathol 3:51–71

Bury RW, Mashford ML (1977) A pharmacological investigation of synthetic substance P on the isolated guinea pig ileum. Clin Exp Pharmacol Physiol 4:453–461

Coons AH, Leduc EH, Conolly JM (1955) Studies of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med 102:49–60

Ersparmer V, Melchiorri P (1973) Active polypeptides of the amphibian skin and their synthetic analogues. Pure App Chem 35:463–493

Gaginella TS, O'Dorisio TM (1979) Vasoactive intestinal polypeptide. Neuromodulator of intestinal secretion? In: Binder HJ (ed) Mechanisms of Intestinal Secretion, Kroch Foundation Series, Vol 12, 231–248

Grimelius L (1968) A silver nitrate stain for A_2 cells in human pancreatic islets. Acta Soc Med Upsala 73:243–270

Kachelhoffer J, Mendel C, Dauchel J, Hohmatter D, Grenier JF (1976) The effects of VIP on intestinal motility. Dig. Diseases 21:957–962

Kataoka K (1974) An electron microscope study on a neuroendocrine complex in the proventricular mucosa of the finch *Uroloncha striata var domestica*. Arch Histol Jpn 35:391–400

Kingsley Noble G (1954) The biology of the Amphibia. Dover Publications, New York

Lane BP, Europa DL (1965) Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. J Histochem Cytochem 13:579–582

Larsson L-I, Rehfeld JF (1977) Evidence for a common evolutionary origin of gastrin and cholecystokinin. Nature 269:335–338

Lechago J, Holmquist A, Rosenquist GL, Walsh JH (1978) Localisation of bombesin-like peptides in frog gastric mucosa. Gen Comp Endocrinol 36:553–558

Matsuo Y, Seki A, Fukada S (1976) Neuro-endocrine cells in the lamina propria of rat stomach. In: Fujita T (ed) Endocrine Gut and Pancreas. Elsevier, Amsterdam, p 159–165

Pearse AGE, Polak JM (1975) Bifunctional reagents as vapour- and liquid-phase fixatives for immunocytochemistry. J Histochem 7:179–186

Polak JM, Pearse AGE, Heath CM (1975) Complete identification of endocrine cells in the gastrointestinal tract. Use of the semithin-thin technique to identify motilin cells in human and animal intestine. Gut 16:225–229

Polak JM, Pearse AGE, Szelke M, Bloom SR, Hudson D, Facer P, Buchan AMJ, Bryant MG, Christofides N, MacIntyre I (1977) Specific immunostaining of CCK cells by use of synthetic fragment antisera. Experientia 33:762–763

Polak JM, Bloom SR (1979) Neuropeptides and the antrum. In: Rehfeld JH (ed) Gastrin and the Vagus, Academic Press

Said SI, Mutt V (1970) Potent peripheral and splanchnic vasodilator peptide from normal gut. Nature 225:863–864

Solcia E, Vassallo G, Capella C (1968) Selective staining of endocrine cells by basic dyes after acid hydrolysis. Stain Technol 43:257–263

Solcia E, Sampietro R, Capella C (1969a) Differential staining of catecholamines, 5-hydroxytryptamine and related compounds in aldehyde fixed tissues. Histochemie 17:273–283

Solcia E (1969b) Lead haematoxylin as a stain for endocrine cells – significance of staining and comparison with other selective methods. Histochemie 20:116–126

Von Euler US, Pernow B (eds) (1977) Substance P. Raven Press, New York