Phylogenetic Aspects of the Occurrence and Distribution of Secretogranin II Immunoreactivity in Lower Vertebrate Gut*

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Summary. A novel monoclonal antibody raised against bovine secretogranin II (Sg II) was used in immunohistochemical studies on amphibian (*Rana esculenta*), reptilian (*Podarcis sicula*) and avian (*Gallus gallus*) gut. Sg II immunoreactivity was detected in epithelial and nervous elements. Cells immunoreactive for Sg II were examined by double immunostainings to determine whether they might also co-store certain previously known bioactive amine/peptide substances. Almost all the endocrine cells immunoreactive for bombesin, substance P, neurotensin, gastrin/cholecystokinin, neuropeptide tyrosine (NPY) and calcitonin gene-related peptide as well as some of those immunostained for serotonin, histamine, and polypeptide tyrosine tyrosine (PYY) also contained Sg II. Sg II-immunoreactive cells varied in number and distribution according to regions of the gut and animal species. The number of Sg II immunoreactive granules notably varied not only according to cell type, but also within the same cell population.

Many histamine-, calcitonin gene-related peptide (CGRP)-, substance P-, PYY-, and neurotensin-immunoreactive neurons also contained Sg II. These were mostly situated in the myenteric plexus; their distribution pattern varied among the three species.

These findings show that, despite being well conserved during phylogeny, Sg II has a heterogeneous distribution.

Secretogranin II (Sg II) (previously also named chromogranin C, see EIDEN et al., 1987, for nomenclature) belongs, together with chromogranins A and B, to a family of structurally related hydrophilic proteins found in high concentrations in a wide variety of neuroendocrine secretory granules. Sg II is an anionic tyrosine-sulphated protein initially found in the secre-

tory vesicles of the anterior pituitary cells (ROSA and ZANINI, 1981; ROSA et al., 1985a, b; RUNDLE et al., 1986) and successively demonstrated in several neuroendocrine tissues as well (FISCHER-COLBRIE et al., 1986, 1987; HAGN et al., 1986; LASSMANN et al., 1986; RINDI et al., 1986; YOSHIE et al., 1987; BUFFA et al., 1988; GRUBE and YOSHIE, 1989; HUTTNER et al., 1991; CETIN and GRUBE, 1991).

Although little is known of the physiological role(s) of the chromogranin/secretogranin family, it has been suggested that these proteins are involved in the organization of the secretory granule matrix (i.e. binding of calcium and ATP), in the processing, packaging or various combining of peptidic, aminic and other molecules within the secretory vesicles, in addition to some regulatory actions during or after exocytosis (GERDES et al., 1989; WIEDENMANN and HUTTNER, 1989; DEFTOS, 1992). Recent studies have indicated that each protein contains multiple pairs of basic residues that are potential sites of posttranslational proteolytic cleavage to yield smaller peptides with possible biological activities. This role as prohormone has been also suggested for Sg II because secretoneurin, a small biologically active peptide derived from its linear sequence, has been isolated from extracts of total frog brain (VAUDRY and CONLON, 1991) and from the mammalian brain and endocrine tissue extracts (KIRCHMAIR et al., 1993).

Because of its potential usefulness as a marker of neuroendocrine tissues both in normal and in pathological conditions, the presence of Sg II has been investigated mainly in mammals (WEILER et al., 1987, 1988; WIEDENMANN et al., 1988; WIEDENMANN and HUTTNER, 1989).

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In agreement with the few previous studies (DEFTOS et al., 1987; RIEKER et al., 1988; REINECKE et al., 1991) indicating that chromogranins A and B are well conserved in the phylogeny, we have already described the occurrence, topography and co-localization patterns of chromogranins A- and B-immunoreactive cells in the mucosa of the alimentary tract of the chicken, lizard and frog (D'ESTE et al., 1992, 1993, 1994). No precise indications appear to have been published on the presence of Sg II in the guts of animals other than mammals. For this reason, using a novel monoclonal antibody directed against bovine Sg II, but also cross-reacting with human protein, we investigated the occurrence and distribution of Sg II-immunoreactive elements in the gut of the same animal species. Using double immunostaining procedures and employing a panel of polyclonal antisera against different amine/peptide signed substances, we also sought possible co-localizations within Sg II-immunoreactive elements.

MATERIALS AND METHODS

Animals

Ten adult specimens of both sexes of each animal species were used for this study. Green frogs (*Rana esculenta*) and lizards (*Podarcis sicula*) were collected at springtime from cultivated areas around Rome and kept unfed in our laboratory for no more than 48 h; adult chicks (*Gallus gallus*, Wyandotte strain) aged about 4/6 months were purchased at a local poultry farm. Frogs were anaesthetized with tricaine (MS222, Sigma, USA), and lizards and chicken with ether. All the specimens were perfused *via* the heart ventricle with 100 ml cold 0.01 M phosphate-buffered saline, pH 7.4 followed by 100 ml of a cold fixative containing 4% paraformaldehyde (FA), 0.2% picric acid (PA), 0.35% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (PB).

Tissue preparation

Digestive organs from the oesophagus to the cloaca were quickly removed and divided into several fragments, each of which was subdivided into two 5-mm thick blocks and post-fixed for 24 h at +4°C, one by immersion in 4% FA, 0.2% PA in PB, and the other in the same mixture with the addition of 1% carbodiimide (Sigma, USA). All the tissue blocks were then cryoprotected by immersion in cold 15% sucrose in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), frozen, and transversally cut with a cryotome (Frigocut, Reichert-Yung, Austria) into serial 12- μ m thick sec-

tions that were mounted on chrome-alum/gelatine-coated slides.

Antisera

Specimens were treated with a new monoclonal antibody raised against bovine Sg II, and produced as previously reported (PELAGI et al., 1992). Briefly, a Sg II-enriched fraction of bovine anterior pituitary was used for mice immunization. Hybridomas producing anti-Sg II antibodies were revealed by ELISA and by two-dimensional immunoblotting and then cloned. Immunoblotting and immunohistochemistry showed that the supernatant labelled as 5A7 recognized Sg II both from the bovine pituitary and from human pheochromocytoma. The relative hybridoma was then sub-cloned and ascite fluids further selected and used for several immunohistochemical investigations.

Immunohistochemistry

Sg II-immunoreactive elements were demonstrated by the indirect immunofluorescent technique. Non-specific binding of the antisera was blocked by a 30-min pre-incubation at room temperature in normal goat or swine serum (Dakopatts, Denmark) diluted 1:30 in PBS containing 1% bovine serum albumin (BSA, Sigma) (PBS/BSA). Slides were then incubated in a moist chamber overnight at room temperature or for 48 h at +4°C, with the monoclonal antibody diluted up to 1:1000 with PBS/BSA. After washing with PBS, they were then incubated for 30 min at room temperature with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dakopatts) or tetraethylrhodamine-isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:50 with PBS/BSA. Slides were then mounted and coverslipped in a glycerol-buffered medium.

To ascertain possible co-localizations with other known biologically active molecules, the polyclonal antisera listed in Table 1 were used in double immunostaining procedures. Sections thus treated were successively incubated with TRITC- or FITC-conjugated swine anti-rabbit IgG (Dakopatts) diluted 1:50 with PBS/BSA, for 30 min at room temperature. To avoid false-positive or false-negative results, all possible staining combinations were tried, using the monoclonal or the polyclonal antibodies in the first immunostaining, with either FITC- or TRITC-conjugated secondary antisera. Results were then observed with a photomicroscope (Polyvar, Reichert-Yung) equipped with appropriate filter combinations for FITC and TRITC. Pairs of photos of the same microscopic field, taken with both filter combinations, were then compared to show double labelled elements.

Controls

Immunohistochemical controls for method-dependent non-specificity and for antibody specificity included the following: 1) the omission of single steps in the immunohistochemical procedure; 2) the substitution of primary antiserum with normal mouse or rabbit serum, or with a buffer; 3) a pre-absorption test using primary antisera pre-incubated (overnight at +4°C) with homologous antigens (up to 50 µg per ml diluted antiserum) or heterologous but structurally related antigens (up to 200 µg per ml); 4) the random omission of the antiserum for second localization or its substitution with normal mouse or rabbit serum in the double immunostaining procedure, in order to avoid false secondary localizations. The corresponding FITC- or TRITC- conjugated secondary antiserum was then used alone.

RESULTS

General remarks

The monoclonal antibody revealed endocrine cells and nervous elements containing a variable amount of Sg II-immunoreactive granules. Their occurrence and distribution pattern varied widely in the gut tracts and among the three animal species.

Tables 2, 3 and 4 summarize the findings and provide a semiquantitative assessment of the occurrence and the topography of Sg II-immunoreactive elements and the co-localization patterns with amines/peptides in the frog, lizard and chicken gut.

Frog

Cells immunopositive for Sg II were found throughout the gut tracts (Figs. 1, 2), a higher concentration being present in the pylorus and the proximal tracts of the small intestine. In the oesophagus, they were scattered within the superficial epithelium of the mucosa and in the glands. Most Sg II-immunoreactive cells had a small diameter and a roundish shape and contained variable quantities of immunopositive granules. In the oxyntic mucosa, Sg II-immunoreactive cells were found among the epithelial cells of the glands, where they occupied two distinct positions: the neck and the bottom of the glands. The cells in the glandular neck were slender and filiform. They often had a bracket-like shape, and almost invariably reached the lumen of the glands ("open" type); the immunopositive granules accumulated particularly in the cell luminal processes. The Sg II-immunoreactive cells in the glandular bottom were small in diameter and roundish and rarely reached the glandular lumen ("closed" type). Towards the pylorus, the "closed" type gradually diminished until they disappeared, being replaced in the glandular body by a new population of flask-shaped cells, with a cytoplasmic process directed towards the lumen. In the small and large intestines, the majority of Sg II-immunoreactive cells appeared slender, filiform or triangular, of the "open" type, and occupied the villi as well as the crypts.

Sg II-immunoreactive nerve cells and fibres were found from the oesophagus to the small intestine. They were predominantly located in the myenteric plexus and formed a denser network in the pylorus and duodenum.

Table 1. Polyclonal antisera used for double immunostaining studies. The working dilution (W. D.) for the immunofluorescent procedure was indicated.

Antigen	W. D.	Source
Serotonin (5HT)	1 : 1000	Dr. KIMURA, (Otsu, Japan)
Histamine (HI)	1 : 500	Milab (Sweden)
Somatostatin (SRIF)	1 : 500	Peninsula (UK)
Gastrin/Cholecystokinin (G/CCK)	1 : 500	Peninsula (UK)
Bombesin (B)	1 : 500	Peninsula (UK)
Substance P (SP)	1 : 500	Amersham (UK)
Peptide tyrosine tyrosine (PYY)	1 : 500	Peninsula (UK)
Pancreatic polypeptide (PP)	1 : 500	Dakopatts (Denmark)
Neuropeptide tyrosine (NPY)	1 : 500	Amersham (UK)
Neurotensin (NT)	1 : 500	Peninsula (UK)
Calcitonin gene-related peptide (CGRP)	1 : 500	Peninsula (UK)

Table 2. Occurrence and distribution of Sg II-immunoreactive elements in the gut of *Rana esculenta*

Gut tracts		Cells		Nerve fibres	
		Sg II	Co-localizations	Sg II	Co-localizations
Oesophagus		+	B-SP	+	CGRP
Stomach	proximal	++	B-PYY-NPY	+	CGRP-HI-SP
	middle	++	B-PYY-NPY	+	CGRP-HI-SP
	pylorus	+++	G/CCK-PYY-NPY-5HT	+	CGRP-HI-SP
Small intestine	proximal	+	—	+	CGRP-HI-SP
	distal	±	—	±	HI
Large intestine		+	—	±	—

Cells: + + +, 6-10/0.2 mm²; ++, 3-5; +, 1-2; ±, not in all the microscopical fields. Nerve fibres: +, delicate network; ±, few or single fibres, scattered.

Table 3. Occurrence and distribution of Sg II-immunoreactive elements in the gut of *Podarcis sicula*

Gut tracts		Cells		Nerve fibres	
		Sg II	Co-localizations	Sg II	Co-localizations
Oesophagus		++++	B-SP-HI-5HT	—	
Stomach	proximal	++++	B-SP-HI-5HT-PYY	++++	CGRP
	middle	++++	B-SP-HI-5HT-PYY-G/CCK	++++	CGRP
	pylorus	++++	PYY-HI-5HT-NT-G/CCK	+	—
Small intestine	proximal	++++	5HT-NT	+	
	distal	+++	—	+	—
Large intestine		++	—	—	—

Cells: + + + +, >10/0.2 mm²; + + +, 6-10; ++, 3-5; +, 1-2; —, none.
Nerve fibres: + + + +, dense network; +, a few fibres.

Table 4. Occurrence and distribution of Sg II-immunoreactive elements in the gut of *Gallus gallus*

Gut tracts		Cells		Nerve fibres	
		Sg II	Co-localizations	Sg II	Co-localizations
Oesophagus		—	—	—	—
Proventriculus		++++	HI	++++	CGRP-SP-NT
Gizzard		+	—	—	—
Antrum		++++	G/CCK-NT-PYY-5HT	++++	CGRP-SP-PYY
Small intestine	proximal	+++	5HT	+++	CGRP-SP-PYY
	distal	+++	5HT	+++	CGRP-SP-PYY-HI
Large intestine	Coecum	++++	—	++++	PYY
	Colon	++	—	++	—

Cells: + + + +, >10/0.2 mm²; + + +, 6-10; ++, 3-5; +, 1-2; —, none.
Nerve fibres: + + + +, dense network; + + +, discrete network; ++, delicate network; +, a few fibres; —, none.

Lizard

As compared with the frog, the lizard gut contained a higher number and a wider distribution of Sg II-immunoreactive cells (Figs. 3, 4). In the caudal part of the oesophagus and in the gastric body, they lay against the basal lamina within the superficial epithelium and at the bottom of the glands. They were roundish, of small diameter and displayed "closed" features. The flask-shaped cells in the pyloric glands and the slender filiform cells in the small intestine were more numerous than those in the frog. They became fewer in the large intestine, but increased again in the cloaca, which contained both "open" and "closed" cell types.

Sg II-immunoreactive nerve elements within the myenteric plexus were detected only in the stomach.

Chicken

Endocrine cells and neurons immunoreactive for Sg II were widely represented throughout the chicken gut tracts (Figs. 5–8) with the exception of the oesophagus. The proventriculus had a rich population, concentrated mainly in the distal third of its composite glands. These cells had diverse morphological forms; the majority were elongated and probably of the "closed" type because their major axis lay parallel to the basal lamina of the glandular epithelium. Rare in the gizzard, the Sg II-immunoreactive cells reached a true peak in the antral mucosa, where they filled the glandular epithelium and usually displayed "closed" features; their numbers then declined in the remaining tracts. In the small and large intestines, slender and filiform cells preponderated. Because their major axis lay perpendicular to the basal lamina, they appeared to be of the "open" type.

With the exception of the gizzard, all the chicken gut tracts contained networks of variable densities of myenteric nerve elements immunopositive for Sg II.

Co-localizations

A series of double immunostainings on the same tissue section made it possible to investigate the coexistence of amine/peptide-immunoreactive granules within the Sg II-immunoreactive elements.

Serotonin (5HT) cells

Cells-immunoreactive for 5HT were widely distributed in almost all the gut tracts. In the frog, some of those situated in the pyloric glands also co-stored Sg II-immunoreactive granules. In the lizard (Fig. 3g, h) and chick (Fig. 5c–f), a variable portion of 5HT cells

throughout the gut tracts displayed co-localization with Sg II. The quantity of positive granules within these cells varied widely, often being reduced to a thin peripheral ring.

Histamine cells

Cells immunoreactive for histamine were observed in the pyloric glands in the frog, from the oesophagus to the small intestine in the lizard, and in the proventriculus in the chicken. None of the frog histamine cells displayed co-localization with Sg II. In the lizard, only the histamine cells situated in the oesophagus and the stomach also contained a variable amount of granules immunoreactive for Sg II (Fig. 3a, b), whereas the more caudally situated cells contained none. In the chicken proventriculus, the distal third of the composite glands was filled with numerous histamine-immunoreactive cells, most of which contained granules also immunoreactive for Sg II (Fig. 5a, b).

Gastrin/cholecystokinin (CCK) cells

Gastrin/CCK-immunoreactive cells were grouped mostly in the pyloric region and in smaller amounts in the small intestine. In frog and lizard, they were predominantly flask-shaped, with "open" features, whereas in the chicken antrum they tended to be roundish and apparently devoid of contact with the lumen. Double-staining experiments showed that, in the frog and lizard, the majority of these cells also contained variable amounts of Sg II-immunoreactive granules (Figs. 2c, d, 3e, f). Conversely, some of the abundant gastrin/CCK-immunoreactive cells that filled the chicken antrum lacked any Sg II-immunopositive content (Fig. 6a–d). In the small intestine, the coexistence with Sg II declined to only a few gastrin/CCK cells.

Bombesin cells

In the frog and lizard, cells immunoreactive for bombesin were found in the oesophagus and the stomach; they were small and roundish and had "closed" features. Virtually all these cells additionally co-localized granules also immunoreactive for Sg II (Fig. 1a, b). Bombesin-immunoreactive cells were also found in the glands of the chicken proventriculus, but none displayed co-localization with Sg II.

Substance P (SP) cells

Although SP-immunoreactive cells were widely distributed throughout the gut tracts, they preponderated in the oesophagus and the stomach of the frog and lizard and had the same features as the bombesin-immunoreactive cells. Like the bombesin cells, the

Fig. 1. *Rana esculenta* proximal (a, b) and middle (c-h) portion of the stomach. Double fluorescence immuno-staining for bombesin (B) (a), PYY (c, e) and NPY (g) and then for Sg II (a, d, f, h). Note the heterogeneity of Sg II-immunoreactive granular content within the same cell population. Arrow indicates a solely PYY-immunoreactive cell. $\times 416$

Fig. 2. *Rana esculenta* pylorus. Double fluorescence immunostaining for PYY (a), gastrin/CCK (G/CCK) (c) and histamine (HI) (e) and then for Sg II (b, d, f). Note the heterogeneity of the peptide/Sg II co-existence within the endocrine cells and the nearly complete co-localization of histamine and Sg II in the myenteric nervous network. *m* Circular muscular layer. a, b: $\times 104$; c, d: $\times 416$; e, f: $\times 260$

Fig. 3. *Podarcis sicula* stomach (a-f) and small intestine (g, h). Double fluorescence immunostaining for histamine (HI) (a), SP (c), gastrin/CCK (G/CCK) (e) and 5HT (g) and then for Sg II (b, d, f, h). a and b. All the histamine-immunoreactive cells store a variable quantity of Sg II. c and d. Two double immunostained cells buried against the basal lamina within the lining epithelium. e and f. Gastrin/CCK-immunoreactive cells co-storing Sg II within the gastric gland epithelium. g and h. Three intestinal 5HT-immunoreactive cells store different amount of Sg II-immunoreactive granules. Arrow points a solely 5HT-immunoreactive cell. a-f: $\times 416$; g, h: $\times 260$

Fig. 4. *Podarcis sicula* stomach (a-f) and distal intestine (g, h). Double fluorescence immunostaining for CGRP (a, c), and PYY (e, g) and then for Sg II (b, d, f, h). a-d. Both the glands and the superficial epithelium present some double immunostained cells. e-h. Two PYY-immunoreactive cells subtypes having opposite features towards the Sg II-immunoreactive granular content are shown: one, in the stomach (e, f), displaying almost complete co-existence and the other, in the distal portion of the small intestine (g, h), lacking in co-localization. a, b: $\times 325$; c, d: $\times 520$; e, f: 260; g, h: $\times 104$

Fig. 5. *Gallus gallus* proventriculus (**a, b**) and antrum (**c-f**). Double fluorescence immunostaining for histamine (HI) (**a**) and 5HT (**c, e**) and then for Sg II (**b, d, f**). Almost all the histamine-immunoreactive cells contain variable quantity of Sg II-immunoreactive granules (**a, b**). 5HT-immunoreactive cells co-storing different amount of Sg II are present both in the glands (**c, d**) and in the superficial epithelium (**e, f**). a, b: $\times 416$; c, d: $\times 260$; e, f: $\times 416$

Fig. 6. *Gallus gallus* antrum. Double fluorescence immunostaining for gastrin/CCK (G/CCK) (a, c) and NT (e) and then for Sg II (b, d, f). The distribution pattern clearly suggests the co-existence of gastrin/CCK, NT and Sg II in the same cell population within the antral glands. *Arrows* indicate some solely gastrin/CCK-immunoreactive cells. a-d: $\times 260$; e, f: $\times 416$

Fig. 7. *Gallus gallus* antrum. Double fluorescence immunostaining for PYY (**a**, **c**) and CGRP (**e**) and then for Sg II (**b**, **d**, **f**). The coexistence of PYY with Sg II is more complete in the glands (**a**, **b**) than in the superficial epithelium (**c**, **d**). Arrow indicates a solely PYY-immunoreactive cell. $\times 416$

Fig. 8. *Gallus gallus* proventriculus (a, b), antrum (c, d) and coecum (e, f). Double fluorescence immunostaining for CGRP (a, c) and PYY (e) and then for Sg II (b, d, f) to show the presence of double immunostained nervous network within the myenteric nerve plexus. Arrows follow a long double immunostained nerve fiber. $\times 416$

SP-immunoreactive cells all contained Sg II-immunopositive granules (Fig. 3c, d).

Poly peptide tyrosine tyrosine (PYY) cells

PYY-immunoreactive cells were widely distributed in almost all the gut tracts, with the exception of the oesophagus in the three animal species and the proventriculus of the chick. As a rule, they displayed "open" features and occupied various positions within the mucosa. Double immunostaining distinguished two PYY cell subtypes. The first also co-stored variable amounts of Sg II-immunopositive granules and was situated mainly in the frog (Figs. 1 c-f; 2a, b) and lizard (Fig. 4e, f) stomach and in the chicken antrum (Fig. 7a-d). The second subtype had no granules immunoreactive for Sg II and preferentially occupied the small and large intestines (Fig. 4 g, h). Both subtypes were common, in the duodenum, the first situated within the villi and the second within the crypts.

Neuropeptide tyrosine (NPY) cells

Cells immunoreactive for NPY, resembling local PYY cells in morphology and topography, were observed only in the frog stomach. Like the PYY cells, these cells displayed the co-localization of Sg II-immunopositive granules in their cytoplasm (Fig. 1g, h).

Neurotensin (NT) cells

All the gut tracts contained NT-immunoreactive cells. Several of them, mainly those in the lizard duodenum and chicken antrum, also co-stored Sg II-immunopositive granules (Fig. 6e, f). The number of co-localizing cells consistently declined in the more caudal gut tracts.

Calcitonin gene-related peptide (CGRP) cells

The antiserum raised against rat CGRP specifically stained a discrete number of endocrine cells both in the lizard stomach (Fig. 4a-d) and in the chicken antrum (Fig. 7e, f). They tended to occupy both the superficial epithelium and the proximal third of the glands, and preferably displayed "open" features. All of them had a variable granular content immunoreactive for Sg II.

Pancreatic polypeptide (PP) cells

PP-immunoreactive cells were found in the small and large intestines of the three species. Despite being frequently in close contact with Sg II-immunoreactive cells, these PP cells never co-localized Sg II.

Somatostatin cells

Although cells immunoreactive for somatostatin were widely distributed in all regions of the gut, none of them immunoreacted with Sg II.

Hormonally non-identified cells

Double immunostaining disclosed, mainly in the small and large intestine, a discrete number of cells immunoreactive for Sg II but devoid of other immunoreactivity with the antisera panel of Table 1.

Neurons

Double immunostaining demonstrated that almost all the Sg II-immunoreactive nervous elements were also aminergic or peptidergic and had different distribution patterns according to the regions of the gut and animal species.

The frog gut contained only a delicate network of Sg II-immunoreactive neurons and sometimes only a few or single fibres. They mainly co-localized CGRP, histamine (Fig. 2e, f) and SP and were situated in the myenteric plexus of almost all the regions of the gut (Table 2).

In the lizard, Sg II-immunoreactive nerve fibres formed dense networks only in the gastric myenteric plexus and almost all these fibres co-stored CGRP (Table 3).

In the chicken, a large quantity of nerve elements immunoreactive for Sg II was found in the myenteric plexus of all the gut regions; many neurons co-localized CGRP (Figs. 8a-d), SP and PYY (Fig. 8e, f), whereas only in the proventriculus did a moderate number also co-store NT. Only in the duodenum did a few nerve fibres co-store histamine.

DISCUSSION

The use of a novel monoclonal antibody produced against bovine Sg II, but also cross-reacting with human protein (PELAGI et al., 1992), has demonstrated the widespread presence of Sg II-immunoreactive endocrine cells and nerve elements throughout the guts of the frog, lizard and chick.

As previously shown for chromogranins A and B (DEFTOS et al., 1987; RIEKER et al., 1988; REINECKE et al., 1991; D'ESTE et al., 1992, 1993, 1994), this study has provided evidence that Sg II is well conserved during phylogeny.

Double immunostainings performed on the same tissue sections yielded the following findings:

1) A certain number of Sg II-immunoreactive endocrine cells, situated mainly in distal gut tracts, failed to immunoreact with any of our polyclonal antisera. We cannot exclude other possible co-localizations with known peptides not investigated in this study or with other unknown molecules.

2) In contrast, many Sg II-immunoreactive endocrine cells and nerve elements were identified by the co-localization of amine/peptide-immunoreactivities in their granules:

(a) A serotonin cell subtype, co-storing a variable amount of Sg II-immunoreactive vesicles, was found in the frog stomach and more widely distributed in the lizard and chicken gut mucosa. Their Sg II content frequently consisted of a few immunoreactive granules, sometimes situated in a perinuclear zone or sometimes packed in the most peripheral cytoplasm. Our earlier findings (D'ESTE et al., 1992, 1993, 1994) that the 5HT cells of these animals almost always contain chromogranin A and less frequently chromogranin B, suggest that a discrete number of 5HT-immunoreactive cells in lower vertebrates could co-store chromogranin A and Sg II, and that a few of them probably co-store all three proteins. These findings disagree with results obtained in mammals indicating that Sg II is never present in 5HT cells (CETIN and GRUBE, 1991).

(b) The different co-localization pattern displayed by the same endocrine cell population suggests that Sg II has an organ-specific distribution. All the PYY-immunoreactive cells situated in the gastric mucosa, for example, co-stored Sg II-immunopositive granules in their cytoplasm, whereas those of the intestine did not. Supporting our earlier observations on the co-existence of PYY and chromogranin A in the lizard (D'ESTE et al., 1993) and frog gut (D'ESTE et al., 1994), these findings clearly suggest that the gastric PYY cells contain both proteins while intestinal PYY cells contain none. PYY cells that positively immunostained both for chromogranin A and for Sg II have also been described in the guinea pig, although exclusively in the ileum and colon (CETIN and GRUBE, 1991).

(c) Some findings of this study suggest that Sg II has a species-specific distribution.

All the frog and lizard bombesin-immunoreactive cells co-stored Sg II, whereas those of the chicken proventriculus never did. In the frog and lizard, the bombesin cells were small, roundish, "closed" cells, buried in the epithelium lining the oesophagus and stomach and at the bottom of the gastric glands. Virtually all these cells showed bombesin- and substance P-immunoreactivity. Similar findings have already been reported in the stomach of another

lizard species (*Lacerta lepida*) (PEREZ-THOMAS et al., 1989). All performed controls allowed us to exclude possible cross-reactions between bombesin- and substance P-antisera. In addition, substance P-immunoreactive nerve fibres were never immunostained by the bombesin-antiserum, and conversely, the numerous cells of chicken proventriculus immunoreactive for bombesin were never stained by the substance P-antiserum. Hence, the frog and lizard cells may contain, in addition to Sg II, a molecule that probably differs from both substance P and bombesin, but shares with them a common epitope.

None of the cells immunoreactive for histamine observed in the frog pylorus showed Sg II-immunoreactivity, whereas almost all those of the lizard oesophagus and stomach and of the chicken proventriculus did. Because we noted the same features in our study of the co-existence of histamine and chromogranin A (D'ESTE et al., 1992, 1993), we presume that histamine-immunoreactive cells in the lizard and chicken contain both proteins. These findings correspond with the results obtained in the guinea pig gut (CETIN and GRUBE, 1991). In addition, the cells immunoreactive for histamine situated in the lizard small intestine, none of which immunostained for either protein, could again reflect the organ-specific distribution of the chromogranins. Finally, in the frog, where the histamine-immunoreactive endocrine cells were completely devoid of chromogranin content, myenteric histamine-immunoreactive nerve cell bodies and fibres co-storing Sg II were found throughout the gut tracts. Apart from the few histamine-immunoreactive nerve fibres in the chicken duodenum, no histamine-immunoreactive nerve elements were found. Histamine and chromogranins may therefore initially co-exist in nervous elements in the amphibian gut. From reptiles onwards, this co-existence occurs in epithelial endocrine cells. The possible physiological significance of these findings is unclear.

(d) The presence or absence of the co-existence with Sg II in gastrin/CCK-immunoreactive cells agrees with previous biochemical and immunochemical studies indicating that the gastrin/CCK-immunoreactive cells of lower vertebrates constitute a heterogeneous family. They probably contain a peptide structurally closer to mammalian CCK than to mammalian gastrin (for references see JOHNSEN and REHFELD, 1992). Since our method is not ideal for double immunostaining using two polyclonal antisera, by comparing the morphology and topography of the cells in adjacent sections, we noted that some of these cells also co-stored PYY, and others NT. Other reported data support our observations. The coexis-

tence of gastrin/CCK with PYY has already been shown in mammals (for references, see SUNDLER et al., 1993) and in the lizard stomach (DIAZ DE RADA et al., 1992) and intestine (BURRELL et al., 1992). The coexistence of gastrin/CCK with NT agrees with previous data reported in the chicken (RAWDON and ANDREW, 1981), mammals (RIX et al., 1986) and frogs (FLÜCHER et al., 1988). Furthermore, the number of the gastrin/CCK-immunoreactive cells that also contained Sg II-immunoreactive granules was notably higher in the gastric regions than in the small intestine.

(e) In the frog stomach and duodenum, yet never in the lizard or chick, almost all the PYY-immunoreactive/Sg II-immunoreactive cells were also immunostained by NPY antiserum. These data fully agree with those obtained in *Rana temporaria* (BÖTTCHER et al., 1985), and suggest that both antisera recognize a product encoded by the ancestral gene of the pancreatic polypeptide family, already duplicated for PP, but not yet for PYY/NPY (CONLON et al., 1992). In the frog gut, a distinct population of PP-immunoreactive cells has already been demonstrated; it differs from PYY-immunoreactive cells in morphology, in topography and in its lack of Sg II immunoreactive content.

(f) A certain number of endocrine cells immunostained by the anti rat CGRP serum were found in the lizard stomach and in the chicken antrum. At both sites, all these cells also co-stored a variable amount of Sg II. To our knowledge, only one previous study (DIAZ DE RADA et al., 1992) has reported the presence of CGRP-immunoreactive endocrine cells in the lizard *Podarcis hispanica* stomach.

(g) Almost all the nerve elements positively immunostaining for SP, histamine and PYY also co-stored Sg II, whereas only a part of CGRP-immunoreactive elements did so. In addition, the fact that none of the numerous CGRP-immunoreactive nerve cell bodies in the myenteric ganglia showed Sg II immunoreactivity clearly indicates that the CGRP-/Sg II-immunoreactive nerve fibres must be of extrinsic origin.

3) The immunoreactivity for Sg II was always irregular not only among different endocrine cells or peptidergic nerve elements, but also within the same epithelial or nervous cell population. These data closely agree with those obtained in several normal and pathological mammalian tissues (for references, see CETIN and GRUBE, 1991). In accordance with these authors, we believe that this heterogeneity might equally arise from a real variation of the intragranular protein content, and from the differing availability of the epitopes recognized by our antibody.

Because much evidence shows that in the secretory vesicles these proteins undergo a continuous metabolic turnover, the proteolytic processes may produce a series of break-down fragments which could differ among various cells, organs and animal species, and could be differently recognized as epitopes by our antibody. The extreme variability of Sg II-immunoreactive content may therefore reflect the actual physiological state of each cell at the moment of tissue fixation. On the other hand, as previously demonstrated for chromogranins (BARBOSA et al., 1991; WATKINSON et al., 1991), recent data from frogs and mammals have shown that Sg II is also an important source of small peptides (VAUDRY and CONLON, 1991; KIRCHMAIR et al., 1993), with some of them, such as secretoneurin, also possessing biological activities.

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