Amylin-Immunoreactivity is Co- Stored in a Serotonin Cell Subpopulation of the Vertebrate Stomach and Duodenum*

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Summary. Amylin (or islet amyloid polypeptide) is a 37 amino acid peptide originally isolated from amyloid deposits in the pancreas of non-insulin dependent diabetic patients. It has already been immunohistochemically localised within the B and D cells of pancreatic islets and in endocrine cells of the rat and human stomach and duodenum. In this phylogenetic study, a polyclonal antiserum raised against the carboxy-terminal tridecapeptide amide of human amylin was used to demonstrate and examine the distribution of amylin-immunoreactivity in the stomach and duodenum of various vertebrate species. Except for fish, gastrointestinal tracts of all the species studied contained amylin-immunoreactive endocrine cells. They were located chiefly in the lower half portion of the distal gastric body and pyloric glands, and in the lining epithelium of the duodenal villi and crypts. Many cells were elongated, triangular or oval, and had a cytoplasmic process that extended from the cell base along the basement membrane. Others had a bipolar feature that gave them a so-called “open” appearance. Double and triple staining procedures on the same tissue section showed that almost all the amylin-immunoreactive cells present in the gastro-duodenal region also co-stored serotonin and chromogranin A, and displayed argyrophilia in Grimelius impregnation. On the other hand, almost all the serotonin-immunoreactive cells of this region co-stored amylin, whereas those in more distal gut regions did not. This finding suggests that those amylin-containing cells correspond to a subtype of gastroduodenal serotonin cells.

The 37-amino-acid peptide amylin, also named islet amyloid polypeptide (IAPP), or diabetes associated polypeptide (DAP), was first isolated from the pancreatic islet amyloid of Type II (non-insulin-dependent) diabetic patients as well as from diabetic cat islets and human insulinomas (Westermark et al., 1986, 1987a, b, c; Cooper et al., 1987; Johnson et al., 1989; Nishi et al., 1990). It shares a 43% homology with the primary amino acid sequence of human α-calcitonin gene-related peptide (h-αCGRP) and 46% with h-βCGRP (Westermark et al., 1986, 1987a, b; Cooper et al., 1987). The amylin gene is located in chromosome 12, whereas calcitonin, α-CGRP and β-CGRP genes have been assigned to chromosome 11 (Moselmann et al., 1988).

Amylin-immunoreactivity was shown by radioimmunoassay to exist both in the pancreas and in the gut of normal mammals (Nakazato et al., 1989; Asai et al., 1990; Miyazato et al., 1991). Immunohistochemical studies in normal and pathological pancreatic islets of man and various mammalian species have demonstrated that amylin is co-stored in the B cells (Westermark et al., 1987a, b, Johnson et al., 1988; Clark et al., 1989; Lukinius et al., 1989; Toshimori et al., 1990), or in both B and D cells of rats and man (Mulder et al., 1993; Ohitsuka et al., 1993). In mammals, amylin mRNA was detected in the rat pancreas both by Northern blot analysis (Ferrier et al., 1989; Leffert et al., 1989; Nishi et al., 1990) and by in situ hybridization (Denijn et al., 1992; Mulder et al., 1993). Only three previous studies have demonstrated amylin-immunoreactive endocrine cells in the rodent and human antral region (Toshimori et al., 1990; Ohitsuka et al., 1993; Mulder et al., 1994). Ohitsuka et al. (1993)
also observed small quantities of amylin-immunoreactive cells in the gastric body, and described the co-localisation of gastrin with amylin in the same cells. In addition, Toshimori et al. (1990) also demonstrated the presence of amylin-immunoreactive cells in other gastrointestinal regions such as the duodenum and rectum. MÜLDER et al. (1994) confirmed the gastric localisation of amylin by *in situ* hybridisation and reported its partial co-localisation with somatostatin, gastrin and PYY.

Various experiments have indicated that amylin may exert a series of biological activities, such as a) the inhibition of insulin-stimulated glucose synthesis or transport in skeletal muscle (COOPER et al., 1988; LEIGHTON and COOPER, 1988; HOTERSHALL et al., 1990), b) the inhibition of glucose-stimulated insulin secretion from pancreatic islets (OHSAWA et al., 1989), c) calcitonin-like actions (DATTA et al., 1989; MACINTYRE, 1989; ZAIDI et al., 1990), d) vasodilatory effects similar to CGRP (BRAIN et al., 1990), and e) anorectic effects after peripheral (MORLEY and FLOOD, 1991) and intrahypothalamic injections (CHANCE et al., 1991). Some of the biological effects of amylin are thought to be mediated by adenylate cyclase activation via CGRP receptors (MORISHITA et al., 1990). The presence of receptors for amylin distinct from CGRP-receptors has already been demonstrated in one human hepatoblastoma line (SHERIF et al., 1992), but is still unclear in normal human tissues.

This phylogenetic study used a polyclonal amylin antiserum to investigate the presence and topographic distribution of amylin-immunoreactive cells in the stomach and duodenum of various vertebrate specimens, attempting to identify them by their immunohistochemical and histochemical features.

**MATERIALS AND METHODS**

**Animals**

Five adult specimens of each studied animal species were used. Two sea-water fish species (stickleback, *Lepidosteus aculeatus*; sea bass, *Dicentrarchus labrax*) were caught in the Tyrrhenian Sea, newts (*Triturus cristatus carnifex*) were supplied by the local comparative anatomy station; green frogs (*Rana esculenta*) and lizards (*Podarcis sicula*) were collected in 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 to 6 months were purchased at a local poultry farm. Adult male Wistar rats, weighing about 200 g, were obtained from the local animal house and received food and water *ad libitum*. Human gastric and duodenal specimens were obtained from endoscopic biopsies or surgery.

Fish, newts and frogs were anaesthetized with tricaine (MS222, Sigma, USA), lizards and chicken with ether and rats with sodium pentobarbital (50 mg/kg, i.p.; Abbott, Chicago, USA). All animal specimens were perfused via the cardiac ventricle with 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), and 0.35% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (PB). Small fragments of the stomach and duodenum were rapidly dissected out and postfixed in the same cold mixture without glutaraldehyde for 12 h. Gastric and duodenal human specimens were fixed by immersion for 12-24 h in this latter fixative.

**Tissue preparation**

All specimens were processed either for paraffin embedding or for cryotomic sectioning. Specimens for cryotomy were rinsed with cold saline PB (PBS) containing 15% sucrose, then frozen and cut in a cryotome (Frigocut, Reichert-Jung, Austria) into 15 µm-thick sections that were collected on gelatin-chrome alum-coated slides and stored till use at −70°C. Paraffin-embedded specimens were cut on a rotary microtome (Biocut, Reichert-Jung) into 5 µm-thick sections that were collected on albuminized slides.

**Antisera**

Three polyclonal antisera (AM 10F, AM 37L and AM R37C) were initially examined in this study. These preliminary studies showed that the antiserum AM 10F was superior because it yielded better immunohistochemical results (data not shown). This antiserum was raised in three rabbits against a synthetic carboxyterminal amidate fragment of human amylin (AM 25-37-NH₂). This peptide fragment, like whole amylin, was synthesised by solid-phase technology with an ABI automated peptide synthesiser (430A), using t-Boc chemistry. The peptide fragment was conjugated to thyroglobulin with carbodiimide. Each rabbit was immunized every two weeks with the peptide-thyroglobulin conjugate emulsified with incomplete Freund’s adjuvant. The blood was collected 5 days after every booster injection. The three antisera were able to fully cross-react (100%) with both human and rat amylin and their respective carboxy-terminal tridecapeptide amide. Practically no cross-reactivity (<0.1%) was shown against other peptides examined, including adenomendullin, CGRP (8-CGRP; hβ-CGRP and rat-CGRP), calcitonin (human, salmon and eel), insulin (porcine and human), katacalcin, neuropeptide tyrosine (NPY),
gastrin, cholecystokinin, and bombesin.

**Immunohistochemistry**

Both immunofluorescence and avidin-biotin-complex (ABC)—labeled with horseradish peroxidase—methods were used in this study. To avoid non-specific tissue binding, sections were first incubated for 30 min at room temperature with normal goat serum diluted 1:30 with PBS containing 1% bovine serum albumin (BSA, Sigma, USA) (PBS/BSA).

For immunofluorescence, sections were then incubated in a moist chamber overnight at room temperature (or for 48 h at 4°C) with anti-amylin serum (1:500 dilution of AM10F with PBS/BSA). After washing with PBS, they were incubated for 30 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Dakopatts, Denmark), diluted 1:50 with PBS/BSA. Slides were then coverslipped with a glycerol-buffered medium and observed with a photomicroscope (Polyvar, Reichert-Jung) equipped for incident fluorescence by the appropriate filter combination for FITC.

In order to block endogenous peroxidase activity in the ABC method, the sections were pre-incubated for 20 min at room temperature with 0.5% H2O2 in PBS. The sections were also pre-treated with normal goat

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**Fig. 1.** Amylin-immunoreactive cells demonstrated by the ABC method (a, c, d, f) and immunofluorescence (b, e) in various animal species. 

- **a.** Newt pyloric mucosa. 
- **b.** Frog pyloric mucosa. 
- **c.** Lizard pyloric mucosa. 
- **d.** Chick proximal duodenum. 
- **e.** Rat juxtapyloric region. 
- **f.** Human antral biopsy. 

a: ×720, b: ×580, c: ×900; d: ×450; e: ×360, f: ×560
serum, as described above, and then incubated in a moist chamber overnight at room temperature (or for 48 h at 4°C) with amylin antiserum diluted 1:8,000. After washing with PBS, they were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (Vector, USA), diluted 1:1,000 with PBS/BSA and then for 1 h with avidin-biotin-peroxidase complex (Vectastain Elite, Vector, USA), diluted 1:2,000 with PBS/BSA. Peroxidase activity was revealed by immersion for 3 min at room temperature in 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Fluka, Switzerland) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.005% H2O2 and 0.3% nickel ammonium sulphate.

All the required controls were performed, including the omission of the primary antiserum, its substitution by either buffer or rabbit pre-immune serum, and the pre-absorption test. The last of these was carried out overnight at 4°C pre-incubating the primary antiserum with an excess of either carboxy-terminal tridecapeptide amide or intact amylin (AM 1-37) (10–25 μg/ml diluted antiserum) or of human- or rat-CGRP (up to 100 μg/ml diluted antiserum).

In order to know whether the amylin-immunoreactive cells also co-stored proteins of the chromogranin/secretogranin family, the same histological section, first immunostained with FITC-conjugated secondary antibody, was exposed to a second immunostaining with one of the monoclonal antibodies raised against human chromogranin A (All). Characteristics of this antibody, which has been used in our previous studies (D’ESTE et al., 1993, 1994), were previously reported (PELAGI et al., 1989). The sections were then incubated for 30 min at room temperature with a tetraethylrhodamine-isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Dakopatts), diluted 1:50 with PBS/BSA. Pairs of photos of the same microscopic field, taken with FITC and TRITC filter combinations, were then compared to show double labelled cells.

In addition, to ascertain whether amylin-immunoreactive cells might constitute a distinct endocrine cell type or whether amylin is co-stored in certain already known amine/peptide-immunoreactive endocrine cells, the same section was double immunostained with a monoclonal serotonin antiserum (Dakopatts), while strictly neighbouring sections were alternately immunostained with polyclonal antibodies against various other antigens, including serotonin (gift of Prof. KIMURA, Otsu, Japan), histamine (Milab, Sweden), gastrin/CCK, bombesin, somatostatin, and CGRP (all from Peninsula, UK). In the second localisation, either double immunofluorescent or double ABC methods with the ABC-alkaline phosphatase complex and its revealing kit (Vector) for the second localisation, were used randomly.

**Histochemistry**

In order that amylin-immunoreactive elements could be classified as argyrophil, after single or double immunofluorescence, some tissue sections were stained by the Grimelius silver method (GRIMELIUS, 1968). For the control, the same staining was also used on neighbouring sections which had not been pre-immunostained.

**RESULTS**

**Fish.** Neither of the two investigated fish species contained amylin-immunoreactive cells.

**Newts.** A discrete number of amylin-immunoreactive cells were found in the pyloric region; they were situated both in the lining epithelium of the villi and in the glandular epithelium (Fig. 1a). Amylin-immunoreactive cells appeared bipolar or unipolar, and had a basal extension that gave them the so-called “open” appearance. Double immunostainings demonstrated that approximately 60% of the amylin-immunoreactive cells co-localised serotonin, whereas almost all the serotonin-immunoreactive cells present in this region were also positive for amylin. Immunoreactivity for chromogranin A was never demonstrated. The Grimelius silver method revealed that almost all amylin-immunoreactive cells were argyrophil (Fig. 2a-c).

**Frogs.** Although amylin-immunoreactive cells were scattered throughout the digestive tract to the cloaca (data already published, D’ESTE et al., 1994), their number increased notably in the gastric and duodenal mucosa (Fig. 1b). They were of the so-called “open” type and occurred both in the lining epithelium of the villi and in the glandular epithelium, though more frequently in glands. Double immunostainings combined on neighbouring sections showed that the amylin-immunoreactive cells nearly always displayed also the immunoreactivity for serotonin and chromogranin A. In addition, they were also stained by the Grimelius silver method (Fig. 2d-f).

**Lizards.** Amylin-immunoreactive cells were present in all gastrointestinal portions (data not shown). They were particularly numerous in the pyloric region where they were of the “open” type and often occupied the epithelium of the glandular base (Fig. 1c). Double and triple staining procedures revealed that amylin-immunoreactive cells co-stored both serotonin and chromogranin A and displayed argyrophilia.
Fig. 2 a-c. Newt pyloric mucosa. The same cell situated in the lining epithelium of the mucosa was immunoreactive for both amylin (AM; a) and serotonin (5HT; b) and displayed argyrophilia with the Grimelius silver method (GRIM; c). d-f. Frog pyloric mucosa. Several cells immunoreactive for both amylin (AM; d) and chromogranin A (CgA; e) also displayed argyrophilia (arrows) (GRIM; f). g-h. Chick antrum. Almost all the cells co-localised both amylin (AM; g) and serotonin (5HT; h). a-c: ×900, d-h: ×580
Chicks. Both the antrum (gizzard-duodenal junction) (Fig. 1d) and the duodenum contained a discrete number of amylin-immunoreactive cells. Often displaying an “open” feature, they tended to occupy both the lining epithelium of villi and that of glands. Most of them co-localised serotonin (5HT; d) and displayed argyrophilia (GRIM; b, e). a-b: ×900, c-e: ×360

Rats. Amylin-immunoreactive cells were scattered in the pylorus and in the duodenum, whereas they became particularly numerous in a certain narrow zone of the distal body of the stomach neighbouring the pyloric region (Fig. 1e). Although the amylin-immunoreactive cells in this region mainly occupied the basal third of the glandular epithelium, some also lay scattered within the lining epithelium of the mucosa (Fig. 3a-b). Double immunostainings demonstrated that all these cells also co-stored both serotonin and chromogranin A and were argyrophil (Fig. 3a-e). Double immunostainings performed on the same tissue sections by using polyclonal anti-gastrin/CCK and monoclonal anti-serotonin (Fig. 4a-d) showed that the two antisera stained two different cell populations.

Man. Amylin-immunoreactive cells were found in distal gastric and duodenal biopsies (Fig. 1f). Never high in number, they preferably occupied the epithelium of the distal third of the glands. Almost all these cells, which co-stored serotonin and chromogranin A,
DISCUSSION

The presence of amylin-immunoreactive cells in the pancreatic islets, both in healthy subjects and in diabetics, has been demonstrated in various mammalian specimens, including humans (Westermark et al., 1987a, b; Johnson et al., 1988; Clark et al., 1989; Lukinius et al., 1989; Toshimori et al., 1990; Mulder et al., 1994). Only a few studies have previously described the presence of amylin-immunoreactive cells in the alimentary tract of rodents and man; the cells were found mostly in the pyloric region (Toshimori et al., 1990; Ohtsuka et al., 1993; Mulder et al., 1994). Toshimori et al. (1990) found, besides the pylorus, scattered positive cells in the distal gastric body, duodenum and rectum, whereas Ohtsuka et al. (1993) and Mulder et al. (1994) did not confirm these secondary locations. Moreover, by light and electron microscopy, Ohtsuka et al. (1993) demonstrated that these cells co-stored gastrin in their secretory vesicles, while Mulder et al. (1994) showed co-localisations with several peptides, including somatostatin, gastrin and PYY. No reports are available demon-

Fig. 4 a-d. Rat juxtapyloric region. Two pairs of sections of the superficial (a-b) and deep (c-d) zones of the region double immunostained for serotonin (5HT; a, c) and gastrin (G; b, d). The two antisera labelled two different cell populations. In each pair of photographs, the same cell is indicated by an arrow of the same size. a-b: ×820, c-d: ×580
demonstrating the presence of amylin-immunoreactive cells in animal species other than mammals.

Using a polyclonal antiserum, that recognizes the carboxy-terminal tridecapeptide amide of h-amylin, we here demonstrated amylin-immunoreactive cells in all the animal species studied, except in fish.

The results obtained in the rat and in humans agree on the whole with previously reported studies (Toshimori et al., 1990; Ohtsuka et al., 1993; Mulder et al., 1994), as they confirm the major cell concentration in the distal stomach. In confirming the presence of amylin-immunoreactive cells in other parts of the digestive tracts, such as the gastric body and duodenum, and the apparent lack of co-localisation with gastrin, they agree only with the data from Toshimori et al. (1990).

However, by studying longitudinal sections of the rat antro-pyloric region, we found that amylin-immunoreactive cells are not concentrated in the pyloric mucosa, but in a narrow region of the distal gastric body just preceding the pylorus sensu strictu. By contrast, the gastrin/CCK-immunoreactive cells, which abounded in the pylorus, were not so numerous in this narrow juxta-pyloric zone. This inverse ratio of the distribution pattern apparently disagrees with the co-localisation of amylin with gastrin shown by Ohtsuka et al. (1993) and Mulder et al. (1994). We did not follow the protocol of Ohtsuka et al. (1993) of submitting the specimens to an acidic fixation to preserve gastrin better within the secretory granules, and therefore we cannot exclude the possibility that

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**Fig. 5 a-d.** Human pyloric biopsy. Triple staining of the same section demonstrated that the glandular epithelium contained several cells that were immunoreactive for both amylin (AM; a) and serotonin (5HT; b) and were argyrophil (GRIM; c). In the neighbouring section, the same cells also showed chromogranin A-immunoreactivity (CgA; d). × 720.
Amylin-cells might also co-store gastrin. However, since the comparison of neighbouring sections immunostained for amylin and gastrin, respectively, was not always satisfactory, and indicated that all the amylin cells co-store serotonin, we could claim an indirect support of our view. Alternatively, the neutral fixation we used could cause only a certain amount of gastrin to be lost. Hence, our conditions might allow us to recognize only those cells containing large quantities of gastrin and not those containing smaller quantities, as it might occur in the multiple antigens storing cells. Furthermore, we could not confirm the co-localisation with somatostatin and PYY described by Mulder et al. (1994). The different quality of antisera used must also be taken into due account.

On the other hand, our double immunostaining studies clearly demonstrated that some endocrine cells situated in the gastro-duodenal region co-stored amylin with serotonin and chromogranin A. This is the first demonstration of the co-localisation of these three antigens. Moreover, almost all the serotonin-immunoreactive cells of this gut region co-localised amylin and chromogranin A, whereas the numerous serotonin-immunoreactive cells found more distally in the intestinal mucosa always co-localised chromogranin A, but never amylin. Serotonin-containing cells or EC cells have also been reported to contain other peptides, including substance P, motilin and enkephalins. They therefore constitute a heterogeneous population. According to the different molecules co-stored and the various regions occupied, they were distinguished as EC1, or an intestinal type, and EC2, or a duodenal type. Those of the stomach, whose peptide content was unknown, were named ECn or gastric type EC cells (Sölcia et al., 1978). Thus, the present findings nominate amylin to represent the much debated peptidic component of the ECn cells. Ultrastructural studies must be undertaken to confirm this hypothesis.

In vivo experiments have already shown that amylin plasma levels increase in response to the administration of glucose or to mixed meal (Butler et al., 1990; Mitsukawa et al., 1990). Intraluminal infusion of 50% glucose solution, on the other hand, has been known to cause a sudden and dramatic degranulation of gastro-duodenal serotonin (EC) cells (Kobayashi and Fujita, 1974). These data seem to be accounted for by our findings showing that amylin was co-stored with serotonin in the same cell type.

The tendency for amylin-immunoreactive cells to preponderate in the gastro-pyloric zone and be scattered in other regions was confirmed in almost all the other species investigated, except fish. The latter species always resulted negatively for amylin-immunoreactivity. Frog specimens showed the highest number of amylin-cells among all the digestive tracts already observed (D'Este et al., 1994). Almost the whole amylin population of the gastroduodenal regions of the frog, lizard, chick, rat and man typically displayed co-localisation with serotonin and chromogranin A and argyrophilia. These findings differed only in the newt specimens, where most of the amylin-immunoreactive cells displayed argyrophilia, but only about 60% of cells co-stored serotonin, and none chromogranin A. Yet, because our human chromogranin A monoclonal antiserum never stained positive cells in any of the investigated gut tracts of either the newt or fish, we believe that the chromogranins in these lower vertebrates may differ structurally from the human molecule. This may explain the negative results we obtained with the chromogranin antibody.

In conclusion, the present study demonstrated the presence of amylin-immunoreactive cells in the gut of various animal species, with a particular concentration in the gastro-duodenal region where almost all the amylin-immunoreactive cells co-stored serotonin and chromogranin A, and displayed argyrophilia. These features indicate that the amylin cells present in this region possibly correspond to the gastric serotonin cell subtype.

REFERENCES


Amylin-immunoreactivity in a Serotonin Cell Subpopulation


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