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Regulatory peptides in gut endocrine cells and nerves in the starfish *Marthasterias glacialis*

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Abstract. The endocrine cells of the starfish digestive tract are spindle-shaped, contacting both the lumen and the basiepithelial plexus. Silver impregnation labels the basiepithelial and subcoelomic plexuses as well as these cells. Twenty antisera have been tested using the avidin-biotin method, in order to identify the regulatory substances involved in this system. Endocrine cells and nerves immunoreactive to GFNSALMFamide- (S1), FMRFamide-, peptide tyrosine-tyrosine- (PYY), pancreatic polypeptide- (PP), melanocyte stimulating hormone- (αMSH) and peptidylglycine alpha-amidating monooxygenase- (PAM) specific antisera have been found in the epithelium. The antibodies against S1, a peptide isolated from the nervous system of a starfish, and αMSH, stain both the basiepithelial plexus and the subcoelomic plexus, but the others react only with nerves in the basiepithelial plexus. Absorption controls show that antibodies for S1 and FMRFamide totally cross-react recognizing the same molecule, possibly S1. The other antibodies do not show cross-reactivity to any of the rest, and thus we conclude that these regulatory peptides are present in starfish. This is the first report of the presence of FMRFamide, PYY, αMSH and PAM in the Echinodermata. Under the electron microscope the endocrine cells exhibit secretory granules, microtubules and mitochondria. Direct contact with the subcoelomic plexus can be observed.

Key words: Immunocytochemistry – Gut – Innervation – Regulatory peptides – Endocrine cells – *Marthasterias glacialis* (Echinodermata)

Introduction

The innervation of the alimentary tract of the starfish, *Marthasterias glacialis*, consists of two nervous plexuses, separated by a connective layer: a basiepithelial plexus derived from the ectoneural system, and a subcoelomic plexus, related to the entoneural system, which innervates the muscle cells (Cobb and Raymond 1979). Although some authors have described the ultrastructure of the nervous system in different echinoderms (Bachmann and Goldschmid 1978; Cobb 1969, 1978; Doyle 1967; Fontaine 1962; Welsch et al. 1989), a systematic study of the involved peptides does not exist.

In a previous study, we described, for the first time, a diffuse endocrine system in the epithelium of the pyloric caeca of *M. glacialis*. This consists of endocrine cells connected to the basiepithelial plexus (Martínez et al. 1989), which are positive to pancreatic polypeptide (PP), glucagon and somatostatin. A positive reaction to PP is also given by the basiepithelial plexus. Some immunoreactive products have also been reported in crinoids (Welsch et al. 1989a, b), and CCK-positive cells have been demonstrated in an holothurian (García-Arrarás et al. 1991). However, a study on calcitonin and CGRP distribution in echinoderms (Sasayama et al. 1991) has failed to show any immunoreactivity in starfish. Recently, an octapeptide, the GFNSALMFamide (S1), has been sequenced (Elphick et al. 1990, 1991a, 1991b) and immunocytochemically located (Moore et al. 1990) in the central nervous system of the starfish *Asterias rubens*.

The present work has been undertaken to: (1) complete our former study using new antibodies raised against regulatory substances, in order to identify new starfish-specific peptides; and (2) try and immunocytochemically detect the enzymes that are involved in the peptide-amidating process.

Materials and methods

Twelve adult specimens of the starfish *Marthasterias glacialis* were collected from the Cantabrian sea (Spain). After anaesthesia with magnesium chloride, a careful dissection of the digestive tract was performed. Pieces of cardiac and pyloric stomachs, pyloric caeca, intestine and rectal caeca were processed.

The fragments to be embedded in paraffin were fixed in isotonic GPA (glutaraldehyde, picric and acetic acids) as described in Martínez et al. (1989). Tissues were sectioned at a thickness of 3 μm,

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Table 1. Antisera used in this study

Antisera	Source	Code
Starfish S1	MC. Thorndyke[1]	S1
Synthetic FMRFamide	Peninsula	61009
Human PYY	JM. Polak[2]	1234
Human PP	INC	64-711-1
Avian PP	JR. Kimmel[3]	
Porcine NPY	Amersham	RPN-1702
α-MSH	JM. Polak	1463
Human PAM (PHM-CC)	F. Cuttitta[4]	2281
Human PAM (PHM-HTHH)	F. Cuttitta	2284
Human PAM (PAL)	F. Cuttitta	2287
Human galanin	JM. Polak	1152
Neurofilament	JM. Polak	1472
PGP 9.5	Ultraclone[5]	RA95103
POMC	JM. Polak	888
ACTH 1–24	JM. Polak	1371
AVP	JM. Polak	1640
Adipokinetic H.	JM. Polak	796
C-PON	JM. Polak	2234
Human calcitonin	JM. Polak	813
Human CGRP	JM. Polak	1210

[1] Royal Holloway and Bedford New College, Egham, UK;
[2] Hammersmith Hospital, London, UK;
[3] Sent by Professor Rawitch, University of Kansas Medical Center, USA;
[4] Biomarkers and Prevention Research Branch, DCPC, NCI, NIH, Bethesda, USA;
[5] Cambridge, UK

Table 2. Absorption controls. +, Staining remains in the tissue. −, Staining has been precluded. (*) Homologous absorption for PP precluded staining in mouse pancreas but not in starfish tissue. All antigens were supplied by Sigma Chemical Company

Antigen	Antisera				
	S1	FMRFamide	PYY	PP	αMSH
Starfish S1	−	−	+	+	+
Synthetic FMRFamide	−	−	+	+	+
Porcine PYY	+	−	−	+	+
Human PP	+	−	+	+(*)	+
Synthetic αMSH	+	+	+	+	−

then stained with hematoxilin-eosin, Masson's trichromic, or by the Grimelius silver impregnation technique (Grimelius 1968).

In order to identify the distribution of the regulatory peptides, the avidin-biotin complex method (Hsu et al. 1981) was applied: after removal of paraffin, intrinsic peroxidase was blocked by treatment of the sections with a solution of methyl alcohol containing 3% H_2O_2 for 30 min. Background blocking was performed with 1:20 normal swine serum for 30 min prior to incubation with the specific antiserum (Table 1), which was carried out for 16–20 h at 4° C. After rinsing in TRIS-buffered saline (TBS, 0.05 M, pH 7.36, 0.55 M NaCl), the sections were incubated in biotinylated swine antirabbit IgG (Dakopatts E353, Glostrup, Denmark) at a 1:200 dilution for 30 min. Following a second rinse in TBS, the sections were further incubated with the avidin-biotinylated peroxidase complex (1:100 dilution, Dakopatts K355) for 30 min. After a final rinse, peroxidase activity was visualized using diaminobenzidine (50 mg per 100 ml) and H_2O_2 (60 µl per 100 ml). Avian and mammalian tissues (central nervous system and gastroenteropancreatic tract) were used as positive controls, and absorption controls were carried out incubating the antisera with 1 to 10 nmol of the corresponding antigen (Table 2) per ml of optimally diluted antiserum for 16 h at 4° C.

Small fragments of tissue were processed for electron microscopy. Fixation was carried out with 4% glutaraldehyde for 6 h, and postfixation with 2% osmium tetroxide for 2 h. After dehydration, the pieces were embedded in Epon 812 (Luft 1961). Thin sections were cut using a glass knife and stained with uranyl acetate and lead hydroxide.

A Zeiss EM10CR electron microscope was used to examine the sections.

Results

Endocrine cells are usually long and slender, like the rest of the cells that comprise the epithelium, and have the nucleus in a basal or medial position. Usually, a long apical cytoplasmic process reaches the lumen, and the basal region of the cell is in direct contact with the nerve plexus. Silver impregnation stains both endocrine cells and some nerve processes in the basiepithelial and the subcoelomic plexuses (Fig. 1).

The immunocytochemical results for the different regions of the starfish digestive system are summarized in Table 3. All regions have S1-positive material. S1 positivity (Fig. 2) follows a similar pattern to the Grimelius staining, showing endocrine cells and nerves belonging to both plexuses.

FMRFamide- (Fig. 3), PYY- (Fig. 4) and PP-immunoreactivity (Fig. 5) is widely distributed in the endocrine cells and in the basiepithelial plexus throughout

Fig. 1. Argyrophilic cell in the cardiac stomach epithelium. The nucleus appears in a basal position. Some nerve processes of both plexuses are also positive (*arrows*). Grimelius silver impregnation. ×1500. *Bar*: 10 µm

Fig. 2. Slender cell positive for S1 antiserum. Basiepithelial and submucous plexuses appear strongly stained. ×600. *Bar*: 20 µm

Fig. 3. The FMRFamide antiserum stains cells and nerves in the pyloric stomach. ×600. *Bar*: 20 µm

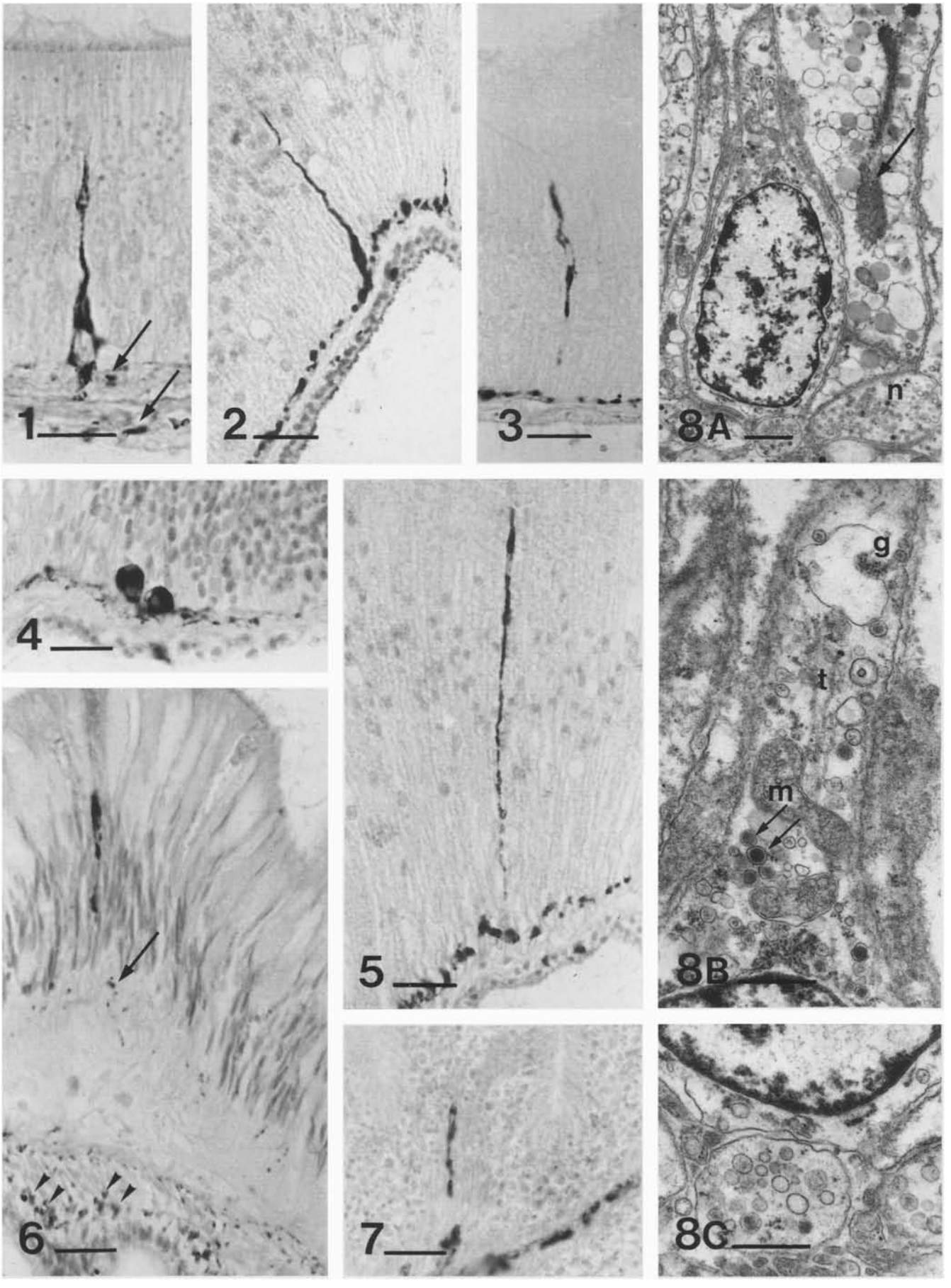
Fig. 4. Two rounded cells located in a basal position are labeled with the PYY antiserum. The basiepithelial plexus is also positive. Pyloric caeca. ×600. *Bar*: 20 µm

Fig. 5. A very tall and slender cell of a pyloric caecum immunoreactive for PP and contacting the basiepithelial plexus. ×600. *Bar*: 20 µm

Fig. 6. αMSH immunoreactivity in the mucosecretory portion of the cardiac stomach. An epithelial cell, few nerves in the basiepithelial (*arrow*) and subcoelomic plexuses (*arrowheads*) can be observed. ×600. *Bar*: 20 µm

Fig. 7. PAM-like material in a cell and in the basiepithelial plexus of the rectal caeca. ×600. *Bar*: 20 µm

Fig. 8A. An endocrine cell is located among the other epithelial cells of the cardiac stomach in direct contact with the basiepithelial plexus. A contact between a nerve ending (*n*) and a myoepithelial cell (*arrow* pointing to the filament bundle) can be observed. **B.** Detail of the apical process of the endocrine cell containing mitochondria (*m*), microtubules (*t*), glycogen (*g*) and specific granules (*small arrows*). **C.** Detail of the basal membrane and an adjacent naked nerve of the basiepithelial plexus. **A** ×9400; *Bar*: 1 µm. **B** ×28800; *Bar*: 0.5 µm. **C** ×28800; *Bar*: 0.5 µm


1
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3
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Table 3. Distribution of immunoreactivities throughout the starfish digestive tract. B, Basiepithelial plexus; S, subcoelomic plexus; E, endocrine cells in the epithelium. Immunoreactive structures: +++, very numerous; ++, moderately numerous; +, small quantity; −, absent

Region of digestive tract		Antisera					
		S1	FMRFamide	PYY	PP	αMSH	PAM
Cardiac stomach[1]							
Mucosecretory region	B	+++	++	+	+	++	++
	S	+	−	−	−	+++	−
	E	+	+	+	+	++	++
Current zones	B	+++	++	+	+	+	++
	S	+	−	−	−	++	−
	E	++	++	+	+	+	++
Pyloric stomach	B	+++	++	+	+	+	+
	S	+	+	+	−	++	−
	E	++	+	−	−	+	−
Pyloric caeca[1]							
Pouches	B	++	+	+	+	−	−
	S	+	−	−	−	+	−
	E	++	+	+	+	−	−
Current zones	B	+++	++	++	++	+	+
	S	++	−	−	−	+	−
	E	+++	++	++	+	+	−
Rectal caeca	B	+	+	+	+	+	++
	S	+	−	+	−	++	−
	E	+	−	+	−	+	+
Intestine	B	+	+	+	+	+	++
	S	+	−	−	−	++	−
	E	−	−	−	−	−	+

[1] Two regions have been distinguished in cardiac stomach and pyloric caeca according to Anderson (1960)

the digestive tract, the PYY-like material being less abundant than the others. Although occasionally some of the PYY-positive cells reach the lumen most of them are located in a basal position (Fig. 4).

Immunoreactivity for αMSH is found in some endocrine cells and in the nerves of both plexuses, being more abundant in the subcoelomic one (Fig. 6). This finding contrasts with the staining pattern of the rest of antibodies, which preferently stain the basiepithelial plexus. Positivity for αMSH is higher in the cardiac stomach than in the rest of the digestive tract. No immunoreactive response is seen for other peptides belonging to the proopiomelanocortin (POMC) family with the respective specific antisera used in the present study (Table 1).

The three antibodies raised against different regions of the PAM molecule, label cells and nerves of the basiepithelial plexus (Fig. 7).

Absorption controls (Table 2) show that S1 and FMRFamide totally cross-react; the peptides preclude the staining with the heterologous antiserum. The homologous absorption for anti-PP does not preclude the staining when applied to starfish tissue, but when it is applied to mouse pancreas no positive cells are found.

In the posterior regions of the digestive tube fewer cells and nerve fibers show immunoreactivity for the peptides and enzymes studied (Table 3).

The endocrine cells can be easily distinguished ultrastructurally because of the presence of secretory granules with different electron densities (Fig. 8 A–C). The cytoplasm also contains some microtubules, mitochondria and glycogen (Fig. 8 B). The basal plasmatic membrane is in direct contact with the basiepithelial plexus (Fig. 8 C).

Discussion

The function of the subcoelomic plexus is clearly the innervation of the muscle layer, but the role of the basiepithelial plexus has not yet been elucidated. Cobb and Raymond (1979) suggest a cilio-effector activity for this plexus. This hypothesis is in agreement with the recent finding of cells bearing contractile filament bundles attached to the flagellar complex in the luminal epithelium of this starfish, and of the correlation between the number of these cells and the thickness of the plexus (Martínez et al. 1991). One of these cells and its contact with a nerve ending is seen in Fig. 8 A.

The existence of enteroendocrine cells and their relationship with the naked nerve processes of the basiepithelial plexus have been previously described in the pyloric caeca (Martínez et al. 1989). In the present work, the same structures are described for the rest of the starfish gut.

The role of the RFamide-terminal peptides in invertebrate muscle contraction is well known (Robb and Evans

1990; McFarlane and Grimmelikhuijzen 1991; McFarlane et al. 1991; Anderson et al. 1992), and possibly the molecule revealed with the FMRFamide and S1 antisera share the same function, being involved in the contraction of the filament-containing epithelial cells.

Even though the anti-FMRFamide totally cross-reacts with anti-S1, the distribution of the respective immunoreactivities is slightly different (Table 3), the FMRFamide being negative in the subcoelomic plexus in most regions. This difference could mean that both of them recognize the same antigen and that the antibody for S1 is more sensitive, but the distribution of the FMRFamide immunoreactivity is more similar to that displayed for the peptides of the PP family.

The absorption of anti-FMRFamide with PYY and PP is due to the sequence identity of the FMRFamide and the terminal tetrapeptide of the PP family. The lack of homologous absorption for the PP immunoreactivity suggests that the PP-like peptide present in starfish tissues is different from the mammalian hormone. Recently, a peptide belonging to the same family has been isolated from the central and peripheral nervous systems of a parasitic flatworm (Maule et al. 1991), suggesting that these PP-like invertebrate peptides could be the phylogenetic precursors of the PP family. A recent study (Conlon et al. 1992) compares the primary structures of different mammalian peptides of this group and also points to the existence of an ancestral peptide from which the rest of them may have originated. The isolation and sequencing of these peptides will be necessary to understand their phylogenetic position.

α-MSH immunoreactivity has been found in several tissues of other invertebrates (De Loof and Schoofs 1990; Prado et al. 1992). In all the reported cases, no colocalization with other POMC-derived peptides has been observed (Schoofs et al. 1987). Although several functions have been proposed for αMSH in invertebrates (Schoofs et al. 1988), so far there is very little conclusive evidence. The characteristic distribution and the immunological behaviour observed in this study suggest that an αMSH-related peptide is also present in the neuroendocrine system of echinoderms.

This is the first immunocytochemical evidence of the presence of C-terminal amidating enzymes (PAM) in invertebrates. The mammalian amidating enzymes were first isolated and sequenced from the pituitary gland (Eipper et al. 1987), but it is now known that these enzymes have a wide distribution throughout the peptide-producing systems (Von Zastrow et al. 1986; Mackin et al. 1987; Spindel et al. 1987; Noguchi et al. 1988). PAM is involved in the amidation of the glycine-extended precursors of peptides, a post-translational process essential for the physiological role of these regulatory substances (May et al. 1990).

It is likely that the sequences of such important enzymes might have been well conserved throughout phylogeny. This is why the antibodies raised against the mammalian proteins crossreact with their counterparts in invertebrates.

Most of the general markers for the mammalian nerve system, such as neurofilaments or the peptide gene product 9.5 (PGP 9.5), are negative in this species of starfish, suggesting differences in the chemical nature of the molecules recognizes by these markers.

In conclusion, the present results suggest that a well developed neuroendocrine system is present in the gut of echinoderms, probably related to the regulation of the digestive processes. Further studies are needed to establish the exact chemical nature of these peptides as well as the role of endocrine cells in the digestive physiology.

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