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Ultrastructural study of the presence of vasoactive intestinal polypeptide and serotonin in mucosal nerve fibres and endocrine cells of the intestine of goldfish (*Carassius auratus*) and tilapia (*Oreochromis mossambicus*)

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Abstract. In order to establish a possible role of vasoactive intestinal polypeptide (VIP) and serotonin as (neuro)transmitters involved in the regulation of fish intestinal epithelium, we studied the presence of VIP and serotonin at the ultrastructural level in the intestinal mucosa of tilapia and goldfish. A low percentage of varicosities near the basal membrane of the tilapia intestinal epithelium was found to label for VIP or for serotonin, whereas in the goldfish, this percentage was much higher. The varicosities usually contained large granular and small clear vesicles. Immunogold labeling indicated that serotonin and VIP were localized in the large granular vesicles. Unlabeled large granular vesicles and small clear vesicles were usually also present in varicosities with serotonin- or VIP-labeled vesicles. In the goldfish, the serotonin-labeled varicosities were close to the epithelial cells, and direct contacts between serotonin-labeled nerve fibres and epithelial cells could sometimes be visualized. However, synaptic membrane specializations were never observed. In tilapia, the distance between the VIP- or serotonin-labeled varicosities and the epithelial cells was large (more than 2 μm).

Key words: Vasoactive intestinal polypeptide (VIP) – Serotonin – Ultrastructure – Immunogold label – Mucosal nerves – Goldfish, *Carassius auratus* – Tilapia, *Oreochromis mossambicus* (Teleostei)

Introduction

The basic components of the enteric nervous system of fish are similar to those of mammals, namely, ganglia containing intrinsic nerve cell bodies that send processes toward the muscle layers and that form networks of nerve bundles in the mucosal layer. There are, however, some differences, the most apparent of which is the

smaller number of neurons and the near absence of nerve cell bodies in the submucous plexus of fish; this plexus in mammals consists of two layers with different transmitter contents (Timmermans et al. 1990).

Most enteric nerve fibres have varicose nerve terminal regions, the appearance of which is similar in non-mammalian and mammalian vertebrates (Burnstock 1978a,b; Furness and Costa 1979a,b; Hökfelt et al. 1980a,b; Schultzberg et al. 1980). The beaded fibres are endowed with varicosities appearing every few micrometres.

In both fish and mammals, synaptic contacts on mucosal epithelial cells have been found only rarely (Newson et al. 1979) or not at all (Halasy et al. 1988; Mestres et al. 1992; Furness and Costa 1982; Wade and Westfall 1985; Watson 1981; Wong and Tan 1978; Anderson 1990). This near absence of synaptic contacts raises questions regarding the manner in which epithelial functional processes are coordinated. One obvious possibility is that transmitters are released from the varicosities and that they exert their action after diffusion over a large distance. This so-called volume transmission has been reviewed (Agnati et al. 1993).

The evidence that vasoactive intestinal polypeptide (VIP) serves as a neurotransmitter between the enteric nervous system and the enterocytes in mammalian intestine is now strong: (1) its presence in nerve fibres in close proximity to the epithelial cells has been established (Costa et al. 1987), (2) the binding of VIP and the uptake of the VIP-receptor complex has been shown in a number of epithelial cells (Izzo et al. 1991), (3) the effect of VIP on cultured cells has been studied and correlates well with the effect on in vivo and on in vitro intestinal preparations (Mandel et al. 1986; Laburthe et al. 1989). In contrast, little is known about VIP in fish intestine (see Bakker et al. 1993). In goldfish and tilapia intestine, relatively abundant VIP-like immunoreactive (IR) nerve fibres have been found in the lamina propria at the light-microscopical level, and the presence of VIP receptors on tilapia enterocytes has been demonstrated (Kiliaan et al. 1989a, b, 1990, 1992, 1993).

Serotonin (5-HT) is present in enterochromaffin cells in the intestinal epithelium of mammals, in mast cells in the lamina propria, and in neurons of the myenteric plexus, but not in nerve fibres in the lamina propria (Cooke 1986). The function of serotonin as a neurotransmitter between the enteric nervous system and epithelial cells is therefore questionable in mammalian intestine. In contrast, in fish, 5-HT-like immunoreactivity is present in nervous tissue in the lamina propria (Kiliaan et al. 1989a). The 5-HT-induced change in the ion selectivity of the tight junctions of fish intestine can not be blocked by the neurotoxic agent tetrodotoxin. This suggests a direct action of 5-HT on the enterocytes (Kiliaan et al. 1989a). The receptors involved in the 5-HT effect on fish enterocytes appear to be different from the 5-HT receptor types described so far for higher vertebrates (Bakker et al. 1993). In this study, we have used ultrastructural immunocytochemistry to investigate the possible presence of VIP or 5-HT in nerve fibres located just below the epithelium and in the endocrine cells in the intestinal epithelium.

Materials and methods

Routine electron microscopy

Fish were fed daily in the morning with Trouvit fish food (Trouw, The Netherlands), except for the morning when they were killed. Male goldfish, *Carassius auratus* ($n=4$, about 15 cm in length, r), and male tilapia, *Oreochromis mossambicus* ($n=4$, about 20 cm in length), were kept in well-aerated fresh water at 18°C and 27°C, respectively. They were anesthetized in tricaine methanesulfate (0.3 g/l) for 10 min and then perfused through the ventricle (10 ml/min) with 50 ml ice-cold 0.1 M sodium phosphate buffer (PB), pH 7.3, followed by 250 ml ice-cold fixative (2% paraformaldehyde and 2% glutaraldehyde dissolved in the same buffer). We used the part of intestine located directly behind the intestinal bulb of the stomachless goldfish and the stomach of the tilapia. The intestinal bulb is morphologically considered as an analog of the stomach and it functions as food reservoir like a true stomach (Kapoor et al. 1975); we therefore regard the first part of the intestine directly behind the stomach, and the intestinal bulb as comparable. The intestine was dissected out and the first 7 cm immersed in the same fixative (1 h at 4°C) followed by postfixation in 1% OsO_4 in the buffer for 1 h at room temperature. The tissues were dehydrated in ethanol and embedded in resin (812 resin Taab, England).

Immuno-electron microscopy

Fish (four tilapia and four goldfish) were anesthetized (see above) and perfused through the ventricle with 50 ml ice-cold Ca^{2+} -free Tyrode's buffer followed by 250 ml ice-cold fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3). The proximal intestine was dissected out and postfixed in the same fixative (1 h at 4°C).

After postfixation, pieces of the intestine, taken randomly from the first 7 cm, were cryoprotected by consecutive immersion in 10%, 20%, and 30% glycerol in phosphate buffer (PB) (30 min per concentration). Subsequently, the tissues were placed on Thermanox (LAB-TEK DIV, USA), snap-frozen in liquid propane, and put in a precooled chamber (-90°C) of a quick-freezing apparatus (Reichert-Jung, Germany). The tissue was freeze-substituted in methanol containing 0.5% uranyl acetate and warmed at a rate of 4°C per hour to -45°C. Embedding in Lowicryl (HM20) resin (Biorad, UK) was carried out in three steps at -45°C, while progressively increasing the ratio of resin to methanol. Polymerization of Lowicryl was performed by UV radiation (360 nm) for 16 h at -45°C and then for 24 h at 20°C.

Ultrathin sections (70–80 nm) were placed upon uncoated 300 mesh nickel grids. The grids were consecutively placed on drops of (1) 0.1 M phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.2% gelatine (PBG) for 10 min, (2) primary antiserum for 72 h at 4°C, (3) PBG for 30 min at room temperature, (4) Protein-A gold ($\varnothing$ 10 nm; Janssen Biotech NV, Belgium) diluted in PBG for 1 h at room temperature, (5) PBG for 30 min with six changes, (6) PBS, and (7) distilled water.

VIP antiserum (raised in rabbit against natural porcine VIP; code B34-1, Milab, Sweden) was diluted 1:4000, and 5-HT antiserum (raised in rabbit; gift of Dr. H. Steinbusch, Amsterdam; Steinbusch et al. 1983) was diluted 1:400. Controls were carried out by replacing the antiserum with normal rabbit serum, or by pre-adsorption of the antiserum with VIP (10^{-6} M) or 5-HT (10^{-6} M). No immunoreaction could be observed in the control experiments.

Sections were stained with lead citrate for 45 s for contrast. Observations were made with a Philips 201 c electron microscope.

Pre-embedding immunohistochemistry

After postfixation (see immuno-electron microscopy), tissue pieces were rinsed in 5% sucrose dissolved in 0.1 M PB (pH 7.3) for 24 h at 4°C. Using a Vibratome, 50- μm sections were cut and collected in 0.05 M TRIS-buffered saline (TBS) at pH 7.6 and then incubated for 24 h at 4°C in the antiserum (a-VIP 1:8000, a-5-HT 1:20000) diluted in TBS containing 0.1% Triton X-100, 0.5% BSA and 1% normal goat serum. The tissue was rinsed 3 $\times$ 10 min in 0.05 M TBS at room temperature, immersed in goat anti-rabbit (Fc-specific) immunoglobulins (1:100, 1 h), rinsed in 0.05 M TBS, immersed in peroxidase-antiperoxidase (PAP, 1:600, 90 min) and 3,3'-diaminobenzidine (DAB; 0.2 mg/ml 0.05 M TRIS-buffer, pH 7.6, 15 min), and then in the same solution to which 0.01% H_2O_2 was added (15 min). The tissue was then post-fixed in OsO_4 for 30 min, dehydrated, and embedded in Epon on slides, and examined with the light microscope to identify appropriate pieces, to be cut for electron microscopy.

Quantification

We counted the labeled and unlabeled varicosities present in a counting window with an area of 100 μm^2 , covering 50 μm along the basal membrane of about 10 enterocytes and 2 μm from the basal lamina into the lamina propria (Fig. 1). The varicosities were counted directly from the screen of the electron microscope. Windows were chosen in various parts of the proximal intestine and at various locations in the folds. The diameters of varicosities and vesicles, the intervaricosity length, and the distance of vari-

Fig. 1. Scheme of the intestinal mucosa of tilapia and goldfish to illustrate the area in which varicosities were counted. The counting window covers 100 μm^2 ; 50 μm along the basal lamina, and 2 μm into the lamina propria

cosities from the epithelium were measured from electron micrographs of control and pre- or postembedded immunolabeled tissue.

Results

Technical aspects

For the preservation of the antigenicity of 5-HT in the fish gut, we had to adjust the fixation and embedding procedures used for routine electron microscopy. Fish intestine fixed even in low concentrations of glutaraldehyde totally lost its 5-HT antigenicity. Therefore, we used 4% formaldehyde as fixative and no glutaraldehyde. Postfixation with 1% OsO₄ improved the membrane structures of organelles, but totally quenched 5-HT antigenicity. Thus, we had to omit postfixation. Another adaptation concerned the type of resin: with epoxy resin, all antigenicity was lost, but Lowicryl (HM20) proved to be a good substitute.

Glutaraldehyde or the use of resin did not totally quench the immunoreactivity for VIP; it was still preserved in the endocrine cells but could not be found in nerve fibres. We therefore used the same methods for both antisera. Thus, although the ultrastructure of the tissue in general, and the plasma membranes in particular, was better preserved following fixation in glutaraldehyde and osmium, and embedding in resin, we had to choose the freeze-substitution technique (without using glutaraldehyde and osmium), so as to have sufficient antigenicity in the sections to study the presence of VIP and 5-HT.

Nerve fibres

Within the counting window, twice as many nerve fibres were found in tilapia as in goldfish (Table 1A). All nerve fibres showed numerous varicosities containing many vesicles. In tilapia, the majority of the varicosities contained small clear vesicles (SCV) and the minority predominantly possessed large dense-cored granular vesicles (LGV) together with some SCV (Fig. 2a). In the goldfish, equal numbers of varicosities containing only SCV and varicosities containing LGV plus SCV were found (Fig. 2b–f, Table 1A). Directly underneath the epithelium, the nerve fibres occurred singly, but deeper in the lamina propria they lay together in groups, often next to capillaries. Scarce synaptic contacts between varicosities were found in the lamina propria and in the myenteric plexus (Fig. 2a, b). No synaptic contacts were observed between nerve fibres and epithelial or muscle cells or other cells in the lamina propria, and usually the varicosities were separated from the epithelial cells by the basal lamina, which was 0.2–0.4 µm thick. However, enterocytes sometimes extended through the basal lamina and formed close contacts with a varicosity but without real synaptic specializations (Fig. 2c–f).

The VIP-IR and serotonin-IR nerve fibres in both tilapia and goldfish possess varicosities with a diameter of

Table 1. Numbers of varicosities underlying the basal lamina

	Tilapia	Goldfish
<i>A. Fixation and embedding for routine electron microscopy^a</i>		
Number of windows	19	19
Total number of varicosities	126	62
Varicosities with SCV	102	30
Varicosities with LGV	24	32
Mean number of varicosities per window ^b :		
Varicosities with only SCV	5.4±0.6 (19)	1.6±0.3 (19)
Varicosities with LGV	1.3±0.2 (19)	1.7±0.2 (19)
<i>B. Fixation and embedding for immuno-electron microscopy^c</i>		
Total number of varicosities ^b :		
Varicosities with unlabeled SCV	nd	nd
Varicosities with LGV	33 (24)	32 (24)
VIP-IR varicosities	1 (12)	19 (19)
5-HT-IR varicosities	1 (12)	22 (19)
Mean number of varicosities per window ^b :		
Varicosities with unlabeled LGV	1.30±0.30 (24)	1.60±0.30 (24)
VIP-IR varicosities	0.08±0.02 (12)	0.95±0.22 (19)
5-HT-IR varicosities	0.08±0.02 (12)	1.15±0.32 (19)

^a Varicosities containing small clear vesicles (SCV) and varicosities containing large granular vesicles (LGV) plus SCV were counted in a window directly underneath the epithelium, 2 µm into the lamina propria and 50 µm along the basal lamina (covering the basal side of about 10 enterocytes), in four animals per species

^b The number of windows is indicated in brackets

^c VIP- and 5-HT-labeled varicosities and varicosities with unlabeled LGV were counted in tissues after processing for immuno-electron microscopy (four tilapia and four goldfish)

0.58±0.04 µm (from 15 randomly chosen varicosities) and are repeated at regular intervals of about 2 µm. Examples of immunogold labeling of VIP- or 5-HT-containing varicosities in the intestine of the goldfish and tilapia are shown in Fig. 3a–d, and an example of pre-embedding DAB staining of VIP-containing varicosities near the basal membrane is shown in Fig. 3e.

The varicosities containing only SCV could not be recognised with certainty because of the fixation procedure for immunogold labeling, and therefore their number in the counting window was not determined. The mean number of LGV-containing varicosities in the windows was not different in the two fixation procedures (Table 1A, B). No gold particles were observed in cell structures other than those containing LGV, but not all of the LGV in a varicosity or endocrine cell were labeled with immunogold. Both VIP-IR and 5-HT-IR nerve fibres predominantly had LGV and only some SCV in their varicosities. The SCV were never labeled, suggesting that VIP and 5-HT are not located in SCV. In goldfish, the vesicles in the serotonergic nerves were round and dense-cored with a diameter of 79±4 nm (*n*=19), but they were larger in tilapia, viz., 133±5 nm (*n*=19). The vesicles of the VIP-containing fibres had the same diameter in tilapia and goldfish (78±8 nm, *n*=19 and 73±4 nm, *n*=19, respectively).

Fig. 2a, b. Examples of synaptic contacts (*arrows*) between varicosities of two nerve fibres in the lamina propria near to the tilapia intestinal epithelium (**a** $\times 20700$) and between the varicosities of two nerve fibres in the myenteric plexus of the goldfish intestine (**b** $\times 39500$). Both large granular vesicles and small clear vesicles

are present within the varicosities. **c–f** Basal extensions of enterocytes of the goldfish pass through the basal lamina toward varicosities (*arrows*) containing large granular vesicles and small clear vesicles (**c** $\times 27900$; **d** $\times 19250$; **e** $\times 14700$; **f** $\times 26300$). *bl* Basal lamina; *ep* epithelium; *cap* capillary

Fig. 3a–e. VIP-IR varicose nerve fibres in fish intestine. **a, b, c, d** Freeze substitution. Gold particles (*arrows*) on some of the electron-dense vesicles in the circular muscle layer of the goldfish (**a, b** $\times 39500$) and tilapia (**c** $\times 20800$; **d** $\times 14700$). **e** 5-HT immunore-

activity is shown by immunogold labeling on the vesicles (*arrows*) of a varicosity in the lamina propria of the goldfish intestine. DAB-staining of IP-immunoreactive vesicles (*arrows*) in nerve fibres in the lamina propria of goldfish. $\times 39500$. *ep* Epithelium

In tilapia, VIP- or 5-HT-containing varicosities represented less than 2% of the population observed in the counting window taken directly underneath the basal lamina, whereas in goldfish, they constituted about 30%. Most of the 5-HT- and VIP-containing nerve fibres were situated more than $2\ \mu\text{m}$ from the tilapia entero-

cytes, usually lying next to the capillaries. In contrast, in the goldfish, many 5-HT-containing nerve fibres were found within $2\ \mu\text{m}$ of the epithelium or even in apposition to the enterocytes (Fig. 4). VIP-IR nerve fibres appeared to be located less frequently close to the epithelium.

Fig. 4. Varicose 5-HT-immunoreactive nerve fibre apposed to an enterocyte of the goldfish intestinal epithelium. Note the gold particles on some large granular vesicles in the varicosities (arrows). Freeze substitution. *ep* Epithelial cell; *bl* basal lamina. $\times 28400$

Fig. 5a, b. 5-HT-immunoreactive endocrine cells in the tilapia intestine. **a** Immunogold label (arrows) is found on the round granules, characteristic of endocrine cells; freeze substitution. $\times 39500$.

b Dense reaction product on the granules (arrows) following pre-embedding DAB labeling for 5-HT. $\times 14700$. *bl* Basal lamina; *ec* endocrine cell

Fig. 6. VIP-ergic endocrine cell in tilapia intestinal epithelium; freeze substitution. Many gold particles are found on the granules. *bl* Basal lamina; *ec* endocrine cell; *ep* epithelial cells. $\times 24100$

Endocrine cells

VIP-ergic and serotonergic endocrine cells in the tilapia intestine were also identified. 5-HT-IR was found both with immuno-electron microscopy (Fig. 5a) and with pre-embedding DAB staining (Fig. 5b) in round dense-cored granules with a similar diameter as those in the nerve fibres (140 ± 8 nm, $n=19$). Labeled VIP-ergic endocrine cells with immunogold-labeled granules were observed in the intestinal epithelium of tilapia (Fig. 6). The vesicles of the VIP-IR endocrine cells ranged from small, round dense-cored (62 ± 2 nm, $n=19$), resembling the vesicles in the varicosities, to large polymorphic dense-cored vesicles (not shown) with a halo (247 ± 17 nm, $n=19$).

No VIP- or 5-HT-containing endocrine cells were found in the intestinal epithelium of goldfish.

Discussion

In tilapia, approximately twice as many varicosities can be found underneath the intestinal epithelium as in goldfish. Most of the varicosities in the tilapia contain SCV. These vesicles presumably do not contain 5-HT or VIP because they are not immunogold-labeled.

The localization of VIP- and 5-HT-containing nerve fibres in the goldfish and tilapia intestinal epithelium is quantitatively different: goldfish has about ten times more 5-HT- and VIP-containing varicosities within a distance of about 2 μ m from the basal lamina. Both types of immunoreactivity are found in varicosities containing LGV and some SCV. None of the SCV and only some of the LGV are immunogold-labeled. This suggests that, in the same varicosity, other neurotransmitters are localized together with VIP or 5-HT. In mammals, VIP-labeled LGV with a diameter of 98 ± 19 nm are found together with medium-sized SCV (Larsson 1977; Stead et al. 1989; Costa et al. 1987; Feher and Lrnth 1983; Llewellyn-Smith et al. 1984; Maeda et al. 1985; Loesch and Burnstock 1985) that sometimes also show VIP immunoreactivity (Feher and Lrnth 1983; Llewellyn-Smith et al. 1984; Loesch and Burnstock 1985).

As in our study, only some of the LGV are labeled for 5-HT in the teleost *Aldrichetta forsteri* (Anderson 1990), and these vesicles are not different in appearance from the unstained LGV. The percentage of 5-HT-containing varicosities in this fish, as determined by the chromaffin reaction, is about 50% of the total number of mucosal varicosities, whereas in goldfish, using immuno-electron microscopy, about 30% of the nerves stain for 5-HT (this study).

The tilapia intestinal mucosa contains far fewer serotonergic varicosities than the goldfish mucosa or the mucosa of *A. forsteri*. However, although *A. forsteri* and goldfish do not possess 5-HT-like endocrine cells, many 5-HT-like endocrine cells are present in the tilapia epithelium. Likewise, mammalian intestinal mucosa possesses a large population of serotonergic endocrine cells but does not have serotonergic nerve fibres (Furness and

Costa 1982). We have found a similar distribution of VIP between endocrine cells and nerve fibres: the tilapia mucosa contains a small percentage of VIP-positive fibres directly underneath the epithelium but has many VIP-ergic endocrine cells, whereas the goldfish (and also the mammalian intestinal mucosa) has a large population of VIP-ergic nerve fibres but no VIP-IR endocrine cells have been described. It appears to be a general feature that, when endocrine cells are immunolabeled, few or no nerve fibres contain the same transmitter, whereas when many IR nerve fibres are present, no IR endocrine cells with the same transmitter can be visualized.

We have only rarely found synaptic structures between neurons in the lamina propria of the intestine of goldfish and tilapia. They are more abundant in the myenteric plexus, as has also been reported by Wong and Tan (1978), and Watson (1981). Similarly, ultrastructurally recognizable synaptic structures in mammals are predominantly present between the nerve cells in the myenteric plexus and in the submucosal plexus (Baumgarten et al. 1970; Gabella 1972). Enteroendocrine cells often have irregular cytoplasmic extensions through the basal lamina and true synaptic specializations have rarely been found between epithelial endocrine cells and nerve terminals in some mammals (Gabella 1979; Stead et al. 1989; Lundberg et al. 1978), but they are not found in mice (Wade and Westfall 1985) or teleosts (this study; Anderson 1990; Benedeczy and Halasy 1988).

In previous studies, we have shown direct effects of VIP and 5-HT on the epithelium of the tilapia and the goldfish intestine (Kiliaan et al. 1989a, 1993; Bakker et al. 1993), and we have postulated that these transmitters have a regulatory role in the functioning of epithelial cells. The scarcity of 5-HT- and VIP-containing varicosities in tilapia intestinal mucosa implies that these messengers do not play a significant role in the regulation of epithelial function by nerves in this species. In contrast, VIP and 5-HT may act as messengers between nerve fibres and epithelial cells in the goldfish. If so, they have to cross a distance of about 1 μ m. This is comparable to the distance VIP or 5-HT have to traverse when released from endocrine cells to influence neighboring enterocytes in tilapia intestine.

In conclusion, from the previously shown direct action on enterocytes (Kiliaan et al. 1989a; Bakker et al. 1993) and the data presented in this paper, we suggest that, in goldfish, 5-HT and VIP regulate the function of enterocytes via "en passant" release from varicosities during the conduction of an action potential. The distance between 5-HT- and VIP-containing nerve fibres near epithelial cells in the tilapia is so large that one has to assume that 5-HT and VIP are endocrine regulators in this fish.

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