Exocytotic release of vasoactive intestinal polypeptide and serotonin from mucosal nerve fibres and endocrine cells of the intestine of the goldfish (*Carassius auratus*) and the tilapia (*Oreochromis mossambicus*): an ultrastructural study

A. J. KILIAAN, G. SCHOLTEN and J. A. GROOT

Institute of Neurobiology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands

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Summary

In earlier studies we determined the effect, presence and ultrastructure of vasoactive intestinal polypeptide (VIP) and 5-hydroxytryptamine (5-HT)-containing nerve fibres in the tilapia and goldfish intestinal mucosa. 5-HT-labelled varicosities were found close to the epithelial cells; however, synaptic membrane specializations have never been observed. VIP-like immunoreactive nerve fibres appear to be located less frequently close to the goldfish epithelium, as in the tilapia intestine, in which the distance between the VIP- or 5-HT-labelled varicosities and the epithelial cells was also rather large (more than 2 µm). To establish a possible role of VIP and 5-HT as neurotransmitters involved in the regulation of fish intestinal epithelium, both electron microscopical and immunoelectron microscopical methods were used to visualize the release of 5-HT and VIP from nerve fibres. We found exocytoses from VIP-ergic and serotonergic varicosities in the muscle layers of both fish. Directly underneath the intestinal epithelium of the goldfish, it was demonstrated that 5-HT could be released from scarce varicosities. The release of 5-HT in the tilapia intestinal mucosa could only be observed from endocrine cells.

Introduction

The enteric nervous system of fish is similar to that of mammals (ganglia with intrinsic nerve cell bodies that give off processes towards the muscle layers and the mucosal layer), although the number of neurons in fish intestine is lower and nerve cell bodies in the submucous plexus are nearly absent. Several immunohistochemically identified nerve fibres have been found very close to the fish epithelium (Kiliaan *et al.*, 1989, 1992, 1993) but no synaptic contacts with mucosal epithelial cells could be observed (Kiliaan *et al.*, 1996). It is likely that these enteric nerve fibres exert their action on the epithelium via so-called volume transmission by diffusion of the messenger over a longer distance (Agnati *et al.*, 1993).

In this study, we used the combination of the tannic acid in Ringer (TARI) method (Buma *et al.*, 1984) with immunoelectron microscopy in an attempt to find exocytotic activity from vasoactive

intestinal polypeptide (VIP) and 5-hydroxytryptamine (5-HT)-like as immunoreactive varicosities directly underneath the intestinal epithelium. The role of VIP and 5-HT as messengers to regulate epithelial functions by neurons or endocrine cells upon certain stimuli to the animal would become more acceptable when one could show its exocytosis. With the methods used, at the time of exocytosis, the vesicular contents can be captured and made visible as a densely stained granule. Vesicles can be sectioned in such a plane that the dense granule is partially surrounded by an omega-shaped invagination of the neuronal membrane. We have chosen these two transmitters because of their relative abundance in the lamina propria as shown with light microscopy, their close proximity to the epithelium as shown with electron microscopy (Kiliaan *et al.*, 1996), the apparent presence of VIP receptors on fish enterocytes (Kiliaan *et al.*, 1990)

and the effects of the addition of VIP or 5-HT on isolated fish intestinal epithelial *in vitro* (Kiliaan *et al.*, 1989, 1993; Bakker *et al.*, 1993).

The TARI method has previously been used in combination with immunoelectron microscopy to show the non-synaptic release of identified peptides from nerve fibres in brain tissue (Van Putten *et al.*, 1987; Buma & Nieuwenhuys, 1987, 1988; Pow & Morris, 1991) and from neurosecretory cells in the cerebral ganglia of *Lymnaea stagnalis* (Schmidt & Roubos, 1988). As far as we know, the combined method has not been applied to peripheral tissue containing fewer neuronal fibres with a small proportion of immunoreactive fibres containing vesicles much smaller and with a less dense appearance than the vesicles in brain tissue.

Materials and methods

ROUTINE ELECTRON MICROSCOPY

Male goldfish, *Carassius auratus* ($n = 4$, about 15 cm in length), 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. These fish were used because of earlier anatomical and electrophysiological data obtained in our group (Bakker *et al.*, 1993; Kiliaan *et al.*, 1989, 1990, 1992, 1993, 1996).

The fish were fed Trouvit fish food (Trouw, The Netherlands); feeding was discontinued 1 day before experimentation. Fish were anaesthetized in tricaine methanesulphate (0.3 g l^{-1}) for 10 min. Then, 50 ml of ice-cold 0.1 M sodium phosphate buffer solution, pH 7.3, was perfused through the ventricle at a rate of 10 ml min^{-1} . This was followed by 250 ml of ice-cold fixative (2% paraformaldehyde and 2% glutaraldehyde dissolved in the same buffer). The proximal third part of the intestine (directly behind the intestinal bulb of the goldfish and directly behind the stomach of the tilapia) was dissected out and the first 7 cm immersed in 2% paraformaldehyde and 2% glutaraldehyde in the phosphate buffer (1 h at 48°C), followed by post-fixation in 1% osmium tetroxide (OsO_4) in the buffer for 1 h at room temperature. The tissues were dehydrated in ethanol and embedded in TAAB 812 resin (TAAB, UK).

TANNIC ACID IN RINGER (TARI) METHOD

We used the TARI method in order to visualize exocytoses in subsequently immunolabelled tissue. Because of the scarcity of exocytoses with the standard TARI method, we had to enhance exocytosis by increasing the potassium concentration of the perfusion solution from 5.7 mM to 61 mM ('high K^+ ') by replacing 55.3 mM of the sodium chloride (NaCl) concentration with potassium chloride (KCl). Four goldfish and four tilapia (about 15 and 20 cm in length respectively) were anaesthetized and perfused through the heart with a normal Ringer's solution for 5 min, followed by a 'high K^+ ' solution containing 2% tannic acid for 15 min and a short perfusion (3 min) with 2% paraformaldehyde and 2% glutaraldehyde dissolved in the same buffer.

The proximal intestine was dissected out and fixed by immersion in the same fixative (1 h at 48°C). Post-fixation followed in 1% OsO_4 in the buffer for 1 h at room temperature. The tissues were dehydrated in ethanol and embedded in TAAB 812 resin.

TARI METHOD PLUS IMMUNOELECTRON MICROSCOPY

Three goldfish and two tilapia (same length as before) were prepared as above, but 4% paraformaldehyde in 0.1 M phosphate buffer was used as a fixative and post-fixation with OsO_4 was omitted.

After immersion-fixation, pieces of the proximal intestine (taken randomly) were cryoprotected by consecutive immersion in 10%, 20% and 30% glycerol in phosphate buffer (30 min per concentration). Subsequently, the tissues were placed on Thermanox (LAB-TEK Division, 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 48°C per hour to -45°C . The 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 mounted on uncoated 300-mesh nickel grids. The grids were consecutively placed on drops of 0.1 M phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.2% gelatine (PBG) for 10 min, on drops of the primary antiserum for 72 h at 48°C, PBG for 30 min at room temperature, protein-A gold (10 nm diameter; Janssen Biotech NV, Belgium) diluted in PBG for 1 h at room temperature, PBG 30 min with six changes, PBS and finally on distilled water.

Vasointestinal polypeptide (VIP) antiserum (raised in rabbit against natural porcine VIP; code B34-1, Milab, Sweden) was diluted 1:4000 and 5-hydroxytryptamine (5-HT) antiserum (raised in rabbit, gift from Dr H. Steinbusch, Amsterdam; Steinbusch *et al.*, 1983) was diluted 1:400. Control experiments were carried out by replacing the antiserum with normal rabbit serum, or by preadsorption 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 201c electron microscope.

Estimation of exocytotic events near to the epithelial cells

To obtain an impression of the number of immunoreactive varicosities showing exocytosis, a window with an area of $100 \text{ } \mu\text{m}^2$, covering $50 \text{ } \mu\text{m}$ along the basal membrane of about ten enterocytes and $2 \text{ } \mu\text{m}$ from the basal lamina into the lamina propria, was used as a grid to count varicosities near to the basal membrane. The varicosities were counted directly from the screen of the electron microscope. We studied 18 windows per species (two tilapia, three goldfish, similar length as before), and one window per section.

Windows were chosen in different parts of the proximal intestine and at different locations in the folds.

Preliminary technical analyses

To preserve the antigenicity in the fish gut, we had to adjust the fixation and embedding procedures because fish intestine fixed in low concentrations of glutaraldehyde totally lost their 5-HT antigenicity. Therefore, we used 4% formaldehyde as fixative and no glutaraldehyde at all. Post-fixation with 1% OsO₄ to improve the membrane structures of organelles totally quenched the 5-HT antigenicity. Furthermore, with TAAB resin all antigenicity was lost, but Lowicryl (HM20) proved to be a good substitute. After glutaraldehyde or the use of TAAB resin, some immunoreactivity for VIP was still preserved in the endocrine cells but not in nerve fibres. Therefore, the same method was used for both antisera. Thus, we had to choose the freeze-substitution technique (without using glutaraldehyde and osmium) as a compromise to have sufficient antigenicity in the sections to study the presence of VIP and 5-HT, although the ultrastructure of the tissue in general, and the plasma membranes in particular, was meagre.

Results

Varicosities and immunoreactivity

We have reported in another study (Kiliaan *et al.*, 1996) that in the counting window underneath the

epithelial cells twice as many nerve fibres were found in the tilapia as in the goldfish (with 126 varicosities in 19 windows in tilapia versus 62 varicosities in 19 windows in the goldfish). Fig. 1 shows some examples of nerve fibres near the basal membrane, with varicosities containing large granular vesicles (LGVs) and small clear vesicles (SCVs). In the goldfish, 50% of the varicosities contained only SCVs, in the tilapia 80% contained only SCVs, in the other varicosities both vesicle types could be observed with routine electron microscopy.

Owing to the fixation procedure for immunogold labelling in this study, the varicosities containing only SCVs could not be recognized as varicosities with certainty. Gold particles were only observed in cell structures containing LGVs, but not all of the LGVs in a varicosity or endocrine cell were labelled with immunogold. Both VIP-immunoreactive (VIP-IR) and 5-HT-immunoreactive (5-HT-IR) nerve fibres have predominantly LGVs and only some SCVs in their varicosities. The SCVs were never labelled. Using the immunoelectron microscopical method, we observed that in tilapia VIP- or 5-HT-containing varicosities represent less than 2% of the population of varicosities observed in the counting window. Most of the 5-HT-IR and VIP-IR nerve fibres were situated more than 2 µm away from the tilapia

Fig. 1. Examples of nerve fibres directly underneath the intestinal epithelium (ep) of tilapia (a and b) and goldfish (c and d). Sections prepared as described for 'Routine electron microscopy'. In a and c the varicose nerve fibres are cut longitudinally showing that the vesicles are concentrated in the varicosities of the nerve fibres. Both large granular vesicles and small clear vesicles are present in the varicosities. A cross-section through the intervaricose region of axons just underneath the basal lamina (bl) of tilapia intestine is shown in b. Note the microtubules (arrow). (d) Both large granular vesicles (arrow) and small clear vesicles (arrowhead) are present within one varicosity very near to the goldfish intestinal epithelium. Bars represent 1 µm.

enterocytes. In contrast, in the goldfish many 5-HT-containing nerve fibres were found within 2 μ m of the epithelium or even in apposition to the enterocytes.

Exocytoses

Exocytoses in nerve fibres were found just underneath the basal lamina of goldfish epithelium (Fig. 2), in nerve fibres in the myenteric plexus and in epithelial endocrine cells (Fig. 3). The exocytosed granules appeared as compact electron-dense material enclosed by the omega-shaped plasma membrane. Because of the relatively small number of nerve fibres seen in the electron micrographs, the chance of finding exocytotic activity from neurons in the lamina propria near to the epithelium is rather small. In principle, the amount of visible exocytoses would be larger upon increasing the tannic acid perfusion time and depolarizing the membranes with 'high K^+ ' (Buma *et al.*, 1984). But, because prolonged perfusion with Ringer reduced the im-

munoreactivity of the tissue, we did not extend the perfusion with tannic acid-containing Ringer beyond 15 min. Nevertheless, as shown in Fig. 4, exocytotic activity combined with immunogold labelling could be observed in endocrine cells of the tilapia (goldfish do not have VIP- or 5-HT-containing endocrine cells; Kiliaan *et al.*, 1989, 1993), in 5-HT-IR varicosities in the lamina propria of the goldfish (Fig. 5) and in varicosities within the muscle layers or situated close to the muscle layers (Fig. 6).

An estimate of the numbers in the counting window near the basal membrane is given in Table 1. The table summarizes the results of the counts of varicosities, varicosities with exocytosing activity, the number of IR varicosities and the number of IR varicosities showing exocytoses. Tilapia and goldfish taken together, we found that from a total of 24 varicosities in the counting window only two showed VIP-IR and only three out of 47 showed 5-HT-IR. In only one of these IR-labelled varicosities was exocytotic activity observed

Table 1. The number of varicosities and exocytoses in the lamina propria of the fish intestine, processed for the combined method of TARI and immunogold labelling

	<i>Tilapia</i>	<i>Goldfish</i>
Total number of windows	18	18
Unlabelled sections (TARI method)		
Varicosities with LGVs	30	40
Varicosities showing exocytosis	2	7
Immunogold-labelled sections (TARI plus immunoelectron microscopy)		
VIP-IR varicosities	1(15)	1(9)
VIP-IR varicosities + exocytosis	–	–
5HT-IR varicosities	1(15)	2(32)
5HT-IR varicosities + exocytosis	–	1(32)

Numbers in parentheses indicate number of varicosities found in immunogold-stained sections (tilapia, $n = 2$; goldfish, $n = 3$).

Fig. 2. Omega-shaped exocytosis (arrow) in a varicosity of a nerve fibre containing large vesicles in the goldfish intestinal epithelium (ep); TARI-method. bl, basal lamina. Bar represents 0.5 μ m.

Fig. 3. Omega-shaped exocytosis (arrow) in the basolateral membrane of an endocrine cell (ec) of the tilapia intestinal epithelium. TARI method. Bar represents 0.5 μ m.

Fig. 4. 5-HT immunogold-labelled endocrine cell in the tilapia intestinal epithelium. (Bar represents 2 μ m). An enlargement of the basal part of the cell is displayed in a. Omega-shaped exocytoses (arrows) are visible on the basal side of the 5-HT immunogold-labelled endocrine cell. Note the scarce gold particles on the LGV (arrowheads). bl, basal lamina; ec, endocrine cell. TARI method plus immunoelectron microscopy. Bar represents 0.5 μ m.

Fig. 5. Exocytosis (arrow) from a serotonergic varicosity lying in a group of nerve fibres underneath the goldfish intestinal epithelium. TARI method plus immunoelectron microscopy. ep, epithelium; bl, basal lamina. Bar represents 0.5 μ m.

Fig. 6. VIP-IR immunogold labelled nerve fibre located in the myenteric plexus (a, bar = 0.25 μ m) and near the circular muscle layer in the lamina propria (b, bar = 0.5 μ m) of the goldfish intestine. Exocytoses of gold-labelled granules (arrows). TARI method plus immunoelectron microscopy.

as well. Thus, when the search for IR and exocytosis was confined to the 2- μ m region near to the basal membrane, the yield was extremely low.

Discussion

In previous studies we have discussed the direct effects of VIP and 5-HT on the epithelium of the tilapia and the goldfish intestine (Kiliaan *et al.*, 1989, 1993; Bakker *et al.*, 1993) and we postulated that these transmitters may have a regulatory role in the function of the epithelial cells. The relative scarcity of 5-HT- and VIP-containing varicosities in tilapia compared with the relative abundance of VIP-IR and 5-HT-IR endocrine cells (Kiliaan *et al.*, 1996) may imply that these messengers do play a role in the regulation of epithelial function by endocrine cells, but not 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 similar to the distance VIP or 5-HT have to traverse when released from endocrine cells to influence neighbouring enterocytes in tilapia intestine.

We therefore tried to visualize the exocytotic release of VIP and 5-HT from the varicosities with the TARI method in combination with immunoelectron microscopy. However, because of the loss of immunoreactivity when glutaraldehyde and OsO₄ were applied, we had to use another fixation method that did not preserve the membranes very well, making it more difficult to identify the omega-shaped surrounding of the granules.

In addition, the TARI method strongly reduced the immunoreactivity. For instance, in the goldfish about 20% of the varicosities within 2 μ m of the basal lamina contain 5-HT and a similar percentage of varicosities contained VIP, as judged from electron microscopy of sections prepared directly from Tyrode- and fixative-perfused intestine (Kiliaan *et al.*, 1996). After perfusion with the tannic acid solution, this percentage was reduced to less than 10% (Table 1), and the appearance of the vesicles in the varicosities was less dense. The decrease of immunoreactivity was also apparent from immunogold labelling of endocrine cells. When the standard immunoelectron microscopy procedure was used, endocrine cells in the epithelium of tilapia showed sufficient immunoreactivity against 5-HT. The decrease of immunoreactivity as a result of perfusion with tannic acid made the chance of identifying the neurotransmitters in the varicosities with exocytotic activity very small.

Nevertheless, from the very limited amount of data one may suggest that 5-HT-IR vesicles can be exocytosed near the basal membrane in the goldfish intestine. The chance of observing exocytosis of

5HT-IR or VIP-IR vesicles in the tilapia mucosa is even smaller, because IR fibres near the basal membrane are much less dense than in the goldfish (Kiliaan *et al.*, 1996). Interestingly, in tilapia, we identified endocrine cells containing 5-HT-IR and VIP-IR and also exocytoses from 5-HT-IR endocrine cells. This suggests that 5-HT and VIP may play a role in the regulation of the epithelial cells in tilapia as well.

In conclusion, we have shown that occasionally it is possible to demonstrate exocytosis from sub-epithelial nerve fibres and epithelial endocrine cells and to identify the nature of the released transmitters. However, the method is very insensitive and the chances are small in less dense innervated tissue. Together with the previously shown direct effects on enterocytes (Kiliaan *et al.*, 1989; Bakker *et al.*, 1993), we suggest that in goldfish 5-HT and VIP may 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 in this fish 5-HT and VIP are endocrine regulators.

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