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Influence of forskolin and carbachol on intestinal absorption of horseradish peroxidase in the goldfish (*Carassius auratus*)

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Abstract. The transepithelial route for mucosa-to-serosa transport of the tracer macromolecule horseradish peroxidase (HRP; MW 40 kDa) and modulation of this transport by forskolin and carbachol have been studied in vitro in stripped goldfish intestinal epithelium mounted in Ussing-type chambers. Uptake and transport have been investigated by measuring the HRP flux from the mucosal to serosal sides by an enzymatic method and by visualising HRP reaction products in the mucosa with electron-microscopical techniques. Both the cholinergic agonist carbachol (which is thought to increase intracellular Ca^{2+} and activate protein kinase C activity) and forskolin (a direct activator of adenylylcyclase) affect the amount of enzymatically active HRP in the tissue. In control tissue, HRP product is found only within the epithelial cells, the transepithelial flux reaching a constant value of about 1.5 pmoles/cm² per h. Carbachol increases the amount of HRP product in the cells, but has no significant effect on the HRP flux compared with control values. Forskolin decreases the amount of HRP product in the cells; however, in the presence of forskolin, the lateral intercellular spaces become filled with HRP product. HRP is found in the lamina propria and the transepithelial protein flux increases more than 2.5-fold. In the presence of forskolin plus carbachol, the results are no different from the control. It is concluded that carbachol increases the endocytotic uptake of HRP, whereas forskolin inhibits the uptake but increases the paracellular permeability for HRP in goldfish intestine.

Key words: Intestine – Endocytosis – Tight junctions – Carbachol – Forskolin – Transport – *Carassius auratus* (Teleostei)

Introduction

The intestinal epithelium acts as a barrier to the passive diffusion of ions and (macro)molecules between the lumen and the internal environment of the body. This barrier is physically formed by intestinal epithelial cells that are joined at their apical sites by a continuous network of tight junctions to prevent the paracellular leak of ions and (macro)molecules.

A number of physiological activities coordinate the selective uptake of luminal material and excretion into the lumen and protect the “milieu interieur” from antigenic overload. These mechanisms include the proteolytic breakdown of proteins and peptides in the acid environment of the stomach and the formation of an immunological barrier by immunoglobulins in the mucous layer at the luminal side of the epithelium. In mammals, intact proteins can be absorbed by specific transport processes in specialized epithelial cells, the so-called M-cells that overlie Peyer’s patches (follicles of lymphocytes). In addition, a limited amount of undigested proteins can be taken up via endocytosis by the enterocytes. Most of these proteins are hydrolyzed in the lysosomes, but a small amount can also be presented to the lymphocytes in the lamina propria. This transport is thought to be beneficial for the information of the local immune system and normally leads to oral tolerance (Crowe and Perdue 1992; Heyman and Desjeux 1992; Sire and Vernier 1992; Sanderson and Walker 1993). In studies with sensitized guinea pigs and rats, the secretory response, as elicited by re-challenge is accompanied by an increase in permeability for macromolecules (Ramage et al. 1988; Heyman et al. 1990).

As yet, it is not clear what mechanism or which pathway is involved in the increase of permeability; it may be that the transcellular pathway is activated by increased transcytosis and/or the paracellular pathway may be affected. A relationship between secretion and macromolecular endocytotic uptake in mammals might exist because of the finding that the secretagogue-stimulated increase of the Cl^- conductance of the apical membrane

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is concomitant with the incorporation of vesicles (Bradbury et al. 1992a, b). The recapture of membrane material, following vesicle fusion, may lead to endocytotic uptake of luminal material. Thus, one may ask whether secretagogues can also modulate the intestinal uptake of luminal (macro)molecules. The answer appears to be affirmative for rat and human small intestine (Bijlsma et al. 1993; P.B. Bijlsma et al., in press; Kiliaan et al. 1993).

Comparison of the fish intestine with mammalian intestine is interesting because fish enterocytes lack the Cl^- secretory pathway, although forskolin activates paracellular Cl^- permeability (Bakker and Groot 1989; Kottra et al. 1993). In contrast, addition of protein kinase C (PKC) activators, such as phorbol dibutyrate, Ca^{2+} -ionophore, or carbachol, have no effect on Cl^- permeability in fish enterocytes (R. Bakker and J.A. Groot, unpublished observations, 1989).

We have studied the possibility that, as in rat and human intestine, absorption of the model protein horseradish peroxidase (HRP) can be influenced by the presence of the secretagogue forskolin and/or carbachol in goldfish intestine. We have determined the absorption of HRP in stripped goldfish intestine, mounted in an Ussing-type chamber, by enzymatically measuring the HRP flux across the epithelium and by visualisation of the presence of HRP reaction products in the mucosa with electron-microscopical methods.

Materials and methods

Male goldfish (*Carassius auratus*), 20–25 cm in length, were kept in well-aerated fresh water at 18 °C and were fed daily in the morning with Trouvit food pellets (Trouvit, Putten, NL), except for the day on which they were killed. The fish ($n=4$) were killed by decapitation and the anterior intestine (first part behind the intestinal bulb) was stripped free from the muscle layers.

Flat sheets of the intestinal epithelium (0.2-cm² exposed area) were mounted in tissue holders as described (Groot et al. 1979) and placed in Ussing chambers. Mucosal and serosal compartments were filled with Ringer's solution, continuously stirred and over-gassed with humidified 5% CO_2 and 95% O_2 . The Ringer's composition was 117.5 mM NaCl, 5.7 mM KCl, 25 mM NaHCO_3 , 1.2 mM NaH_2PO_4 , 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 27.8 mM mannitol (mucosal side only), and 27.8 mM glucose (serosal side only). After carbogenation, the pH was 7.3 and the osmolality 320 mOsm. Carbachol (10^{-5} M), forskolin (10^{-5} M) and tetrodotoxin (TTX; 10^{-6} M) were added at the serosal side. TTX was added 5 min before forskolin and carbachol, to reduce the release of endogenous neurotransmitters by the addition of forskolin and carbachol. Forskolin was dissolved in ethanol, TTX in Ringer's solution, and carbachol in water. The final concentration of ethanol was 0.1%, a concentration that had no detectable effects on the electrophysiological parameters as determined in other experiments (Bakker and Groot 1989). After a 15 min equilibration period, the epi-

thelia were exposed to mucosal HRP (10^{-5} M) in the absence or presence of serosal carbachol or forskolin, or the combination of both.

For the flux measurements, serosal samples of 0.125 ml were taken every 30 min (30–180 min) and replaced by oxygenated Ringer. The appearance of HRP in the serosal bath was measured enzymatically. In short, a 0.1 ml sample of test solution was mixed with 1.4 ml phosphate buffer (0.1 M, pH 6.0) containing 0.003% H_2O_2 and 0.009% *o*-dianisidine di-HCl. The linear HRP-concentration-dependent rate of increase in optical absorption at 460 nm was determined with an Eppendorf photometer.

After 30, 60 and 90 min exposure to mucosal HRP and the presence or absence of secretagogues, the tissue was fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for electron-microscopical studies, rinsed overnight (4°C) in 0.1 M sodium-cacodylate buffer (pH 7.4), and then three times for 5 min each in 0.05 M TRIS buffer (pH 7.6) to remove HRP from the luminal side of the tissue. Peroxidase activity was histochemically demonstrated using the method of Graham and Karnovsky (1966) in which the tissue was preincubated for 15 min in 5 mg diaminobenzidine in 10 ml 0.05 M TRIS buffer (pH 7.6, 22°C) and subsequently incubated for 15 min in the same buffer containing 0.01% H_2O_2 . Tissues were then processed for routine electron microscopy.

Electron micrographs were taken from epithelial cells cut transversely. Endosomes filled with HRP product were counted in a window with an area of $6 \times 4 \mu\text{m}^2$, at the apical side of the cells, directly underneath the microvillar border. Their diameters were measured (nm) and the sum of diameters per window was taken as an estimate of the amount of HRP in the window (such measurements are semi-quantitative, because, in any section, not all endosomes are cut through the middle). We compared diameters obtained from measurements in 21 regions per animal in control and experimental tissue and considered that differences in cross-sectioning of the endosomes would be similar in control and experimental tissue.

Results

The number and diameter of HRP-product-filled endosomes appeared to be affected by the presence of carba-

Fig. 7. Semi-quantification of the amount of HRP in epithelial cells after 30 min of exposure. The sum of diameters (nm) of endosomes containing intact HRP per apical region ($24 \mu\text{m}^2$) of goldfish enterocytes were calculated for 40 windows (10 windows per animal, $n=4$, except for the control and carbachol values, which were counted over 21 windows per animal) in the presence or absence of carbachol, forskolin or a combination of both. Bars: Means and standard error of the mean. The amounts of HRP in the presence of carbachol or forskolin are significantly different from the control value. The amount in the presence of carbachol plus forskolin is significantly different from the amount with carbachol alone (* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

Figs. 1–6. Goldfish intestinal epithelium in the absence (control; Figs. 1, 3, 5) or presence (Figs. 2, 4, 6) of carbachol after 10 (Figs. 1, 2), 30 (Figs. 3, 4) and 60 min (Figs. 5, 6) exposure to horseradish peroxidase (HRP) at the mucosal side. Low HRP activity can be detected in control tissue. In carbachol-exposed tissue at 10 min, little HRP activity can be seen but a larger number of HRP-filled endosomes (arrows) is present after 30 and 60 min of incubation. $\times 14\,700$

Fig. 8. HRP is present in the lateral intercellular space and tight junctions of goldfish intestinal epithelium after 30 min of forskolin exposure, but no HRP activity can be detected in the cells. Note the deep invaginations of the basolateral membranes. $\times 14\,700$

Fig. 9. Goldfish intestinal epithelium, exposed for 30 min to a combination of forskolin and carbachol. Only a small amount of HRP (arrows) can be detected in the cell. $\times 14\,700$

Fig. 10. Goldfish intestinal epithelium (EP) after 30 min of forskolin exposure. Dense staining of HRP product is present (arrows) in the lateral intercellular space and the lamina propria (LP). $\times 15\,600$

chol or forskolin. In control tissue, some HRP was found in villus cells, but a larger amount of HRP was found in the presence of carbachol, whereas in the presence of forskolin, the amount was reduced.

HRP activity was examined in the cells in the presence (Figs. 2, 4, 6) and absence (Figs. 1, 3, 5) of carbachol at 10, 30 and 60 min after HRP addition to the mucosal bath. In the presence of carbachol, HRP-product-filled endosomes were more abundant at 30 and 60 min, whereas this was not yet obvious at 10 min (Fig. 2). A semi-quantitative analysis was performed at 30 min (Fig. 7). The amount of HRP in the presence of carbachol appeared to be significantly larger than without car-

bachol. The amount of HRP product in the cells was lower when forskolin was present (Fig. 8) and forskolin could also reduce the effect of carbachol (Fig. 10). The relevant amounts were compared at 30 min (Fig. 7). Interestingly, the filling of the highly contorted lateral intercellular spaces (typical of fish enterocytes) was prominent after the addition of forskolin only (Fig. 8). When forskolin was present together with carbachol, the lateral intercellular spaces and tight junctions showed no HRP activity (Fig. 9). When forskolin was present alone, HRP activity was also found as a dark line along the basal membrane and as dark stained blots in the lamina propria (Fig. 10), indicating that cAMP increased the trans-

transepithelial flux of HRP

Fig. 11. Mucosa-to-serosa HRP flux across the goldfish intestine, in the presence and absence of serosally added secretagogues (carbachol, forskolin). At all time points later than 90 min, forskolin significantly increases the HRP flux ($P < 0.01$, unpaired t -test) compared with control values; the presence of carbachol decreases this effect of forskolin to control values ($P < 0.025$, unpaired t -test). Control, $n=27$, 5 animals; carbachol, $n=6$, 3 animals; forskolin, $n=11$, 5 animals; forskolin+carbachol, $n=5$, 2 animals (n =number of tissue preparations). Values are means $\pm$ SE

port of HRP through the epithelial barrier. This conclusion was confirmed by the results of the flux measurements, as shown in Fig. 11. Forskolin significantly increased the HRP flux from the mucosal to the serosal side to a value of about 3 pmol/cm² per h, significantly larger than control values or when carbachol was present.

Discussion

The appearance of HRP reaction products in endosomes in control fish intestinal epithelium in this study has confirmed earlier observations of endocytosis of macromolecules in fish intestine (Noaillac-Depeyre and Gas 1973; Rombout et al. 1985; Georgopoulou et al. 1986, 1988; 1976; Moriyama et al. 1990; Tamura et al. 1993). The novel finding is that carbachol, a Ca²⁺-related and PKC-related secretagogue in mammals (Bajna et al. 1992), can increase the amount of HRP in the cell, possibly by increased uptake or decreased degradation or transcytosis. Although we have not studied this question in detail, the results of flux measurements do not show an effect of carbachol on transepithelial transport and therefore do not suggest a change in transcytosis. The carbachol-stimulated increase in intracellular Ca²⁺ may be the trigger for exocytosis in enterocytes, as is the case in many other cells. The observed carbachol-induced increase in endocytosis in mammalian (Bijlsma et al. 1993; P. Bijlsma et al., in press; Kiliaan et al. 1993) and fish intestine (this report) may be related to the restoration of the surface area after vesicle fusion, by re-uptake of membrane material. In the human intestinal cell line T84, forskolin inhibits endocytosis (Bradbury et al. 1992a,b). In agreement with these findings, we have observed that forskolin reduces the amount of HRP in fish

enterocytes. The reduction is significant in incubations both without ($P < 0.05$) and with carbachol ($P < 0.01$).

Spontaneous HRP uptake via fluid-phase endocytosis in fish enterocytes has been reported for stomachless fish and for fish with a stomach (Noaillac-Depeyre and Gas 1976; Watanabe 1981; Rombout et al. 1985), these results having been obtained in vivo after the fish had been handled and forced to ingest HRP by oral intubation through the oesophagus into the intestinal bulb and sometimes by anal intubation. Some of the HRP uptake in the in vivo experiments may be attributable to stress-induced release of (neuro-)transmitters. The non-selective absorption of macromolecules via endocytosis has been considered an extension of the normal digestive capacity of the intestine of fish with or without a stomach (Ezeasor and Stokoe 1981; Stroband and Kroon 1981; Georgopoulou et al. 1986). In addition intact HRP crosses the basal membrane and has been found in the lamina propria in macrophages (Georgopoulou et al. 1988; Sire and Vernier 1992). In our in vitro experiments, this can clearly be seen in the presence of forskolin, whereas with carbachol alone or with carbachol plus forskolin, the presence of intact HRP in the lamina propria and the transepithelial flux are no different from that in control tissue. A large number of leucocytes and macrophages in the lamina propria of the fish gut form the gut-associated lymphoid tissue (Sire and Vernier 1992). The observed increased passage of intact macromolecules suggests that cAMP-mediated neurotransmitters and hormones may also have an impact on the fish immune system. This suggestion requires further investigation.

Forskolin appears to reduce the amount of intact HRP in the cells, but the transepithelial flux increases and the filling of the lateral intercellular spaces and lateral infoldings is prominent. These correlations suggest the opening of tight junctions for the macromolecule. Not all lateral spaces are stained with the HRP product, suggesting a heterogeneous reaction of tight junctions to forskolin, i.e. a rise in cAMP. Interestingly, when forskolin and carbachol are present together, no filling of the lateral intercellular spaces or rise in transepithelial flux is observed. The possibility that forskolin decreases the amount of HRP in the cells and increases transepithelial transport by increasing transcytosis can be ruled out by the following reasoning. If the increased transepithelial flux is caused by increased uptake in combination with increased transport of the endosomes to the lateral membrane, one would expect to find that the mannitol flux increases by 2–3 times; however, forskolin does not increase the flux of mannitol significantly (Bakker et al. 1993).

Cyclic AMP can induce a significant increase of the tight junctional Cl⁻ conductance in fish intestine (Bakker and Groot 1989; Kottra et al. 1993) and an increase of the transepithelial polyethylene glycol 4000 flux by 56 $\pm$ 5% (Bakker et al. 1982); nevertheless, as mentioned above, the mannitol flux does not significantly increase (Bakker et al. 1993). As suggested earlier (Bijlsma et al. 1993; P. Bijlsma et al., in press), the discrepancy between increased macromolecular permeability and constancy of mannitol permeability may be explained by assuming that the fusion of small pores gives a larger one

that then allows the passage of large molecules but that does not increase the area for diffusion of the small molecules that can pass through the small pores. This postulated pore-fusion in fish intestine tight junctions is apparently a forskolin-stimulated topical event that can be suppressed by carbachol. The increase of the Cl^- permeability of the tight junctions by forskolin is however not influenced by carbachol (A.J. Kiliaan, unpublished observations); therefore, the forskolin-induced increase of macromolecular permeability and the increase of Cl^- conductance appear to be different mechanisms. Moreover, the observed increase of macromolecular transport might be employed to bring intact proteins into the fish for passive immunisation.

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