



Bile salts regulate ion transport in the intestine of Senegalese sole

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ABSTRACT

The regulatory role of taurocholic acid (TC) and tauroolithocholic acid (TLC) in ion transport was investigated with the short-circuit current (Isc) technique in the intestine of Senegalese sole (*Solea senegalensis*). Under voltage clamp conditions, Isc was stable for over 3 h and averaged $-15.42 \pm 2.75 \mu\text{A}/\text{cm}^2$ and $-65.28 \pm 7.69 \mu\text{A}/\text{cm}^2$ in the anterior and posterior intestine, respectively. Only bumetanide (200 μM), a loop diuretic that inhibits $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporters, inhibited the absorptive Isc, and apical application of NPPB (200 μM), EIPA (200 μM), glibenclamide (200 μM) and DIDS (200 μM) were without effect in the absorptive current. Apical application of TC and TLC induced rapid (< 1 min) increases in the absorptive current in the anterior and the posterior intestine. The responses were dose dependent in the range of physiological levels of TC or TLC previously detected in the intestine of Senegalese sole, 0.4–4 mM, with higher responses in the posterior intestine at the same doses. The response to apical application of TC and TLC was completely abolished by bumetanide (200 μM). In addition, the effect of TC or TLC in preparations with bilateral reduction of chloride (from 146 to 6 mM) in the chamber saline was reduced by 80%. Conclusions of this study indicate that the bile salts TC and TLC alter ionic transport in the intestine of marine fish enhancing the absorptive pathway. These changes revealed by regulation of Isc are characterized by their dose-dependent action, intestinal region dependency, reversibility, chloride dependence and bumetanide sensitivity.

1. Introduction

Senegalese sole is a marine fish species with high commercial value in Southern European countries, with an increasing interest from the aquaculture sector and to which a strong research effort has been dedicated during the last years (Morais et al., 2016). Although feeding mainly on benthic invertebrates such as Polychaeta, crustacean and mollusks at juvenile and adult stages (Cabral, 2000), this species is classified as omnivorous (De Groot, 1971). An acidic digestion in the stomach (pepsin-like activity) normally appears around 40 days after hatching (Ribeiro et al., 2005), but it is a mild acidic digestion, since pH values not lower than 4 are normally found in the stomach of adult Senegalese sole (Yúfera and Darias, 2007). The long anterior intestine (Conceição et al., 2007) resembles the digestive tract of herbivorous species, thus suggesting a digestive strategy different from that of a carnivorous. The Senegalese sole anterior intestine represents around 60% of the total digestive tract, being lined by a columnar epithelium with scattered mucous cells and neuroendocrine cells, like cholecystokinin - CCK (Arellano et al., 1999; Ribeiro et al., 1999, 2008). Primary functions associated to this intestinal region are to conclude the digestion, started in the mouth and stomach, and achieve the subsequent

nutrient absorption (Wilson and Castro, 2010). The region can be greatly distended by the presence of mucosal folds and by apical plasma membrane amplification through brush border microvilli (Wilson and Castro, 2010). The hindgut (i.e. posterior intestine) is caudal to the anterior intestine, with an anatomical transition evidenced by a muscular constriction, similar to ileocecal valve of mammalian intestine. The hindgut is also lined by columnar epithelium, although with a looser matrix and a function which is apparently more related with immune defenses or osmoregulation (Ribeiro et al., 1999; Wilson and Castro, 2010).

Dietary lipids are hydrolyzed in the intestinal lumen by pancreatic lipases. Lipids are the main source of metabolic energy in fish and are constituents of cell membrane bilayers (Sargent et al., 2002). However, the Senegalese sole has been shown to have low tolerance to high levels of dietary lipids (Borges et al., 2009). Bile salts are considered as digestive molecules with a primary function in dietary fat emulsion and absorption of dietary lipids and fat-soluble vitamins. Bile salts are synthesized from cholesterol in the liver and their final synthesis involves the conjugation with an amino acid, usually glycine or taurine (Russell, 2003). In ray-finned fish, all bile acids were found to be conjugated to taurine with few exceptions (Hofmann et al., 2010). Once

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synthesized, bile salts are secreted to the gall bladder and then released in the anterior intestinal lumen (Russell, 2003). Conjugation of bile acids ensures that they become completely ionized and highly soluble. Bile salts are amphipathic, capable of rapidly transform lipid bilayers to mixed micelles at low concentrations, thus solubilizing dietary lipids, after hydrolysis by pancreatic lipases. The predominant bile salts in bile are highly species specific (Hofmann et al., 2010), and in the Senegalese sole bile (and intestinal fluid) have been identified as taurocholic acid (TC), tauroolithocholic acid (TLC) and a minor non-identified bile salt (Velez et al., 2009). Taurocholic acid is present at a higher concentration than TLC in bile (342 vs. 271 mM) and in intestinal fluid (4.13 vs. 0.4 mM; Velez et al., 2009).

In addition to their classic function in fat metabolism, there is recent indication that bile salts may have a role in the movement of water and ions (Keitel et al., 2009), probably related to solute dragged absorption. In marine fish intestine the apical absorption of chloride ions acts as the driving force for intestinal water absorption (Musch et al., 1982; Grosell et al., 2005). The involvement of a bumetanide-sensitive $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC) has been established as one of the driving forces for water absorption (Musch et al., 1982). However, recent studies have established the involvement of apical $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers as likely chloride absorption routes to drive intestinal water absorption (Grosell et al., 2005). In the Senegalese sole intestine, the bumetanide-sensitive mechanism appears to be the main driving of chloride absorption (Ruiz-Jarabo et al., 2017). Therefore, the objective of this study was to understand the regulatory role of bile salts on intestinal ion transport in Senegalese sole (*Solea senegalensis*), using the short-circuit current (Isc) technique.

2. Material and methods

2.1. Fish

Senegalese sole (*Solea senegalensis*) juveniles (80–140 g body weight) were obtained from IPMA's Aquaculture Research Station, in Olhão (Portugal) and transported to the experimental facilities at Centre of Marine Sciences (CCMAR, Faro, Portugal), where fish were adapted for one month to the new environmental and feeding conditions. Fish were maintained in a recirculated water circuit (flat-bottomed tanks supplied with natural seawater at $19.8 \pm 0.5^\circ\text{C}$ and 31 ± 0.5 psu; dissolved oxygen in water always above 85% of saturation; $\text{NH}_3 < 0.1$ mg/L and $\text{NO}_2^- < 0.25$ mg/L; pH = 7.7 ± 0.1 ; 12h:12h light: dark photoperiod; fish density = 4.6 kg/m²). For maintenance, fish were fed to a final ration of 3% of the body weight with a fishmeal-based diet formulated for Senegalese sole juveniles [manufactured at SPAROS Lda., as described by Aragão et al., 2014; 57% protein (DM) and 9% lipids (DM)], during 12 h with automatic feeders. All fish were fasted for 24 h before experimental manipulations to ensure emptiness of the intestine.

For experiments fish were over anaesthetized with 2-phenoxethanol (1:5000 vol/vol, Sigma, Madrid) and sacrificed by pithing. Afterwards fish were dissected out to collect portions from anterior intestine, defined as the region (3–5 cm in length) caudal to the stomach, and the posterior intestine (or hindgut), a distal region delimited by the caudal and the ileo-rectal sphincters. The tissues were transferred to freshly prepared, chilled and gassed saline (see section 2.3).

All animal manipulations were carried out in compliance with the European (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under Group-C licenses from the Direção-Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain). Final concentrations in Ussing chambers of the chemicals used were: 5-

Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) 200 μM , Glibenclamide (GLI) 200 μM , 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS) 200 μM , 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA) 200 μM , Bumetanide (BUM) 200 μM , taurocholic acid sodium salt hydrate (TC) 0.4 or 4 mM and tauroolithocholic acid sodium salt (TLC) 0.4 or 4 mM. All chemicals were added as DMSO concentrated stocks to the Ussing chamber. The amount of DMSO in the solution never exceed 0.2% of the total volume.

2.3. Short-circuit current (Isc) measurements

The anterior and posterior intestine were collected, isolated and mounted in Ussing chambers as previously described (Gregório et al., 2013; Ruiz-Jarabo et al., 2017). Briefly, tissue was placed on a tissue holder of 0.25 cm² and positioned between two half-chambers containing 2 mL of serosal physiological saline, formulated according to previously published plasma values for the Senegalese sole (Arjona et al., 2007): NaCl 140 mM; MgSO₄ 1.5 mM; NaH₂PO₄ 3 mM; CaCl₂ 1.5 mM; NaHCO₃ 5 mM; KCl 3 mM; Glucose 5.5 mM; Na-Hepes 5 mM; osmolality was adjusted to 290 mOsm/kg (Camlab, UK) with mannitol and pH to 7.80. In experiments with low-chloride saline 140 mM NaCl was replaced by 140 mM Na-gluconate. During the experiments, the tissue was bilaterally gassed with 0.3% CO₂ + 99.7% O₂ and the temperature maintained at 22 °C. Transepithelial potential (Vt, in mV) was referenced to mucosa and short-circuit current (Isc, $\mu\text{A}/\text{cm}^2$) was monitored by clamping of epithelia to 0 mV and expressed as negative for absorption of anions. Epithelial resistance (Rt, $\Omega\cdot\text{cm}^2$) was manually calculated (Ohm's law) using the current deflections induced by a 2 mV pulse of 3 s every minute. Voltage clamping and current injections were performed by means of epithelial amplifiers VCC600 (Physiologic Instruments, San Diego, USA). After the tissue achieved a steady state, which occurred usually between 30 and 40 min after mounting, bioelectrical parameters of individual tissues were recorded onto a computer by means of a Lab-Trax-4 acquisition system (World Precision Instruments, Sarasota, FL, USA) using LabScribe3 (iWorx Systems Inc., Dover, NH, USA).

2.4. Statistics

Results are presented as means \pm SEM. All statistics were performed after assessing homogeneity of variance and normality. Effects of bile salts on Isc were assessed with a two-way analysis of variance, considering intestinal region and bile salt dose as main factors, complemented with the post hoc Bonferroni test to identify significant effects. Other statistical analyses were carried out using, as appropriate, Student *t*-test or one-way analysis of variance followed by the post hoc Bonferroni test. All statistical analyses were performed with Prism 6.0 (GraphPad Prism 6.0 for McIntosh, GraphPad Software, San Diego, California, USA) and groups were considered significantly different at $p < 0.05$.

3. Results

3.1. Basal bioelectric properties of the Senegalese sole intestine

Intestinal preparations exhibited stable electrophysiological characteristics for up to 3 h if not manipulated. Under symmetric conditions the intestine of Senegalese sole mounted in Ussing chambers generated open circuit potentials of 1.47 mV and 5.26 mV in the anterior and posterior intestine, respectively (Table 1). After voltage-clamping of the epithelia to 0 mV a short-circuit current (Isc) of -15.42 ± 2.75 $\mu\text{A}/\text{cm}^2$ was observed in the anterior intestine, while Isc in the posterior intestine was significantly higher: -65.28 ± 7.69 $\mu\text{A}/\text{cm}^2$ ($p < .05$, Student *t*-test). The transepithelial resistance was similar in both intestinal sections and averaged between 80 and 90 $\Omega\cdot\text{cm}^2$.

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