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The involvement of H^+-ATPase and carbonic anhydrase in intestinal HCO_3^- secretion in seawater-acclimated rainbow trout

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SUMMARY

Pyloric caeca and anterior intestine epithelia from seawater-acclimated rainbow trout exhibit different electrophysiological parameters with lower transepithelial potential and higher epithelial conductance in the pyloric caeca than the anterior intestine. Both pyloric caeca and the anterior intestine secrete HCO_3^- at high rates in the absence of serosal HCO_3^-/CO_2, demonstrating that endogenous CO_2 is the principal source of HCO_3^- under resting control conditions. Apical, bafilomycin-sensitive, H^+ extrusion occurs in the anterior intestine and probably acts to control luminal osmotic pressure while enhancing apical anion exchange; both processes with implications for water absorption. Cytosolic carbonic anhydrase (CAc) activity facilitates CO_2 hydration to fuel apical anion exchange while membrane-associated, luminal CA activity probably facilitates the conversion of HCO_3^- to CO_2. The significance of membrane-bound, luminal CA may be in part to reduce HCO_3^- gradients across the apical membrane to further enhance anion exchange and thus Cl^- absorption and to facilitate the substantial $CaCO_3$ precipitation occurring in the lumen of marine teleosts. In this way, membrane-bound, luminal CA thus promotes the absorption of osmolytes and reduction on luminal osmotic pressure, both of which will serve to enhance osmotic gradients to promote intestinal water absorption.

Key words: marine fish, osmoregulation, proton pump, water absorption, bicarbonate transport.

INTRODUCTION

For the majority of vertebrates, seawater is a severely dehydrating environment owing to the steep osmotic gradient between the extracellular fluids and the surrounding milieu. Most marine mammals, reptiles and teleost fish meet this environmental challenge by ingesting seawater, with water absorption from the gastrointestinal tract relying on substantial salt absorption, and net water gain being contingent upon subsequent excretion of the absorbed salt load (Skadhauge, 1974; Nedergaard et al., 1999; Larsen, 2000; Larsen and Mobjerg, 2006; Larsen et al., 2006; Larsen et al., 2007).

Intestinal absorption of Na^+ is ascribed to Na^+-co-transporters in the apical membrane of the intestinal epithelium in conjunction with Na^+/K^+-ATPase (NKA) in the lateral membrane. Chloride (Cl^-) absorption occurs *via* both co-transporters and anion exchange in the apical membrane and basolateral Cl^- channels (Marshall and Grosell, 2005; Grosell et al., 2001; Grosell et al., 2005; Grosell, 2006; Grosell and Taylor, 2007; Wilson et al., 2002; Taylor and Grosell, 2006; Taylor et al., 2007), presumably to varying extents depending on the species and environmental conditions. Considering the high rates of intestinal Cl^- absorption of marine fish and the fact that Cl^-/HCO_3^- exchange accounts for as much as 70% of overall Cl^- absorption, high HCO_3^- concentrations in the lumen of the intestine, in some cases as high as 100 mmol l^{-1} (Grosell et al., 2001; Grosell, 2006; Wilson, 1999), are not surprising. However, the apparent high Cl^-/HCO_3^- exchange rates of the marine teleost intestine point to a substantial demand for cellular substrate for this process, raising the question of whether hydration of endogenous epithelial CO_2 or HCO_3^- uptake across the basolateral membrane provides the cellular substrate for apical anion exchange.

It seems that epithelial CO_2 hydration is significant for intestinal Cl^- absorption under physiological conditions (Dixon and Loretz, 1986; Wilson and Grosell, 2003; Grosell, 2006) and, thus, for osmoregulation in marine teleosts. In support of this conclusion are observations of reduced intestinal HCO_3^- secretion by marine teleosts treated with carbonic anhydrase (CA) inhibitors; cytosolic CA (CAc) in the intestinal epithelium catalyzes the hydration of CO_2 to provide HCO_3^- (Dixon and Loretz, 1986; Grosell, 2006; Grosell et al., 2005; Wilson et al., 1996). In further support of a role for CA in marine osmoregulation are observations of increased CAc mRNA expression and enzymatic activity in rainbow trout following transfer from freshwater to 65% seawater (SW) (Grosell et al., 2007). In addition to CAc, a membrane-bound, luminal CA IV isoform also exhibited increased mRNA expression and enzymatic activity following transfer to 65% SW. It was proposed that apically oriented CA IV was facilitating the dehydration of HCO_3^- to CO_2 (Grosell et al., 2007). The consumption of H^+ associated with the dehydration of HCO_3^- in the high HCO_3^-, high pH intestinal fluids would result in enhanced CO_3^{2-} formation. This enhanced CO_3^{2-} formation is involved in $CaCO_3$ precipitation (Grosell et al., 2007), a process critical for marine teleost osmoregulation (Wilson et al., 2002; Grosell, 2006; Genz et al., 2008). However, and in contrast to CAc, direct empirical data supporting a role for CA IV in the intestinal secretion of HCO_3^- is lacking.

Hydration of endogenous CO_2 for epithelial HCO_3^- secretion necessitates cellular H^+ extrusion for intracellular pH (pH_i) regulation. The intestinal epithelium exhibits polarization with respect to HCO_3^- and H^+ extrusion, resulting in net base secretion (Grosell, 2006) and systemic net acid gain associated with the water absorption (Grosell and Taylor, 2007). Such intestinal net acid

absorption appears to be a general feature of marine teleost osmoregulation (Grosell, 2006) and is compensated for by salinity-dependent branchial net acid extrusion in the gulf toadfish (Genz et al., 2008). However, recent observations of apical H^+ pump (V-type H^+-ATPase) localization in the intestine of rainbow trout acclimated to 65% SW, increased mRNA expression and H^+ pump activity following transfer from freshwater to 65% SW, and observations of increased mRNA expression for the apical Na^+/H^+ exchanger 3 (NHE3) isoform point to a potentially important role of apical H^+ extrusion in marine osmoregulation.

Two main objectives of the present study were, therefore, to examine the potential function of membrane-bound, luminal CA activity in intestinal HCO_3^- secretion and to evaluate whether simultaneous luminal H^+ and HCO_3^- secretion occur in seawater-acclimated rainbow trout. In addition, we set out to compare the transport physiology of the pyloric caeca and the anterior intestine, the two most proximal segments of the intestine. The proximal segments of the intestine were chosen because *in vivo* observations of high luminal pH and total CO_2 concentrations in luminal fluids (Wilson et al., 1996) demonstrate that the majority of intestinal HCO_3^- secretion occurs in this region in the rainbow trout intestine as is the case for most other fish examined to date (Grosell, 2006). Seawater-acclimated trout display high $MgSO_4$ and relatively low NaCl concentrations in fluids obtained from the anterior intestine, illustrating that salt and water absorption are also occurring at high rates in the proximal segments of the intestine (Grosell et al., 2007). The motivation for the comparison of the pyloric caeca and the anterior intestine stems from observations of elevated NKA mRNA in pyloric caeca from brown trout during seawater acclimation (Seidelin et al., 2000; Grosell et al., 2007), and observations of stimulated NKA activity and fluid uptake rates in Chinook salmon pyloric caeca in response to seawater acclimation or cortisol implants (Veillette et al., 2005). These responses, combined with measurements of elevated luminal Mg^{2+} concentrations in the pyloric region (suggesting water absorption), implicate this intestinal region in osmoregulation (Wilson et al., 1996). Despite the likely role in osmoregulation and the long recognized role of the pyloric caeca in nutrient absorption (Buddington and Diamond, 1986; Buddington and Diamond, 1987; Collie, 1985), information about the epithelial transport properties of this intestinal segment, which accounts for as much as 70% of the intestinal surface area (Buddington and Diamond, 1986), is sparse. Thus, a third objective of the present study was to assess epithelial electrophysiological parameters and to directly measure epithelial HCO_3^- secretion by pyloric caeca from seawater-acclimated rainbow trout.

MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from the Robertson Creek Hatchery (Fisheries and Oceans Canada), Port Alberni, BC, Canada and transported to Bamfield Marine Sciences Centre (Bamfield, BC, Canada) where they were gradually acclimated to 80% SW over two weeks at 13°C in July 2007 under flow-through conditions. Fish were held in aerated 80% SW in large outdoor aquaria for at least five days prior to experimentation. Food was offered twice daily except for 72 h prior to experimentation.

General experimental protocol for pH-stat experiments

Rainbow trout were killed by a lethal blow to the head. For studies of HCO_3^- secretion by pH-stat titration, the pyloric caeca and the anterior segment of the intestine were obtained by dissection from 150–200 g and 50–70 g rainbow trout, respectively. Individual pyloric caeca were opened by a longitudinal cut and mounted in tissue holders exposing 0.1 cm^2 gross surface area (Physiological Instruments, P2403, San Diego, CA, USA) while segments of the anterior intestine were mounted in tissue holders exposing 0.3 cm^2 (Physiological instruments, P2404). Only one preparation was taken from each individual animal. The tissue holders were then placed between Ussing-style half-chambers (Physiological instruments, 2400), each containing 1.6 ml of appropriate, pre-gassed saline (Table 1). Mixing of salines in the Ussing half-chambers was by gassing with pre-humidified gas as specified below. Stable titration curves were achieved by pre-gassing HCO_3^--free luminal salines with CO_2-free gas for at least 60 min prior to experimentation and by gassing with O_2 in the luminal half-chamber during titrations. Under these conditions, base secretion, as calculated from the amount of acid added over time to maintain constant pH, is comprised largely of HCO_3^- secretion (Grosell and Genz, 2006). The Ussing chambers were placed in chamber holders maintained at 13±1°C throughout experimentation. Current and voltage electrodes (Physiological Instruments P2020-S) connected to amplifiers (Physiological instruments VCC600) recorded the transepithelial potential (TEP) under current clamp conditions (0 μA). At 60 s intervals, 3 s pulses of 30 μA were passed from the mucosal side to the serosal side and TEP measurements were logged on a personal computer using a data acquisition system [Biopac systems interface (Goleta, CA, USA) with AcqKnowledge software, v. 3.8.1 (Goleta, CA, USA)]. TEP values are reported with a luminal reference of 0 mV. For the determination of net base secretion by the pyloric caeca or anterior intestine epithelium, the luminal half-chambers were fitted with a pH combination electrode (Radiometer PHC4000-8, Villeurbanne CEDEX, France) and a micro-burette tip, both of which were connected to a pH-stat titration system (Radiometer, TIM 854 or 856). A common ground connecting the amplifier and the pH-stat titrator allowed for simultaneous current pulsing and pH measurements. All pH-stat titration experiments were performed at a luminal pH of 7.800, and both pH and the rate of acid addition were logged on personal computers using Titramaster software (v. 1.3 and 2.1, Villeurbanne CEDEX, France). Luminal pH was generally maintained within ±0.003 pH units of the 7.800 set point, and base secretion was determined from the rate of addition of acid of known concentration (0.0005 mol l^{-1} HCl). A luminal set-point pH of 7.800 was chosen to avoid pH gradients across the intestinal epithelium and to ensure that any movement of acid–base

Table 1. Composition of salines employed in pH-stat experiments

	Mucosal (mmol l^{-1})	Serosal (mmol l^{-1})	Serosal HCO_3^--free (mmol l^{-1})
NaCl	69	151	151
KCl	5	3	3
$MgSO_4$	77.5	0.88	0.88
$MgCl_2$	22.5	–	–
Na_2HPO_4	–	0.5	0.5
KH_2PO_4	–	0.5	0.5
$CaCl_2$	5	1	1
$NaHCO_3$	–	5	–
Hepes (free acid)	–	3	3
Hepes (Na-salt)	–	3	3
Glucose	–	3	3
Gassing	O_2	0.3% CO_2/O_2	O_2
pH	7.8	7.8	7.8

Osmotic pressures of salines were adjusted to 310 mosmol using mannitol. The pH values of serosal salines were adjusted following >90 min equilibration with 0.3% CO_2/O_2 or O_2 for control and HCO_3^--free salines, respectively.

equivalents was due to transport rather than simple diffusion. Common to all pH-stat experiments was an initial 60 min control period during which base secretion, TEP and conductance stabilized to constant values before experimental manipulations.

Source of base (HCO_3^-) secretion

To determine if the source of base secreted by the pyloric caeca and anterior intestine of rainbow trout is endogenous epithelial CO_2 or serosal CO_2/HCO_3^-, an initial control period was carried out, during which serosal salines contained 5 mmol l^{-1} HCO_3^- and were gassed with 0.3% CO_2 in O_2. This control period was followed by a 60 min period, during which the serosal half-chamber contained a HCO_3^--free (Hepes-buffered) saline gassed with O_2. The HCO_3^-/CO_2-free period was followed by a return to initial control conditions for a final 60 min. No other experimental protocols exceeded the 3 h duration of these experiments. Subsequent experiments were performed with Hepes-buffered serosal salines gassed with O_2.

Apical H^+ extrusion

Amiloride (final concentration 10^{-4} mol l^{-1}) or bafilomycin (final concentration 10^{-6} mol l^{-1}) (Sigma Chemical Co., St Louis, MO, USA and LC laboratories, Woburn, MA, USA, respectively) was added to the luminal saline of preparations of both pyloric caeca and anterior intestine to examine the potential role of Na^+/H^+ exchange and apical H^+ pump activity in overall net base secretion. Amiloride or bafilomycin was dissolved in dimethylsulphoxide (DMSO), yielding a final concentration of 0.1% DMSO in the luminal saline. Previously, this DMSO concentration was found not to influence the intestinal epithelium (Grosell, 2006). Stock solutions of amiloride (10^{-1} mol l^{-1}) in DMSO were alkaline and were therefore neutralized by the addition of 1 mol l^{-1} HCl prior to addition to the luminal saline.

DIDS sensitivity of luminal base secretion

4,4′-diisothiocyanostilbene-2,2′disulphonic acid (DIDS) (2×10^{-4} mol l^{-1}) (Sigma Chemical Co.) was added to the luminal saline of preparations of the anterior intestine to confirm that apical HCO_3^- secretion probably occurs *via* an apical anion exchanger. DIDS stock solutions were prepared daily in DMSO (1 mol l^{-1}), which, when added to the luminal saline, yielded a final concentration of 0.02% DMSO. DIDS stock solutions were highly acidic, and the addition of 1 mol l^{-1} NaOH was used to elevate the pH of these solutions, although the stock remained acidic. Luminal saline displayed a transient pH reduction to 7.2–7.5 upon DIDS addition but returned to 7.800 within a few minutes, owing to base secretion by the tissue, after which pH-stat titration resumed.

Effects of permeant and impermeant CA inhibitors

To evaluate the potential role of membrane-bound, luminal CA in base secretion by the anterior intestine, the impermeant CA inhibitor, polyoxyethylene-aminobenzolamide (F3500) (Conroy et al., 1996), was added to the luminal saline at the end of the initial control period. F3500 consists of the potent CA inhibitor aminobenzolamide covalently linked to a high molecular weight compound and is non-permeating by virtue of size; previous work has demonstrated that F3500 is effective as a selective inhibitor of membrane-bound, luminal CA activity in rainbow trout (Georgalis et al., 2006). A 250 mg ml^{-1} stock solution of F3500 was prepared in luminal saline and was neutralized by the addition of 1 mol l^{-1} NaOH prior to use. The luminal half-chamber was spiked with 16 µl of this stock solution, resulting in a final concentration of 2.5 mg ml^{-1} (approximately 0.7×10^{-3} mol l^{-1}), and pH-stat titrations were continued for an additional 60 min. Subsequently, the permeable CA inhibitor etoxzolamide was added to the luminal saline to give a final concentration of 10^{-4} mol l^{-1} to target both extracellular and intracellular CA isoforms. Etoxzolamide was dissolved in DMSO and neutralized with NaOH prior to addition to the luminal chamber at a final concentration of 0.1% DMSO, and pH-stat titration was continued for a final 60 min period.

Enzymatic activity of the pyloric caeca and anterior intestinal tissue

Pyloric caeca and segments of the anterior intestine were obtained by dissection from seawater-acclimated rainbow trout and flash frozen in liquid nitrogen prior to storage at –80°C. Tissue samples were fragmented under liquid nitrogen, and approximately 25 mg of tissue were transferred into 500 µl SEID assay buffer prior to sonication (Kontes rod sonicator, Pittsburg, PA, USA) in two pulses of 10 s each. Adenosine triphosphatase (ATP) activity of the tissue homogenates were determined as outlined by McCormick (McCormick, 1993), in the absence of ouabain and amiloride (control), in the presence of ouabain (1.0 mmol l^{-1}) or amiloride (0.1 mmol l^{-1}) and in the presence of both ouabain (1.0 mmol l^{-1}) and amiloride (0.1 mmol l^{-1}) in combination.

Statistical analysis and data presentation

Values are means ± s.e.m. of at least five observations. Statistical evaluations were performed by paired Student's *t*-tests with Bonferroni's multisample comparison correction (Sokal and Rohlf, 1995) unless otherwise stated. For Ussing chamber/pH-stat experiments, individual time points after treatment were compared with a mean control value obtained from the last 30 min of the initial control period as previously described (Grosell, 2006). The choice of the last 30 min of the control period as the reference control value reflects that many preparations display unstable base secretion and electrophysiological parameters during the initial 30 min of the control period. Values were considered to be statistically significantly different at $P<0.05$.

RESULTS

Both pyloric caeca and anterior intestinal tissue appeared to be viable for at least 180 min based on stable base secretion rates and near constant electrophysiological parameters (Fig. 1). Despite individual variation among pyloric caeca or anterior intestine preparations, the pyloric caeca and anterior intestine displayed significantly different epithelial transport characteristics (Student's *t*-test). The pyloric caeca displayed higher conductance (11.01±0.65 mSi cm^{-2}, N=17) and lower TEP (–4.01±1.20 mV, N=17) values than the anterior intestine (5.27±0.22 mSi cm^{-2} and 8.21±0.80 mV, N=32, respectively) when measured under identical conditions. Overall, HCO_3^- secretion rates under control conditions were not significantly different between isolated pyloric caeca and anterior intestinal segments (0.48±0.07 µmol cm^{-2} h^{-1}, N=17 for pyloric caeca *versus* 0.42±0.03, N=32 for anterior intestine) (Student's *t*-test).

Under the conditions employed in the present study, resting HCO_3^- secretion rates in both pyloric caeca and the anterior intestine were maintained by the hydration of endogenous epithelial CO_2; neither transepithelial HCO_3^- secretion nor serosal CO_2 contributed to luminal alkalization based on the absence of any effect on HCO_3^- secretion of removing serosal HCO_3^-/CO_2 (Fig. 1). However, the removal of serosal HCO_3^-/CO_2 resulted in a trend (not statistically significant) towards increased magnitude of the

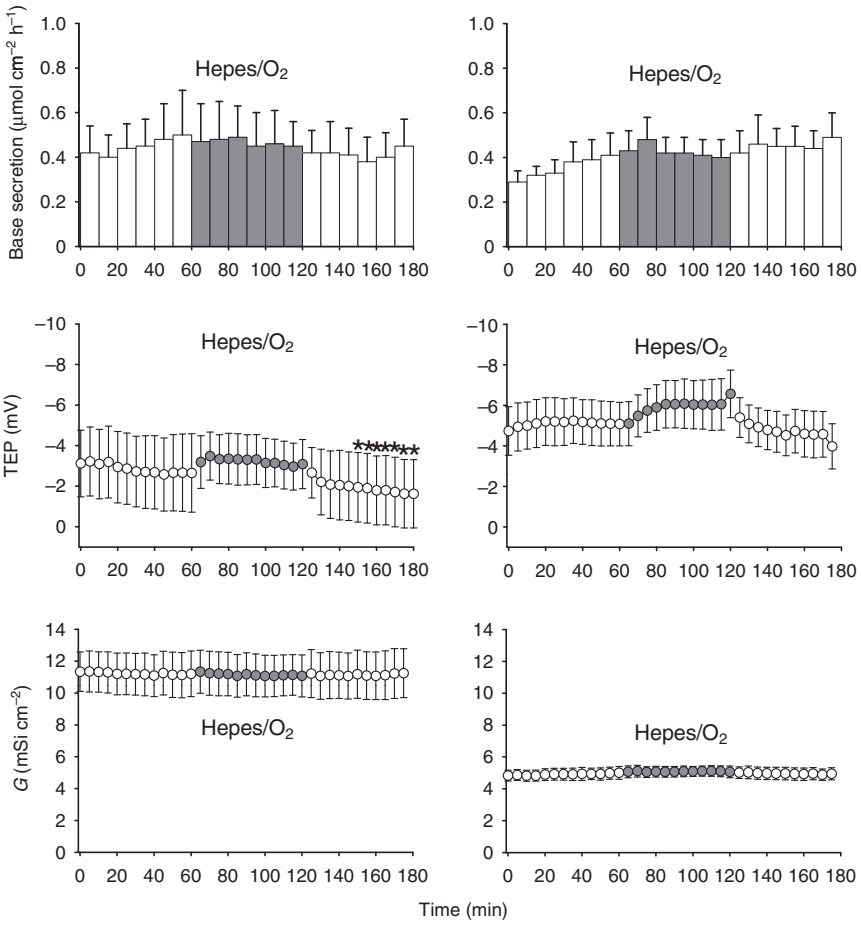

Fig. 1. Base secretion rates (top), transepithelial potential (TEP) (middle) and conductance (bottom) of pyloric caeca (left) and anterior intestine (right) from seawater-acclimated rainbow trout in the presence and absence (shaded bars and symbols) of serosal HCO_3^- and CO_2. Means ± s.e.m., N=5. *Indicates a significant difference from the last 30 min of the initial control period.

serosal side negative TEP in both pyloric caeca and anterior intestine (Fig. 1).

As hypothesized, application of the H^+-pump inhibitor bafilomycin to the luminal saline resulted in a significant (20–30%) increase in net base secretion by the anterior intestinal epithelium (Fig. 2). No significant effect on base secretion was observed for the pyloric caeca or for TEP or conductance in either of the two intestinal segments (Fig. 2). In contrast to expectation, luminal application of amiloride reduced apparent base secretion by the anterior intestinal epithelium (Fig. 3). No statistically significant effect of amiloride addition was observed on pyloric caeca base secretion or on electrophysiological parameters of the pyloric caeca or the anterior intestine (Fig. 3).

As predicted, luminal addition of DIDS significantly reduced luminal HCO_3^- secretion by approximately 40% in the anterior intestine with no effects on electrophysiological parameters (Fig. 4).

Addition of the impermeant CA inhibitor F3500 to selectively inhibit membrane-bound, luminal CA activity resulted in a gradual decrease in HCO_3^- secretion and reduction in the magnitude of the serosal negative TEP (Fig. 5). Subsequent addition of the permeant CA inhibitor etoxzolamide to inhibit CAc as well as membrane-bound, luminal CA further reduced HCO_3^- secretion, as expected, and the gradual reduction of TEP that occurred in the presence of F3500 alone continued after etoxzolamide addition (Fig. 5).

Total ATPase activity was comparable in pyloric caeca and anterior intestine, and ouabain suppressed total ATPase activity, although this effect escaped statistical significance in the pyloric caeca (Fig. 6). Interestingly, the effects of amiloride on total ATPase activity were similar to those of ouabain but the amiloride and ouabain effects were not additive, suggesting that these two drugs inhibited the same fraction of total ATPase activity (Fig. 6).

DISCUSSION

The first measurements of TEP, epithelial conductance/resistance and base secretion rates of isolated pyloric caeca reported in the current study allow comparisons between intestine regions. Our measurements revealed lower electrical gradients and lower resistance in the pyloric caeca than in the anterior intestinal epithelium of rainbow trout in side-by-side measurements and high HCO_3^- secretion rates in both intestinal regions. The high conductance of the pyloric caeca is consistent with the high electrolyte absorption rates proposed for this tissue (see Introduction) and may in part explain the lower TEP as diffusive ion movements could contribute to the depletion of the potential differences. Conductance values for rainbow trout pyloric caeca were higher than those for the anterior intestine of gulf toadfish or European flounder (approximately 10 and 3 mSi cm^{-2}, respectively). However, the rainbow trout anterior intestine displayed a lower TEP (–8 mV) than either the gulf toadfish or European flounder (approximately –20 and –15 mV, respectively) when measured under conditions representative of those *in vivo* (Wilson et al., 2002; Wilson and Grosell, 2003; Grosell et al., 2005; Grosell, 2006). Intestinal HCO_3^- secretion rates observed in the present study were similar to those reported for gulf toadfish at 25°C (approximately 0.45 µmol cm^{-2} h^{-1}) and European flounder at 13°C (approximately 0.35 µmol cm^{-2} h^{-1}), demonstrating that other factors in combination with anion exchange account for the differences in TEP.

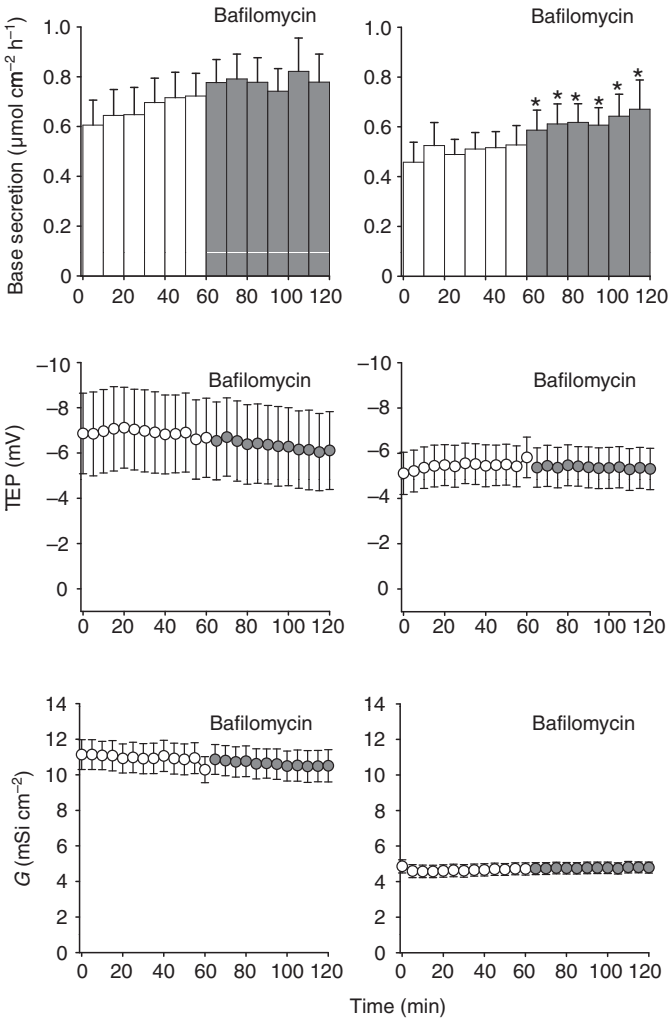

Fig. 2. Base secretion rates (top), transepithelial potential (TEP) (middle) and conductance (bottom) of pyloric caeca (left) and anterior intestine (right) from seawater-acclimated rainbow trout during control conditions and after the luminal addition of the H^+-pump inhibitor, bafilomycin (shaded bars and symbols). Means ± s.e.m., *N*=7 for pyloric caeca and *N*=6 for anterior intestine. *Indicates a statistically significant difference from the mean 30–60 min control secretion rate.

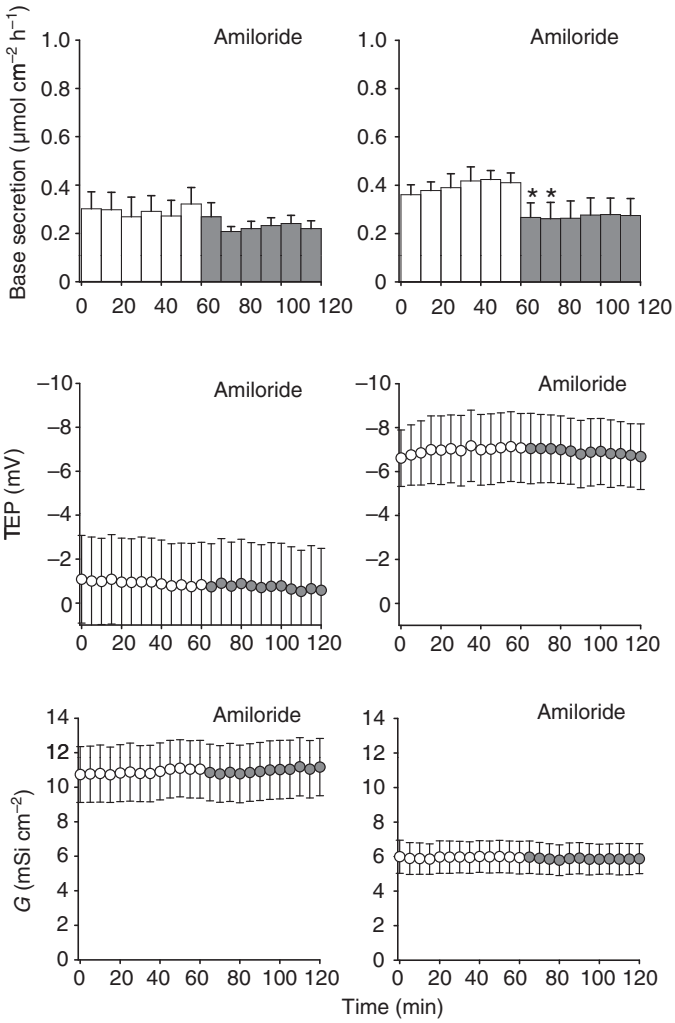

Fig. 3. Base secretion rates (top), transepithelial potential (TEP) (middle) and conductance (bottom) of pyloric caeca (left) and anterior intestine (right) from seawater-acclimated rainbow trout during control conditions and after the luminal addition of the Na^+/H^+ exchanger inhibitor, amiloride (shaded bars and symbols). Means ± s.e.m., *N*=5 for pyloric caeca and *N*=6 for anterior intestine. *Indicates a statistically significant difference from the mean 30–60 min control secretion rate.

The observation of high HCO_3^- secretion rates by pyloric caeca, placed in the context of earlier demonstrations that this intestinal region accounts for approximately 70% of the total intestinal surface area (Buddington and Diamond, 1986), points to a highly significant role played by the pyloric caeca in overall gastrointestinal HCO_3^- secretion as well as Cl^- and thus water absorption. By implication, salmonids and other species with pyloric caeca may have the capacity for higher intestinal salt and water absorption rates than those species lacking pyloric caeca. In agreement with this suggestion are rates of intestinal Cl^- absorption of 2.8 and 1.5 mmol kg^{-1} h^{-1} reported for seawater-acclimated rainbow trout (Shehadeh and Gordon, 1969; Wilson et al., 1996) *versus* only 1.0 mmol kg^{-1} h^{-1} for the gulf toadfish at a considerably higher temperature (Genz et al., 2008) or 0.4 mmol kg^{-1} h^{-1} for lemon sole (Grosell et al., 1999), both of which are species without pyloric caeca.

HCO_3^- secretion continued in both pyloric caeca and anterior intestine in the absence of serosal molecular CO_2 and HCO_3^-, demonstrating that endogenous epithelial CO_2 is sufficient to fuel resting HCO_3^- secretion rates in seawater-acclimated rainbow trout. By contrast, only 30–60% of luminal HCO_3^- secretion can be accounted for by endogenous CO_2 in European flounder and gulf toadfish. When metabolic CO_2 is the only source for HCO_3^- secretion, the relative metabolic rate of the intestinal tissue can be estimated from HCO_3^- secretion rates, assuming complete conversion of CO_2 to HCO_3^- and fully polarized apical HCO_3^- and basolateral H^+ secretion. Under such conditions, rainbow trout displayed HCO_3^- secretion rates of 0.48 and 0.42 µmol cm^{-2} h^{-1} for pyloric caeca and anterior intestine, respectively, values considerably higher than corresponding rates for the anterior intestine of gulf toadfish (~0.2 µmol cm^{-2} h^{-1} at 25°C) (Grosell, 2006) or European flounder (~0.3 µmol cm^{-2} h^{-1} at 13°C) (Wilson et al., 2002; Wilson and Grosell, 2003). Thus, the metabolic rate of trout intestinal epithelium appears to be higher than those of European flounder or gulf toadfish.

The findings of the present study provide direct support for the hypothesis that apical H^+ extrusion into the intestinal lumen contributes

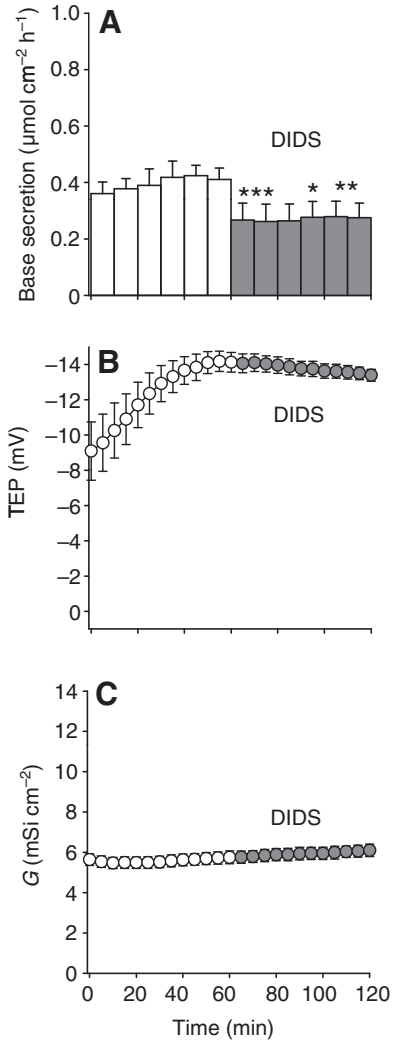

Fig. 4. Base secretion rates (A), transepithelial potential (TEP) (B) and conductance (C) of anterior intestine from seawater-acclimated rainbow trout during control conditions and after the luminal addition of the Cl^-/HCO_3^- exchanger inhibitor, 4,4′-diisothiocyanostilbene-2,2′disulphonic acid (DIDS) (shaded bars and symbols). Means ± s.e.m., *N*=8. *Indicates a statistically significant difference from the mean 30–60 min control secretion rate.

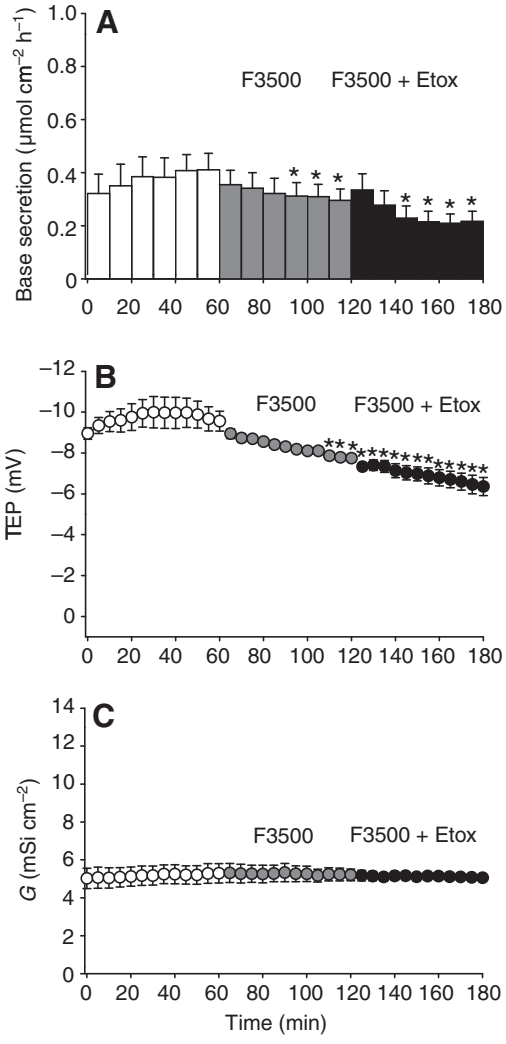

Fig. 5. Base secretion rates (A), transepithelial potential (TEP) (B) and conductance (C) of anterior intestine from seawater-acclimated rainbow trout during control conditions and following luminal exposure to the impermeant CA inhibitor F3500 (gray bars and symbols) and a combination of F3500 and the permeant CA inhibitor etoxzolamide (F3500 + Etox) (black bars and symbols). Means ± s.e.m., *N*=7. *Indicates a statistically significant difference from the mean 30–60 min control secretion rate or TEP values.

to seawater acclimation (at least at the level of the anterior intestine). Intestinal base secretion was elevated in the presence of the H^+-pump inhibitor bafilomycin, implying that simultaneous H^+ and HCO_3^- secretion across the apical membrane under control conditions masks part of the overall HCO_3^- secretion (Fig. 7). The prevention of cellular acidification and thus the protection of cellular substrate (HCO_3^-) for anion exchange results from H^+-pump activity. However, an additional functional significance of the apical H^+-pump localization, probably lies in its capacity to reduce luminal osmotic pressure as two osmolytes (H^+ and HCO_3^-) combine to form osmotically inert molecular CO_2 in a reaction catalyzed by membrane-bound, luminal CA (see below). This reduction of luminal osmotic pressure, in turn, would benefit water absorption (Grosell et al., 2007). Moreover, the lower luminal HCO_3^- concentrations resulting from titration of secreted HCO_3^- by simultaneously secreted H^+ may also provide more favorable gradients for continued HCO_3^- secretion and hence Cl^- absorption *via* anion exchange (Fig. 7). Assuming a luminal concentration of 100 mmol l^{-1} HCO_3^- (Wilson et al., 1996), a 20–30% reduction in HCO_3^- accumulation arising from apical H^+ extrusion would result in a reduction of luminal osmotic pressure of 20–30 mosmol. This value is lower than the estimated effect of $CaCO_3$ precipitation in the order of 70 mosmol (Wilson et al., 2002) but is nevertheless significant.

Demonstration of reduced Cl^- uptake in the presence of a H^+-pump inhibitor can be challenging owing to the presence of several parallel Cl^--uptake pathways in the marine teleost intestine (Marshall and Grosell, 2005). This challenge was overcome in the present study by measuring HCO_3^- secretion as an index of anion exchange and thus Cl^- absorption *via* this pathway. Similar involvement of proton pump in Cl^- uptake *via* anion exchange was previously demonstrated for the freshwater fish gill and amphibian skin (Fenwick et al., 1999; Jensen et al., 2002; Boisen et al., 2003; Jensen et al., 2003).

The relative increase in apparent base secretion in the presence of bafilomycin suggests that approximately 20–30% of the HCO_3^-

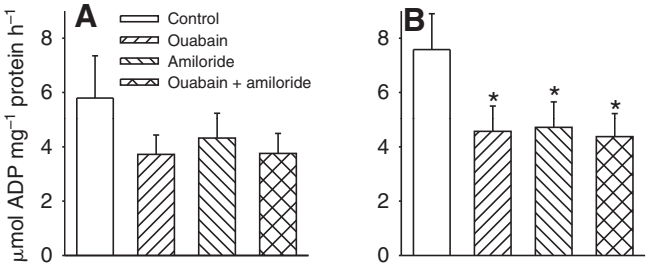

Fig. 6. ATPase activity of pyloric caeca (A) and anterior intestinal (B) mucosa in the absence or presence of ouabain and amiloride or ouabain and amiloride in combination. Means ± s.e.m., *N*=6. *Indicates a statistically significant difference from the inhibitor-free control value. There were no significant differences between ouabain and amiloride applied separately or in combination.

secretion is titrated by simultaneous H^+ secretion in the anterior intestine (Fig. 2). However, this may represent an underestimation as inhibition of the apical H^+ pump will result in reduced cellular HCO_3^- availability and potentially elevated luminal HCO_3^- concentrations in the vicinity of the apical membrane. A similar argument applies for the lack of statistically significant effects of bafilomycin on the pyloric caeca. Interestingly, the lack of significant effect of bafilomycin treatment on HCO_3^- secretion by pyloric caeca suggests differences in HCO_3^- and H^+ transport characteristics between the pyloric caeca and the anterior intestine of seawater-acclimated rainbow trout.

The apical extrusion of H^+ resulting in reduced accumulation of HCO_3^- in the intestinal lumen might seem to counteract the effectiveness of the well-documented $CaCO_3$ precipitate formation known to occur in the intestinal lumen of marine teleosts (Wilson et al., 2002). $CaCO_3$ precipitate formation results in a substantial reduction in osmotic pressure and thus facilitates water absorption while at the same time reduces luminal Ca^{2+} concentration and thereby Ca^{2+} uptake in a hypercalcemic environment (Wilson et al., 2002). However, there is no conflict between $CaCO_3$ precipitation and apical H^+ secretion; Wilson and colleagues measured intestinal fluid pH and total CO_2 concentrations in seawater-acclimated rainbow trout and estimated the CO_3^{2-} concentrations to range from >10 mmol l^{-1} in fluids from pyloric ceaca to >30 mmol l^{-1} in rectal fluids (Wilson et al., 1996). The study by Wilson and colleagues (Wilson et al., 1996) did not report intestinal fluid Ca^{2+} concentrations but in general marine teleost intestinal fluid Ca^{2+} concentrations are approximately 5 mmol l^{-1} (Grosell et al., 2001). Bulk intestinal fluids (all segments combined) collected from the trout used in the present study displayed total CO_2 concentrations of 84.1±5.0 mmol l^{-1} and pH of 8.3±0.1 as well as Ca^{2+} concentrations of 3.8±0.3 mmol l^{-1} (data not shown). An estimate of CO_3^{2-} concentrations in these fluids can be obtained as HCO_3^-=total $CO_2/(10^{(pH-pKii)}+1)$, assuming that total CO_2 is equal to $HCO_3^-+CO_3^{2-}$ (ignoring a small contribution from molecular CO_2) and a pKii of 9.46 (Wilson et al., 2002). The estimated CO_3^{2-} concentration of 5.4 mmol l^{-1} suggests that Ca^{2+} (3.8 mmol l^{-1}) rather than CO_3^{2-} is limiting for $CaCO_3$ formation. It thus appears that the combined action of apical H^+ secretion and membrane-bound, luminal CA-facilitated HCO_3^- dehydration acts to reduce luminal osmotic pressure and favor continued anion exchange without limiting the formation of $CaCO_3$ in luminal fluids.

In contrast to expectations, luminal amiloride reduced rather than increased apparent base secretion by the anterior intestine (Fig. 3). The prediction of increased apparent base secretion was based on the greatly elevated mRNA expression of the apical NHE3 isoform in rainbow trout intestinal segments following transfer from freshwater to 65% SW (Grosell et al., 2007). The lack of increased luminal net base secretion in the presence of amiloride does not exclude the possibility that an apical NHE3 isoform is active in the pyloric caeca and anterior intestine, as certain NHE isoforms and splice variants appear to be insensitive to amiloride in some species (Zerbini et al., 2003; Attaphitaya et al., 2005). However, the reduction in apparent base secretion in the presence of amiloride implies a basolateral action of amiloride, possibly by crossing the apical membrane or the tight junctions. NHE-like proteins in the basolateral membrane are likely to be involved in H^+ extrusion and pH_i regulation and may be important for apical HCO_3^- secretion. For example, in the gulf toadfish, inhibition of basolateral Na^+/H^+ exchange impairs apical HCO_3^- secretion (Grosell, 2006). An additional possible target for basolateral amiloride inhibition is the NKA, for which amiloride sensitivity has been demonstrated (Kleyman and Cragoe, 1990; Ellis-Davies et al., 1996; Kleyman and Cragoe, 1998). Inhibition of NKA by amiloride in the trout intestine could explain reduced apical HCO_3^- secretion because it would act to deplete the cellular Na^+ gradients that drive even amiloride-insensitive Na^+/H^+ exchange across the basolateral membrane. Notably, reduced apical HCO_3^- secretion rates have been reported for toadfish intestinal epithelia in the presence of serosal ouabain, a NKA inhibitor (Grosell, 2006). In support of the suggested NKA inhibition by amiloride were the observations of reduced NKA activity in epithelial homogenates in the presence of amiloride and/or ouabain (Fig. 6), which strongly suggest that the ouabain-sensitive fraction of total ATPase activity, which is normally ascribed to NKA activity, is also sensitive to amiloride.

Anterior intestinal HCO_3^- secretion was sensitive to DIDS, as expected, a finding that is in agreement with earlier reports (Ando and Subramanyam, 1990; Grosell and Jensen, 1999; Grosell et al., 2001). Again, the DIDS effect on isolated epithelia differed from that of the gut perfusion experiment performed on seawater-acclimated rainbow trout, in which no sensitivity to DIDS was observed (Wilson et al., 1996). The apparent lack of sensitivity to DIDS in the gut perfusion experiments performed by Wilson and colleagues may be due to compensatory responses activated in the intact fish, as discussed above for amiloride. An additional possible explanation is the high alkalinity of luminal saline resulting from intestinal HCO_3^- secretion. We noticed in the present study that alkaline DIDS stock solutions were ineffective in inhibiting HCO_3^- secretion by isolated anterior intestinal segments (data not shown). Common to all experiments reporting DIDS effects on isolated intestinal tissue is the fact that DIDS was added to luminal saline of near neutral pH with low HCO_3^- concentrations, perhaps explaining the uniform DIDS response in these experiments.

The present study provides support for the proposed role of membrane-bound, luminal CA isoform(s) in intestinal HCO_3^- secretion (Fig. 7). The involvement of membrane-bound, luminal CA was suggested by observations of elevated CA IV mRNA expression and elevated enzymatic activity of phosphatidylinositol-specific phospholipase C (PI-PLC)-sensitive CA in rainbow trout intestine epithelial homogenates following transfer from freshwater to 65% SW (Grosell et al., 2007). The membrane-impermeant CA inhibitor, F3500 has been employed previously to target extracellular CA activity (Georgalis et al., 2006; Conroy et al., 1996; Maren et al., 1997) but does not distinguish among the multiple membrane-bound, luminal CA isoforms that may be present in the apical membrane of polarized cells (e.g. isoforms IV, XIV and

possibly XV, if expressed in fish) (Hilvo et al., 2005; Purkerson and Schwartz, 2007; Hilvo et al., 2008). However, considering the evidence available for the presence of a CA IV isoform in trout intestine (Grosell et al., 2007), it is highly likely that at least part of the F3500 effect detected in the present study is attributable to CA IV. Regardless of the specific CA isoform(s) present in the apical membrane of the anterior intestinal epithelium of seawater-acclimated trout, it is clear that apical, luminal CA activity is important for HCO_3^- secretion as illustrated by the inhibition of HCO_3^- secretion in presence of F3500. We propose that one or more membrane-bound, luminal CA isoforms catalyzes the dehydration of secreted HCO_3^- to CO_2, thereby reducing the HCO_3^- concentration near the apical membrane to enhance apical anion exchange (Fig. 7). The reduced net base secretion after the addition of F3500 is consistent with this role for CAIV (and other membrane-associated, luminal CA isoforms) as it would result in accumulation of HCO_3^- in the unstirred boundary layer at the epithelial surface opposing further HCO_3^- secretion. Furthermore, in the case of pH-stat titration experiments in which luminal HCO_3^- is continuously titrated, membrane-bound, luminal CA activity would also result in an apparently higher base secretion rate simply because the hydration would consume H^+ from the luminal solutions, which in pH-stat titrations would mask as base secretion.

Although the dehydration of HCO_3^- by membrane-bound CA might result in a reduction of luminal HCO_3^- concentrations, the consumption of protons in the CA-mediated HCO_3^- dehydration would result in the facilitated conversion of excess HCO_3^- to CO_3^{2-}, which in turn would favor the formation of $CaCO_3$ precipitates in the intestinal lumen (Fig. 7). Thus, membrane-bound, luminal CA may act to promote $CaCO_3$ formation by elevating luminal pH and to reduce boundary layer HCO_3^- concentrations thereby promoting further anion exchange and thus Cl^- uptake across the apical membrane. Both the enhanced precipitation reaction and the more favorable conditions for Cl^- absorption would act to promote water absorption.

The simultaneous addition of F3500 and etoxzolamide resulted in a further reduction of HCO_3^- secretion, pointing to the role of CAc in catalyzing the cellular hydration of metabolic CO_2 that fuels apical HCO_3^- secretion. These observations are in agreement with reports from several marine species (Dixon and Loretz, 1986; Wilson et al., 1996; Grosell et al., 2005; Grosell, 2006) and to date the significance of CAc for this intestinal transport process appears to be universal among marine teleosts. Although the underlying mechanisms remain to be elucidated, CAc appears to be compartmentalized within the enterocytes to the apical region (Grosell et al., 2007), suggesting that a primary function of this abundant enzyme in the marine teleost intestine is to serve apical anion exchange.

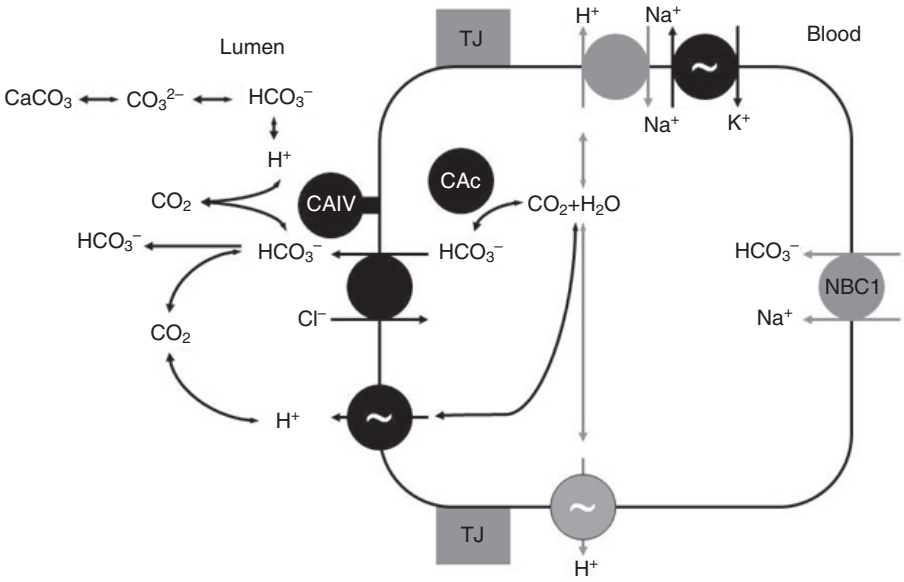

Fig. 7. A transport model illustrating the hypothesized epithelial processes involved in HCO_3^- secretion, $CaCO_3$ precipitate formation, control of luminal osmotic pressure and CO_2 hydration in seawater-acclimated rainbow trout intestine. For completeness, transporters and enzymes beyond those addressed in the present study have been included in the model but are indicated in gray when direct experimental evidence for seawater-acclimated rainbow trout is missing. The depicted transport pathways occur in parallel with sodium co-transport pathways (not shown) [see Marshall and Grosell (Marshall and Grosell, 2005) for an overview]. Basolateral H^+ extrusion, the dependence of this process on serosal Na^+, basolateral H^+-pump localization, lateral Na^+/K^+-ATPase (NKA) localization and the possible involvement of basolateral $Na:HCO_3^-$ co-transporter (NBC) have been demonstrated in previous reports (Grosell, 2006; Grosell et al., 2007; Kurita et al., 2008). Membrane-associated carbonic anhydrase (CA) isoforms in addition to CA IV cannot be excluded (see text for details). The cellular substrate for apical anion exchange in rainbow trout is provided mainly from the hydration of CO_2 produced by the epithelial cell, a reaction catalyzed by cytosolic carbonic anhydrase (CAc). In other species, a significant fraction of cellular HCO_3^- stems from uptake across the basolateral membrane *via* the $Na:HCO_3^-$ co-transporter (NBC1) which is fueled by NKA. After secretion, HCO_3^- is accumulated in the lumen to high concentrations. The high luminal pH and high total CO_2 concentrations result in high levels of CO_3^{2-}, which precipitates as $CaCO_3$. The activity of membrane-bound, luminal CA (CA IV) acts to increase luminal pH and thus the amount of CO_3^{2-} by the consumption of protons to dehydrate a fraction of the HCO_3^- secreted by the apical anion exchange process. Luminal CO_3^{2-} concentrations exceed Ca^{2+} concentrations and are not limiting for precipitate formation (see text for details). Apical H^+ secretion results in the titration of a fraction of the HCO_3^- near the apical membrane and thus reduces the HCO_3^- gradient from the cytosol to the lumen, favoring continued Cl^- uptake *via* anion exchange. Note that Cl^-, once inside the epithelial cell, is above its electrochemical equilibrium and exits basolaterally through Cl^- channels (Loretz, 1995) (not illustrated).

Conclusions

In summary, the first direct measurements of HCO_3^- secretion and electrophysiological properties of pyloric caeca confirm the proposed role of this intestinal region in osmoregulation. We suggest that the majority of HCO_3^- secretion and possibly water absorption by the gastrointestinal tract in seawater-acclimated salmonids occurs in this intestinal segment due to its high conductance and large surface area. Furthermore, we predict that species possessing pyloric caeca will have a higher capacity for intestinal salt absorption and therefore a higher capacity for water absorption than species without pyloric caeca. In addition, we present the first functional evidence for an apical H^+ pump operating in concert with apical anion exchange to facilitate HCO_3^- secretion and Cl^- and thus water absorption, and thereby establish the importance of the H^+ pump for osmoregulation in marine fish. Finally, we demonstrate a role for membrane-bound, luminal CA in the alkalization of the intestinal lumen. Future studies should

examine the effects of H^+ pump and membrane-bound, luminal CA inhibition on water absorption.

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