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Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation

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Grosell, M., and J. Genz. Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *Am J Physiol Regul Integr Comp Physiol* 291: R1145–R1156, 2006. First published May 18, 2006; doi:10.1152/ajpregu.00818.2005.—The gulf toadfish (*Opsanus beta*) intestine secretes base mainly in the form of HCO_3^- via apical anion exchange to serve Cl^- and water absorption for osmoregulatory purposes. Luminal HCO_3^- secretion rates measured by pH-stat techniques in Ussing chambers rely on oxidative energy metabolism and are highly temperature sensitive. At 25°C under in vivo-like conditions, secretion rates averaged 0.45 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, of which 0.25 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ can be accounted for by hydration of endogenous CO_2 partly catalyzed by carbonic anhydrase. Complete polarity of secretion of HCO_3^- and H^+ arising from the CO_2 hydration reaction is evident from equal rates of luminal HCO_3^- secretion via anion exchange and basolateral H^+ extrusion. When basolateral H^+ extrusion is partly inhibited by reduction of serosal pH, luminal HCO_3^- secretion is reduced. Basolateral H^+ secretion occurs in exchange for Na^+ via an ethylisopropylamiloride-insensitive mechanism and is ultimately fueled by the activity of the basolateral Na^+-K^+-ATPase. Fluid absorption by the toadfish intestine to oppose diffusive water loss to the concentrated marine environment is accompanied by a substantial basolateral H^+ extrusion, intimately linking osmoregulation and acid-base balance.

bicarbonate transport; chloride absorption; epithelial water transport; seawater ingestion; pH-stat titrations

OSMOREGULATING MARINE FISH maintain extracellular fluid osmolality at 300–350 mosmol/kg and, as a consequence, experience continuous diffusive water loss to the hypertonic surrounding seawater (~1,000 mosmol/kg). To withstand this osmoregulatory challenge, fish drink seawater in amounts equivalent to ~5% of their body mass per day (25). The ingested fluid is modified along the gastrointestinal tract, and the intestine plays a key role in marine fish osmoregulation by absorbing NaCl and water (25). After desalinization of the imbibed seawater in the water-impermeable esophagus (18, 30), fluid just slightly hyperosmotic to the extracellular compartment enters the intestine (26). Fluid absorption across the intestinal epithelium is driven by the active transport of Na^+ and Cl^- and occurs without or even against net osmotic gradients (15, 24, 26, 32, 42). Uptake of Na^+ and Cl^- from the intestinal lumen across the apical membrane of the intestinal epithelium is mediated in part by two parallel cotransport systems: Na^+-Cl^- and Na^+-K^+-$2Cl^-$ (9, 10, 16, 28), both of which rely on the electrochemical Na^+ gradient established by basolateral Na^+-K^+-ATPase (42).

However, in addition to these cotransport systems, apical anion exchangers have recently been associated with Cl^- and water absorption across the marine teleost intestine (15) and appear to contribute significantly to overall Cl^- absorption. Evidence for intestinal anion-exchange activity, indicated by highly alkaline intestinal fluids with high concentrations of total CO_2 or large cation-anion gaps when total CO_2 was not measured, is abundant in the literature. The earliest report of high total CO_2 in intestinal fluids dates back three-quarters of a century (33), and observations now include a large number of species, including elasmobranchs and sturgeon (14, 31, 39, 43, 46), suggesting that alkaline luminal fluids are common to fish inhabiting marine environments. Experiments on isolated intestinal preparations demonstrate that the source of luminal total CO_2 is HCO_3^- secretion, which may occur along the entire length of the intestine (13–15, 46). A recent study revealed that the intestinal epithelium performs secondary active secretion of HCO_3^-, which accounts for the high luminal total CO_2 concentrations while providing for the active absorption of Cl^- via apical anion exchange in seawater-acclimated European flounder (15). A prediction of strong temperature dependence of this secondary active transport system in the gulf toadfish (*Opsanus beta*) was tested in the present study by direct measurement of HCO_3^- secretion using a pH-stat approach at 15, 25, and 35°C. Furthermore, whether aerobic energy metabolism and, thus, cellular ATP are required for the putative active HCO_3^- transport was examined.

Studies on the source of HCO_3^- secreted by the intestinal epithelium range from demonstrating that extracellular HCO_3^- transported across the intestinal epithelium provides the majority of HCO_3^- secreted into the intestinal lumen (1) to showing that endogenous epithelial CO_2 constitutes the main source of luminal HCO_3^- (15, 45). Therefore, one goal of the present study was to determine the contribution of endogenous metabolic CO_2 and extracellular HCO_3^- to the overall apical HCO_3^- secretion by the intestine of a marine teleost, the gulf toadfish. To the extent that endogenous CO_2 provides for apical HCO_3^- secretion, cytosolic CO_2 hydration must occur. This prompted examination of the potential involvement of carbonic anhydrase (CA) in intestinal HCO_3^- secretion. Our findings demonstrate that hydration of endogenous CO_2 provides for apical secretion of HCO_3^-, which led to investigations of the fate of H^+ from CO_2 hydration. The prediction was that H^+ from CO_2 hydration would be eliminated from the epithelial cells to maintain cellular pH and to prevent reversal of the hydration reaction. Furthermore, because the epithelium exhibits net base secretion, it was predicted that this H^+ extrusion

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would occur across the basolateral membrane. The possibility that basolateral H^+ extrusion by the intestinal epithelium of the gulf toadfish is mediated by Na^+/H^+ exchange (NHE), as in the rat pancreatic ducts (38), was examined by characterization of the dependence of apical HCO_3^- secretion on serosal Na^+ and by pharmacological manipulations of Na^+ gradients. Finally, the high apical HCO_3^- secretion rates and the resulting need to eliminate H^+ produced from the CO_2 hydration reaction prompted direct measurements of basolateral H^+ extrusion. The osmoregulatory role of the intestinal epithelium of marine teleost fish is salt and water absorption to maintain water balance, whereas the gill extrudes salt from the intestine (25). Thus the water absorption across the intestinal epithelium is obligately associated with the need for branchial salt secretion. Our observations of high basolateral H^+ secretion rates in the water-absorbing intestinal epithelium prompted measurement of net water transport by isolated intestinal epithelia, which ultimately allowed for calculation of the pH in the absorbed fluids. These measurements revealed that intestinal fluid absorption in marine teleosts is associated with a significant acid load in addition to the unavoidable salt gain.

MATERIALS AND METHODS

Experimental animals. Gulf toadfish (*Opsanus beta*) from Biscayne Bay, FL, were obtained from shrimp fishermen during spring and early summer 2005 and transferred to holding facilities at the University of Miami Rosenstiel School of Marine and Atmospheric Sciences. Procedures were approved by our Institutional Animal Care and Use Committee. On arrival, the fish received a prophylactic treatment for ectoparasites, as previously described (27). The fish were provided short lengths of polyvinylchloride tubing as shelters in 80-liter glass aquaria that received a continuous flow of filtered seawater (salinity 27–30 ppt, 22–26°C, from Bear Cut, FL). Fish were fed frozen squid twice weekly, but food was withheld 48 h before experimentation.

pH-stat experiments: general experimental protocol. Segments of the anterior intestine of toadfish (25–40 g body wt) were obtained by dissection, cut open, and placed in tissue holders exposing 0.7-cm^2 surface area with the serosal muscle layer intact. The true exposed surface area of the intestinal epithelium clearly exceed 0.7 cm^2 because of the presence of villi and microvilli, but all transport rates are reported as a function of this known gross surface area. The intact intestinal epithelium was subsequently mounted in Ussing chambers (model P2300; Physiological Instruments) containing 1.6 ml of appropriate, pregassed saline in each half-chamber (Table 1). Mixing was achieved by gassing with O_2 through airlifts unless otherwise stated. Pregassing of the luminal saline and continued gassing with CO_2-free gas in the luminal half-chamber ensured stable titration curves throughout the experiments. The Ussing chambers were mounted in chamber holders connected via a pump to a thermostatic water bath maintained at 25°C unless otherwise stated. Current and voltage electrodes connected to amplifiers (model VCC600; Physiological Instruments) recorded the transepithelial potential (TEP) differences under current-clamp conditions at 0 μA. At 60-s intervals, 3-s 50-μA pulses were passed from the mucosal to the serosal side. The TEP measurements were logged on a personal computer using BIOPAC systems interface hardware and Acqknowledge software (version 3.8.1). TEP values are reported with a luminal reference of 0 mV. The luminal half-chamber (unless otherwise stated) was fitted with a combination pH electrode (model PHC4000.8; Radiometer) and a microburette tip, both of which were connected to a pH-stat titration system (model TIM 854 or 856; Radiometer). The pH-stat titration system was grounded to the amplifier to allow for pH readings during current pulsing. The pH-stat titrations were performed on luminal saline solutions at pH 7.800 throughout all experiments, with pH values and rate of acid addition logged on personal computers using Titramaster software (versions 1.3 and 2.1). Luminal pH was generally maintained within ±0.003 pH unit around the set point throughout the experiments. Base secretion rates were calculated from the rate of addition and concentration of titrant (0.0005 N HCl). Common to all experiments was an initial control period of ~60 min with stable TEP and base secretion rates before the manipulations outlined below.

Nature of titrated base secretion. To test the assumption that measured base secretion consisted of HCO_3^-, a separate set of experiments were performed. The luminal chamber was mixed using a peristaltic pump circulating a total of 3 ml of mucosal saline through the mucosal half-chamber with no gassing while the serosal chamber,

Table 1. *Composition of serosal and mucosal solutions*

	Serosal				
	HCO_3^- saline	HCO_3^--free saline	Low Na^+	Buffer-free saline	Mucosal Saline
NaCl, mM	151.00	151.00	0.00	151.00	69.00
N-methyl-D-glucamine, mM			151.00		
KCl, mM	3.00	3.00	3.00	3.00	5.00
$MgCl_2$, mM					22.50
$MgSO_4$, mM	0.88	0.88	0.88	0.88	77.50
Na_2HPO_4, mM	0.50	0.50	0.50	0.50	
KH_2PO_4, mM	0.50	0.50	0.50	0.50	
$CaCl_2$, mM	1.00	1.00	1.00	1.00	5.00
$NaHCO_3$, mM	5.00		5.00		
HEPES, mM					
Free acid	11.00	11.00	11.00		
Sodium salt	11.00	11.00	11.00		
Urea, mM	4.50	4.50	4.50	4.50	
Glucose, mM	5.00	5.00	5.00	5.00	
Osmolality,* mosmol/l	~ 330	~ 330	~ 330	~ 330	
pH	7.80	6.60, 7.00, 7.40 or 7.80‡	7.80	7.80†	7.80†
Gas	0.3% CO_2 in O_2	O_2	0.3% CO_2 in O_2	O_2	O_2

Composition of mucosal saline is based on measured composition of intestinal fluids obtained from unfed toadfish (39). *Adjusted with mannitol to ensure transepithelial isomotic conditions in all experiments. †pH 7.800 was maintained by pH-stat titration. ‡pH was adjusted with HCl under continuous O_2 or N_2 gassing.

containing HCO_3^--free saline, was gassed with O_2. In this preparation, O_2 supply on the serosal side alone is sufficient to maintain normal base secretion rates and electrophysiological parameters (7). Secreted base was allowed to accumulate in the mucosal saline for 3 h without pH-stat titration, then total HCO_3^- and CO_3^{2-} concentrations in the luminal saline were determined using double end-point titrations, which determine the titratable alkalinity of the saline. This method is described in detail elsewhere (12). Briefly, the sample was gassed with CO_2-free gas for 30 min to remove gaseous CO_2 from the solution; pH was titrated to 3.800 with HCl using Gilson microburettes, and the volume of HCl was recorded. At this low pH, all HCO_3^- and CO_3^{2-} will convert to gaseous CO_2, which was removed from the sample by continuous gassing for ≥15 min. Thereafter, pH was titrated back to the initial value during continuous gassing and careful recording of the amount of NaOH added by Gilson microburettes. From the volumes of HCl and NaOH used and the normality of these titrants, the titratable alkalinity (HCO_3^- equivalents) was determined from the difference in amounts of H^+ needed to bring the solution to pH 3.800 and the amount of OH^- needed to return the solution to the starting pH after HCO_3^- and CO_3^{2-} were removed as gaseous CO_2. The rate of excretion of HCO_3^- equivalents by the epithelium in these experiments was calculated from the total HCO_3^- equivalents in the mucosal saline at the end of the 3-h experimental period, the epithelial surface area, and the elapsed time. Because these experiments revealed that the majority of base secreted was HCO_3^-, base secretion will be referred to as HCO_3^- secretion.

Longevity of toadfish intestinal epithelium. Two series of control experiments were performed to assess the temporal performance of the preparation, with gut saline on the luminal side and HCO_3^-/CO_2 saline on the serosal side of the epithelium. In the first series of control experiments, three subsequent 60-min flux periods were separated by a change of the serosal saline. In the second series of control experiments, an initial 60-min control period was followed by two 120-min flux periods. The initial 60-min flux period and the two 120-min flux periods were again separated by serosal saline changes. The serosal saline in the first of the two 120-min flux periods contained 0.1% DMSO, which served as a vehicle control for some of the pharmacology experiments (see below).

Is aerobic energy metabolism required for HCO_3^- secretion? The intestinal HCO_3^- secretion in marine teleosts appears to be of a secondary active nature (15) and can, therefore, be expected to rely on aerobic energy metabolism and, thus, cellular ATP. Cellular ATP levels were suppressed by N_2 gassing of mucosal and serosal saline solutions. Also, for these experiments, the serosal HCO_3^--free saline was replaced after 60 and 120 min with saline preequilibrated for >90 min with N_2 or O_2, respectively. From 60 to 120 min, gassing of mucosal saline solutions was changed from O_2 to N_2.

Temperature dependence of HCO_3^- secretion. A strong temperature dependence of luminal HCO_3^- secretion was hypothesized to be due to the secondary active transport characteristics of luminal alkalinization. To test this hypothesis, measurements were performed after an initial 60-min control period at 25°C and for 120 min at 15 or 35°C, then the temperature was returned to 25°C for an additional 120-min recovery period. Exposure temperatures were verified by measurements of saline temperature in the Ussing chambers, which revealed that up to 30 min were required for a complete change in temperature from 25°C to 15 or 35°C.

Determining the source of luminal HCO_3^- secretion. To assess whether secreted HCO_3^- is derived from endogenous epithelial CO_2 or is dependent on serosal CO_2 and/or HCO_3^-, experiments were performed to compare secretion rates obtained with serosal HEPES-saline gassed with O_2 with rates obtained with serosal saline containing 5 mM HCO_3^- and gassed with 0.3% CO_2 in O_2. In these experiments, an initial 60-min control period with a HEPES-O_2 serosal saline solution was followed by a 60-min period with the above-mentioned serosal HCO_3^-/CO_2-containing saline solution before a final 60-min control period with HEPES-O_2 serosal saline solution. The HCO_3^- concentrations and CO_2 levels resulted in pH and CO_2 partial pressure typical of the extracellular fluids of toadfish.

Does CA mediate CO_2 hydration? Having established that hydration of endogenous CO_2 is the main source of luminal HCO_3^- secretion, the potential involvement of CA was examined by application of the lipophilic CA inhibitor etoxzolamide (10^{-3} M in 0.1% DMSO) to the luminal saline. The CA inhibitor was added after a 60-min control period, and measurements were continued for 120 min thereafter. These experiments were performed with HEPES-O_2-containing serosal saline.

Involvement of serosal H^+ secretion. To test the hypothesis that serosal H^+ export is important for luminal HCO_3^- secretion, luminal HCO_3^- secretion was measured during manipulation of serosal pH and, thereby, H^+ gradients across the basolateral membrane, with the prediction that reduced serosal pH would inhibit luminal HCO_3^- secretion. In these experiments, which were performed with HEPES-O_2-containing serosal saline, an initial 60-min control period at serosal pH 7.800 was followed by a 60-min period with reduced serosal pH and, finally, a 60-min recovery period at serosal pH 7.800. A total of three series were performed on three sets of preparations with serosal pH reduced to 7.4, 7.0, and 6.6.

Rate of basolateral H^+ extrusion. Considering that CO_2 hydration within the intestinal epithelium provides the main source for luminal HCO_3^- secretion and that basolateral proton extrusion is critical for normal rates of apical HCO_3^- secretion, we tested the prediction that basolateral H^+ secretion rates across the basolateral membrane would be high. In these experiments, pH-stat titrations were performed with the electrode and burette tip in the serosal, rather than the mucosal, half-chamber, and 0.0005 N NaOH, rather than HCl, was employed as the titrant. The serosal saline for these experiments contained neither HEPES nor HCO_3^- (but was osmotically compensated with mannitol; Table 1) and was gassed with O_2. These titrations were also, in this case, performed with a titration set point of pH 7.800 (normal teleost fish blood plasma pH). Experiments were continued until stable titration and electrophysiological values were obtained for ≥30 min.

Na^+ dependence of serosal H^+ secretion: involvement of NHE. The possibility that secretion of H^+ across the basolateral membrane occurred via an Na^+-H^+ antiport was examined by measuring luminal HCO_3^- secretion under conditions with no serosal HCO_3^- and reduced serosal Na^+. In these experiments, an initial 60-min control period was followed by a 60-min period in which the serosal saline Na^+ concentration was reduced to 11 mM and a final 60-min recovery period under control conditions. In the low-Na^+ saline, the majority of NaCl was replaced with choline chloride. The pH and osmolality were adjusted to match those of the serosal control saline.

Subsequently, experiments with the Na^+-H^+ antiport inhibitor ethylisopropylamiloride (EIPA) were performed. In these experiments, the initial 60-min control period was followed 120 min during which serosal saline contained 10^{-3} M EIPA in a final concentration of 0.1% DMSO.

Na^+-K^+-ATPase inhibitor studies. To further examine whether serosal H^+ extrusion and, thereby, luminal HCO_3^- secretion rely on Na^+ gradients, experiments with the Na^+-K^+-ATPase inhibitor ouabain were performed. A 60-min control period was followed by a 120-min experimental period in which the serosal saline contained 10^{-3} M ouabain. Ouabain was dissolved by sonication in a low volume of serosal saline.

Rates of fluid absorption. For measurement of rates of fluid absorption in the anterior intestine, the fish were killed by an overdose of tricaine methanesulfonate (MS-222; 0.25 g/l), and the anterior segment of the intestine was obtained by dissection. A short length of heat-flared polyethylene tubing was tied in the anterior end of the intestinal segment with double silk ligatures, and the segment was flushed with 20 ml of mucosal saline (Table 1). The proximal end of the ~30-mm-long segments was subsequently closed with double silk ligatures. These intestinal sacs were then filled with gut saline containing ^{14}C-labeled polyethylene glycol (PEG; 0.01 μCi/ml), and a

sample of the mucosal saline was obtained as follows: the sac preparations were moderately overfilled, and the saline from the preparations was drawn from the sacs through the polyethylene catheter into a syringe and flushed back and forth three times before a sample was obtained. This procedure ensured that the sample was completely mixed and that this initial sample of gut saline was truly representative of the content of the sac preparation. The catheter was sealed, and the preparations were rinsed in serosal saline before they were submerged individually in 20 ml of serosal saline in glass vials. The serosal saline for these studies contained HCO_3^- and was gassed with the 0.3% CO_2-in-O_2 gas mixture for 90 min before and during experimentation. At the beginning and end of a 3-h flux period, samples were taken from the serosal saline. Finally, at the end of the flux, a sample of the [^{14}C]PEG-labeled mucosal saline was obtained, the intestinal sac was cut open, and the gross surface area of the exposed epithelial surface was determined from a trace of its outline on graph paper. This type of preparation has been employed in the past and exhibits stable transport characteristics for ≥6 h (15). Water absorption was determined from the increase in the ^{14}C radioactivity of the luminal saline during the 3 h of experimentation. Measurements of ^{14}C radioactivity in the serosal saline solutions confirmed that all [^{14}C]PEG was retained in the mucosal saline, despite considerable rates of fluid absorption.

Data presentation and statistical analysis. Values are means ± SE of five to eight observations. Statistical evaluation revealed that the data were normally distributed and was performed by paired *t*-tests with Bonferroni's multisample comparison correction to evaluate the difference between individual time points after treatment and an average control value (34). This average control value was based on the last 30 min of the corresponding initial 60-min control flux. Samples were considered statistically significantly different at $P < 0.05$.

RESULTS

On the basis of relatively stable HCO_3^- secretion rates, TEP, and conductance, the anterior toadfish intestine appears viable and stable for >5 h under the conditions employed in the present study and is not influenced by 0.1% DMSO (Fig. 1). Control experiments of 3-h duration with saline changes every 60 min also revealed stable transport rates and electrophysiological parameters (data not shown). In these initial experiments in the presence of serosal HCO_3^-, HCO_3^- secretion rates were 0.3–0.5 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Fig. 1), whereas TEP and conductance consistently were approximately −20 mV and 10 mS/cm^2 for all control experiments. Compared with an overall mean control base secretion rate of ~0.25 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ ($n = 42$) in the absence of serosal HCO_3^-, double end-point titration determination of HCO_3^- equivalents building up in the mucosal saline in the absence of titration revealed an HCO_3^- secretion rate of 0.15 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ (Table 2), accounting for 60% of overall base secretion. For these experiments, perfusion of the luminal chamber, rather than gas-lift mixing, did not influence TEP or conductance (Table 2, Fig. 1).

As predicted, intestinal HCO_3^- secretion was greatly reduced by O_2 deprivation (Fig. 2). A trend toward recovery in secretion rates on return to aerobic conditions suggested reversibility of the anaerobically induced inhibition, although full return to control values was not observed in the 60-min recovery period. The 60-min anaerobic period did not significantly influence TEP or conductance.

Intestinal HCO_3^- secretion rates displayed marked temperature dependence, especially when temperature was reduced from 25 to 15°C (Fig. 3). Elevation of bath temperature from 25 to 35°C appeared less potent and caused a transient and modest elevation of HCO_3^- secretion rates. In the experiments with reduced temperature, HCO_3^- secretion exhibited Q_{10} values of 1.8–3.0. Similar responses to temperature changes were observed for conductance, which was significantly reduced at 15°C and elevated at 35°C (Fig. 3). HCO_3^- secretion and conductance demonstrated the reversibility of the temperature-induced changes, but this was not the case for TEP. The temperature increase from 25 to 35°C caused a gradual decline in TEP and an increased variation among preparations. In contrast, a trend toward increased TEP at the end of 120 min at 15°C was observed also with a clear increase in variation.

Compared with HCO_3^-- and CO_2-free conditions, the presence of serosal HCO_3^- and CO_2 caused an approximately twofold reversible increase in luminal HCO_3^- secretion without a change in conductance or TEP (Fig. 4). However, control HCO_3^- secretion rates in the absence of serosal HCO_3^- and CO_2 were substantial (Fig. 4).

Luminal HCO_3^- secretion rates in the absence of serosal HCO_3^- and CO_2 were greatly reduced on addition of the lipophilic CA inhibitor to the luminal saline (Fig. 5). This effect was accompanied by a significant reduction of TEP. Interestingly, HCO_3^- secretion rates, but not TEP, gradually recovered in the presence of etoxzolamide. Addition of etoxzolamide caused a slight acidification of the luminal saline, but luminal pH returned to 7.800 in all experiments within 10–15 min after addition, at which time pH-stat titrations resumed. Etoxzolamide treatment elevated conductance for some individual time points toward the end of the 120 min of exposure (Fig. 5).

As hypothesized, reducing serosal pH in the absence of HCO_3^- and CO_2 caused a reversible and H^+ concentration-dependent inhibition of luminal HCO_3^- secretion (Fig. 6). Serosal pH of 7.40 resulted in maximal inhibition of 33% after 60 min, whereas serosal pH of 7.0 and 6.6 resulted in maximal inhibitions of 44 and 47%, respectively (Fig. 6). The manipulations of serosal pH did not significantly alter TEP or conductance.

In a separate set of experiments, H^+ secretion across the basolateral membrane was measured directly by pH-stat titration. The H^+ secretion rates measured in six individual preparations were in good agreement with the overall mean control HCO_3^- secretion rates across the apical membrane (Fig. 7). Electrophysiological parameters for these experiments were similar to values obtained for luminal pH-stat titration experiments with TEP and conductance of −22.09 ± 2.63 mV and 8.41 ± 0.47 mS/cm^2.

Having established that serosal H^+ secretion is important for luminal HCO_3^- secretion and that the apical HCO_3^- and basolateral H^+ fluxes occur at approximately equal rates, the Na^+ dependence of the serosal H^+ secretion was investigated. Reduction of serosal Na^+ concentrations from 152 to 11 mM in the absence of serosal HCO_3^- caused a marked reduction in luminal HCO_3^- secretion (Fig. 8). Reduced serosal Na^+ greatly reduced and even reversed TEP and, as could be expected, reduced conductance. Although HCO_3^- secretion showed only a slight trend toward recovery after return to control conditions, TEP and conductance almost full recovered (Fig. 8).

Surprisingly, if we consider the apparent Na^+ dependence of basolateral H^+ extrusion, 10^{-3} M EIPA did not influence luminal HCO_3^- secretion or conductance, whereas TEP was

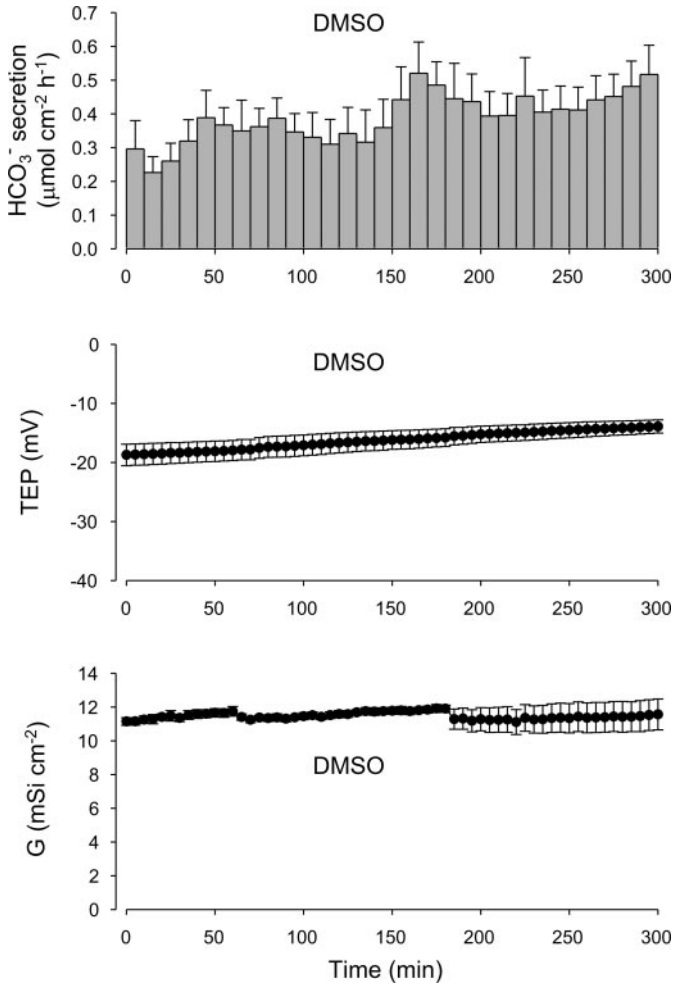

Fig. 1. Bicarbonate (HCO_3^-) secretion, transepithelial potential (TEP), and conductance (G) of isolated anterior toadfish intestine under asymmetric conditions mimicking in vivo intestinal fluid chemistry. Serosal saline solutions were replaced after 60 and 180 min and contained 0.1% DMSO at 60–180 min. On the basis of secretion rates and electrophysiological parameters, preparation appears stable for >5 h. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.37 ± 0.01 μmol·cm^{-2}·h^{-1}, −18.1 ± 0.1 mV, and 11.6 ± 0.1 mS/cm^2. Values are means ± SE (n = 6).

reduced, after 40 min of exposure (Fig. 9). These results are similar to those obtained in preliminary experiments using 10^{-3} M amiloride (results not shown).

Addition of the Na^+-K^+-ATPase inhibitor ouabain (10^{-3} M) caused a gradual reduction of luminal HCO_3^- secretion and a slight reduction of TEP with no change in conductance (Fig. 10).

Water absorption by isolated anterior intestinal sac preparations was 3.92 ± 0.92 μl·cm^{-2}·h^{-1}.

DISCUSSION

The isolated toadfish intestine displays stable HCO_3^- transport and electrophysiological characteristics over 5 h, demonstrating that this preparation, in agreement with previous work on European flounder (15), is suitable for the experimental protocols employed in the present study. Comparisons of base secretion rates obtained using pH-stat techniques with rates obtained by direct double-end-point titration measurements of

Table 2. *HCO_3^- accretion, TEP, and conductance of anterior toadfish intestine mounted in an using chamber*

Parameter	Value
HCO_3^- secretion, μmol·cm^{-2}·h^{-1}	0.15±0.02
TEP, mV	−21.6±2.3
G, mS/cm^2	8.4±0.4

Values are means ± SE (n = 8). TEP, transepithelial potential; G, conductance. HCO_3^- secretion rates were determined from increase in titratable alkalinity, measuring HCO_3^- and CO_3^{2-} equivalents, of mucosal saline over a 3-h period.

HCO_3^- and CO_3^{2-} equivalents revealed that the majority of intestinal base secretion occurs in the form of HCO_3^-. These measurements permit use of the term "HCO_3^- secretion," rather than "base secretion."

As predicted, luminal HCO_3^- secretion appears to rely on aerobic energy metabolism and, thus, ATP. This observation is in agreement with previous observations of reduced H^+ secre-

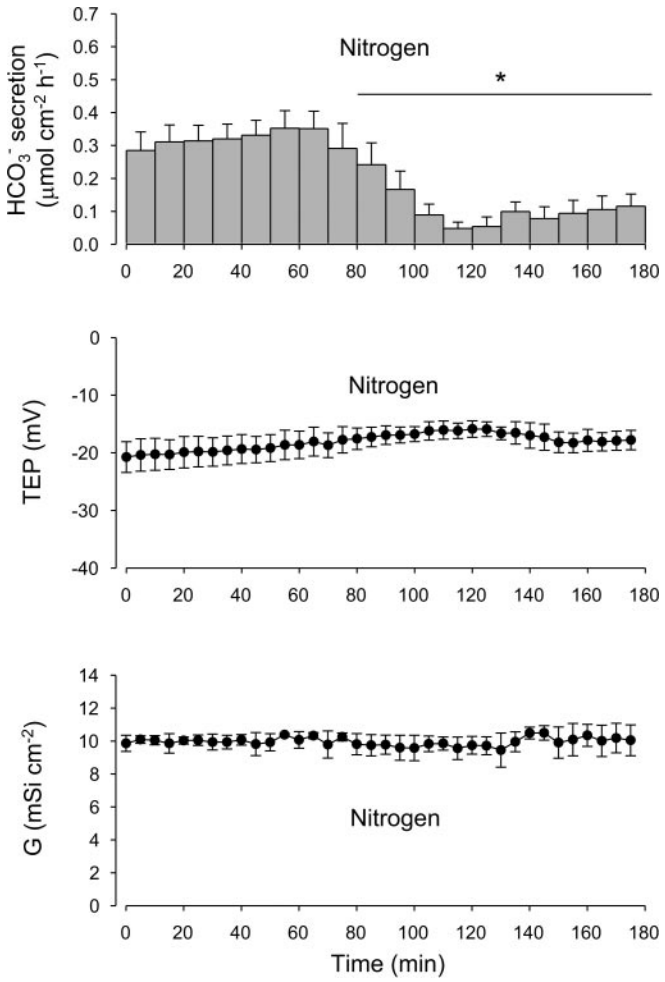

Fig. 2. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under normoxic and anoxic conditions. Serosal and mucosal saline solutions were gassed with O_2 at 0–60 min and 120–180 min and with nitrogen at 60–120 min. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.35 ± 0.01 μmol·cm^{-2}·h^{-1}, −19.1 ± 0.1 mV, and 10.0 ± 0.2 mS/cm^2. Values are means ± SE (n = 5). *Significantly different from control values, i.e., average of the last 30 min of the initial control period (by paired Student's t-test).

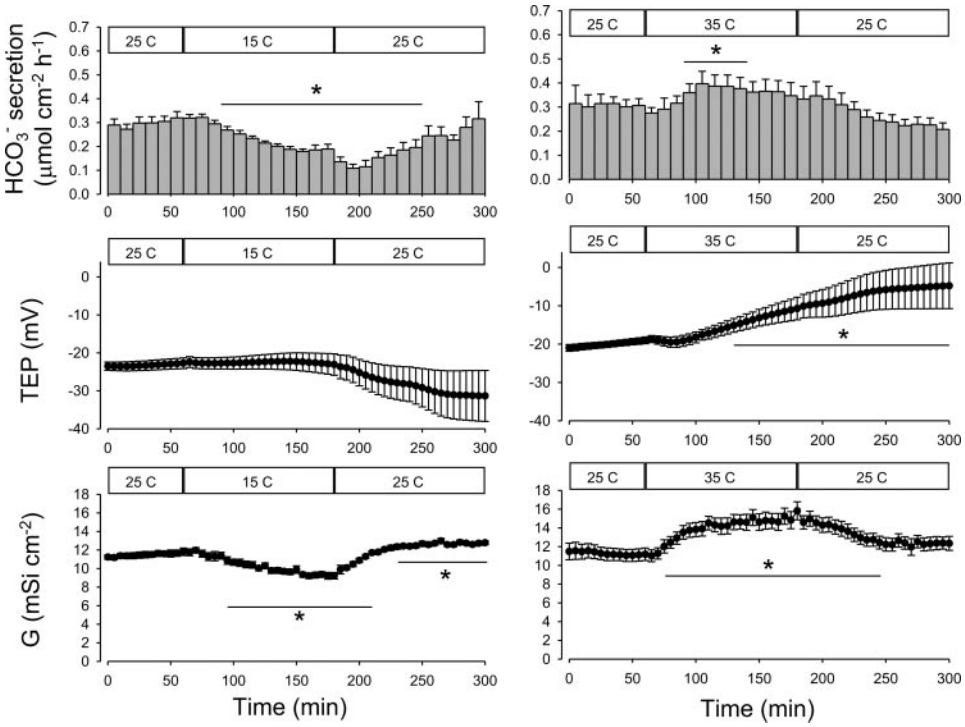

Fig. 3. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine at 15 and 35°C. *Left*: initial 60-min period at 25°C followed by 120 min of gradual temperature decrease to 15°C and a final 120 min of return to 25°C ($n = 7$). *Right*: initial 60-min period at 25°C followed by 120 min of gradual temperature increase to 35°C and final 120 min of return to 25°C ($n = 6$). Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.31 ± 0.01 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, −23.0 ± 0.1 mV, and 11.6 ± 0.1 mS/cm^2 for the 15°C series and 0.31 ± 0.01 $\mu mol \cdot cm^{-2} \cdot h^{-1}$, −19.5 ± 0.3, and 11.1 ± 0.1 mS/cm^2 for the 35°C series. Values are means ± SE ($n = 6$). *Significantly different from control values (see Fig. 2 legend).

tion, which fuels Cl^- uptake (via anion exchange) across anuran skin under anaerobic conditions (21–23), and with findings of HCO_3^- transport in cortical collecting ducts relying on oxidative metabolism (17). Strong temperature dependence is expected for biological reactions mediated by enzymatic reactions and transporters. Typically, increases in reaction velocity of >1.5-fold per 10°C increase are ascribed to biological, rather than physiochemical, processes (19). A 10°C reduction in temperature resulted in a 1.8- to 3.0-fold reduction in luminal HCO_3^- secretion, clearly indicating that intestinal HCO_3^- secretion in the gulf toadfish is biologically mediated. Experiments with increased temperature revealed a lower HCO_3^- secretion response and, perhaps, a general deterioration, as indicated by substantial nonrecoverable reduction in TEP. This latter response may reflect that 35°C is approaching the thermal maximal tolerance for the gulf toadfish, which experience temperatures ranging from 13 to 35°C in its natural environment (3). In any case, the apparent dependence of HCO_3^- secretion on aerobic energy metabolism and, thus, ATP and the strong reduction in secretion rates observed with reduced temperature support previous conclusions that intestinal HCO_3^- secretion in marine teleosts is of a secondary active nature (15).

The continued, although reduced, HCO_3^- secretion in the absence of serosal CO_2 and HCO_3^- demonstrates that endogenous CO_2 provides a significant (~50%) source for luminal HCO_3^- secretion under resting conditions, with transepithelial HCO_3^- transport or serosal CO_2 accounting for the remaining secretion. These observations are in agreement with reports of 30–60% of the luminal HCO_3^- secretion being sustained by endogenous epithelial CO_2 in the European flounder and the goby (8, 15, 45) but are in contrast to findings from the Japanese eel, where serosal HCO_3^- seems to fully account for luminal HCO_3^- secretion (1). However, the studies on the Japanese eel employed hyperphysiological serosal HCO_3^- levels [25 mM vs. 5–8 mM normally present in teleost fish extracellular fluids (25)]. Therefore, it seems that endogenous epithelial CO_2 generally contributes significantly to intestinal HCO_3^- secretion in marine teleosts, at least under resting, nonstimulated conditions. The remainder of the studies presented here were designed to investigate the transport processes associated with HCO_3^- secretion from hydration of endogenous CO_2.

The HCO_3^- secretion rates measured in the present study agree well with previous results from marine teleost fish (1, 8, 15, 45, 46), which seem to be on the order of 0.5 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ under resting conditions in the presence of serosal HCO_3^-. These intestinal HCO_3^- secretion rates from marine teleost fish are ~10-fold higher than the only other resting HCO_3^- secretion rate reported from an exothermic vertebrate, the bullfrog (11), and are comparable to the range (0.5–1 $\mu mol \cdot cm^{-2} \cdot h^{-1}$) of resting duodenal HCO_3^- secretion rates reported from mammals at 37°C (6, 20, 40, 41). The high intestinal HCO_3^- secretion rates in the marine teleost, often at temperatures much below 37°C, and the significant contribution of endogenous CO_2 to luminal alkalinization led to the hypothesis that CA might be involved in accelerating CO_2 hydration to fuel the apical anion exchange. The lipophilic CA inhibitor etoxzolamide clearly inhibited luminal HCO_3^- secretion, although a gradual recovery controlled secretion rates, despite the continued presence of the inhibitor. We interpret this as an immediate depletion of cytosolic HCO_3^- caused by CA inhibition followed by a gradual recovery mediated by noncatalyzed cellular CO_2 hydration, perhaps fueled by an increased cellular partial pressure of CO_2. An increase in partial pressure of CO_2 might be expected in a situation with sustained metabolic CO_2 production and without CA activity. What appears to be a complete inhibition of HCO_3^- secretion in

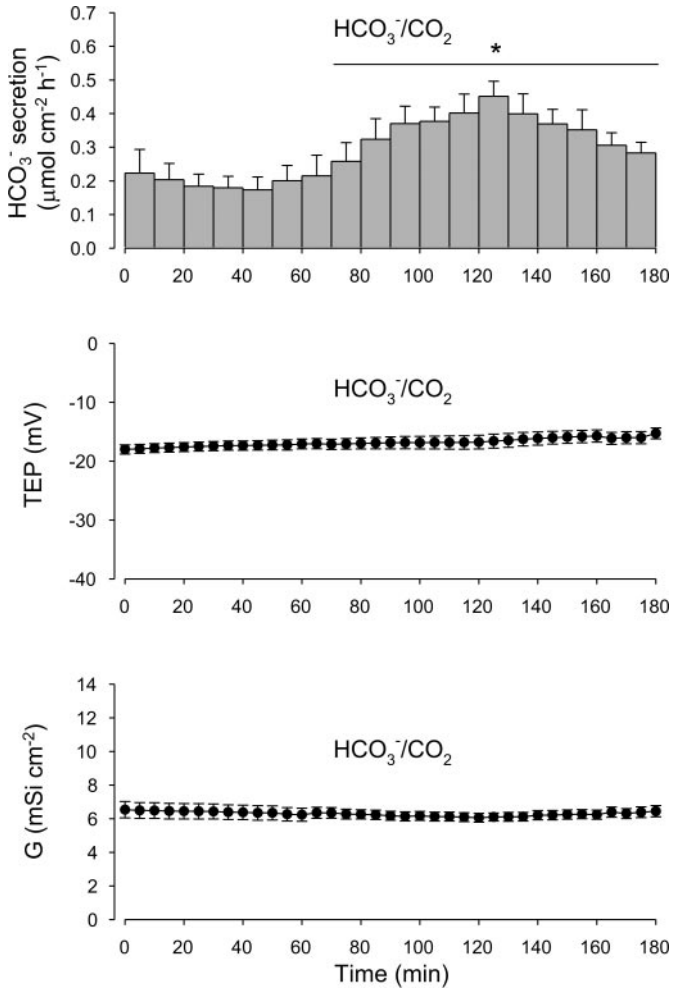

Fig. 4. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine in the presence and absence of serosal HCO_3^- and CO_2. During the initial 60 min and the last 60 min at 120–180 min, serosal saline solutions contained no HCO_3^- and were gassed with pure O_2. At 60–120 min, serosal saline solutions contained 5 mM HCO_3^- and was gassed with 0.3% CO_2 in O_2. Both serosal saline solution contained HEPES and were adjusted to pH 7.800. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.18 ± 0.01 μmol·cm^{-2}·h^{-1}, −17.3 ± 0.1 mV, and 6.3 ± 0.1 mS/cm^2. Values are means ± SE (n = 7). *Significantly different from control values (see Fig. 2 legend).

the first 10 min after etoxzolamide addition is caused in part by a slight acidification of the buffer-free mucosal saline in response to drug addition. However, luminal pH of 7.80 was fully recovered in all preparations after 10–15 min of drug addition, when a 50% reduction in luminal HCO_3^- secretion rates persisted. This observation is in agreement with previous reports of reduced, but not fully inhibited, intestinal HCO_3^- secretion by marine teleosts in the presence of CA inhibitors (8, 44) and with the strong physical and functional association between CA and anion exchangers in mammals (35–37).

The H^+ arising from hydration of CO_2 must be extruded from the epithelial cells to maintain intracellular pH, and furthermore, whereas HCO_3^- is excreted across the apical membrane, the H^+ must be extruded preferentially across the basolateral membrane, because the intestinal epithelium exhibits strong net base secretion. This polarization of HCO_3^- and H^+ secretion is demonstrated for the toadfish intestinal epithelium by the reduced HCO_3^- secretion when serosal H^+ concentration is increased, presumably leading to reduced basolateral H^+ extrusion, and by direct measurements of basolateral H^+ secretion. A similar polarization of HCO_3^- and H^+ secretion is characteristic of the pancreatic duct, which secretes HCO_3^- to reach concentrations of 70–140 mM (38), values similar to HCO_3^- concentrations in marine teleost intestinal fluids (14, 25, 26, 46).

Manipulation of the H^+ gradient across the basolateral membrane by reduction of serosal pH revealed a concentration-dependent inhibition of luminal HCO_3^- secretion that appeared to be at least partly reversible within 60 min after return to control conditions. Serosal pH as low as 6.6 did not influence epithelial integrity, as indicated by unaltered TEP and conductance. A likely explanation for these observations is that basolateral H^+ extrusion is reduced by the increased serosal H^+ concentrations and that this in turn slows the cellular CO_2

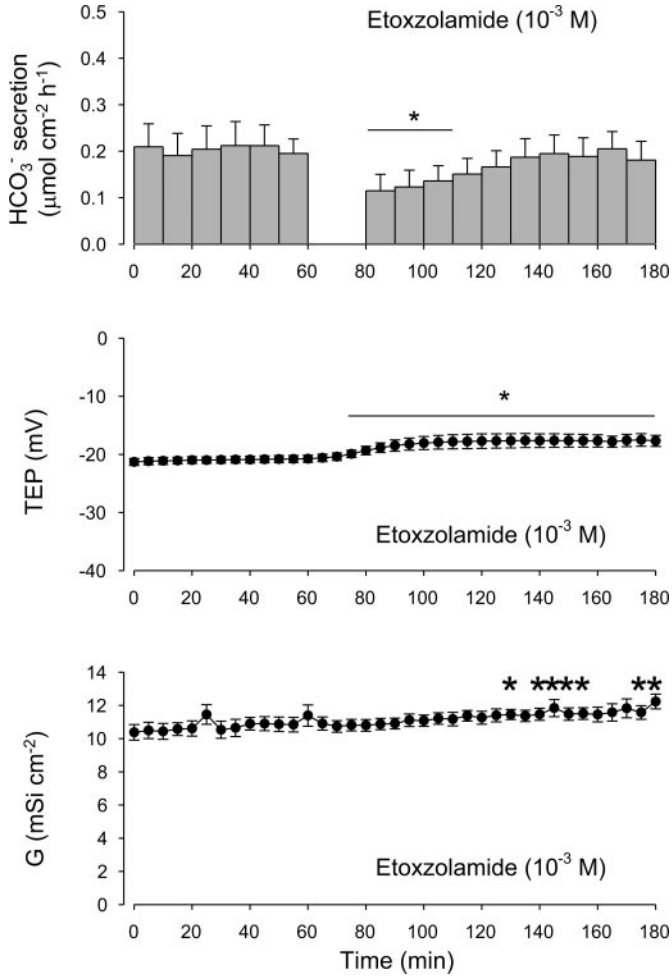

Fig. 5. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under control conditions and in the presence of the carbonic anhydrase inhibitor etoxzolamide in luminal saline. Because etoxzolamide slightly reduced luminal pH (~0.2 pH unit), HCO_3^- secretion could not be measured during the first 10–15 min until luminal pH recovered to 7.8. Thus no HCO_3^- secretion data are reported for the first 2 time intervals. Values are means ± SE (n = 7). Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.21 ± 0.01 μmol·cm^{-2}·h^{-1}, −17.3 ± 0.1 mV, and 6.3 ± 0.1 mS/cm^2. *Significantly different from control values (see Fig. 2 legend).

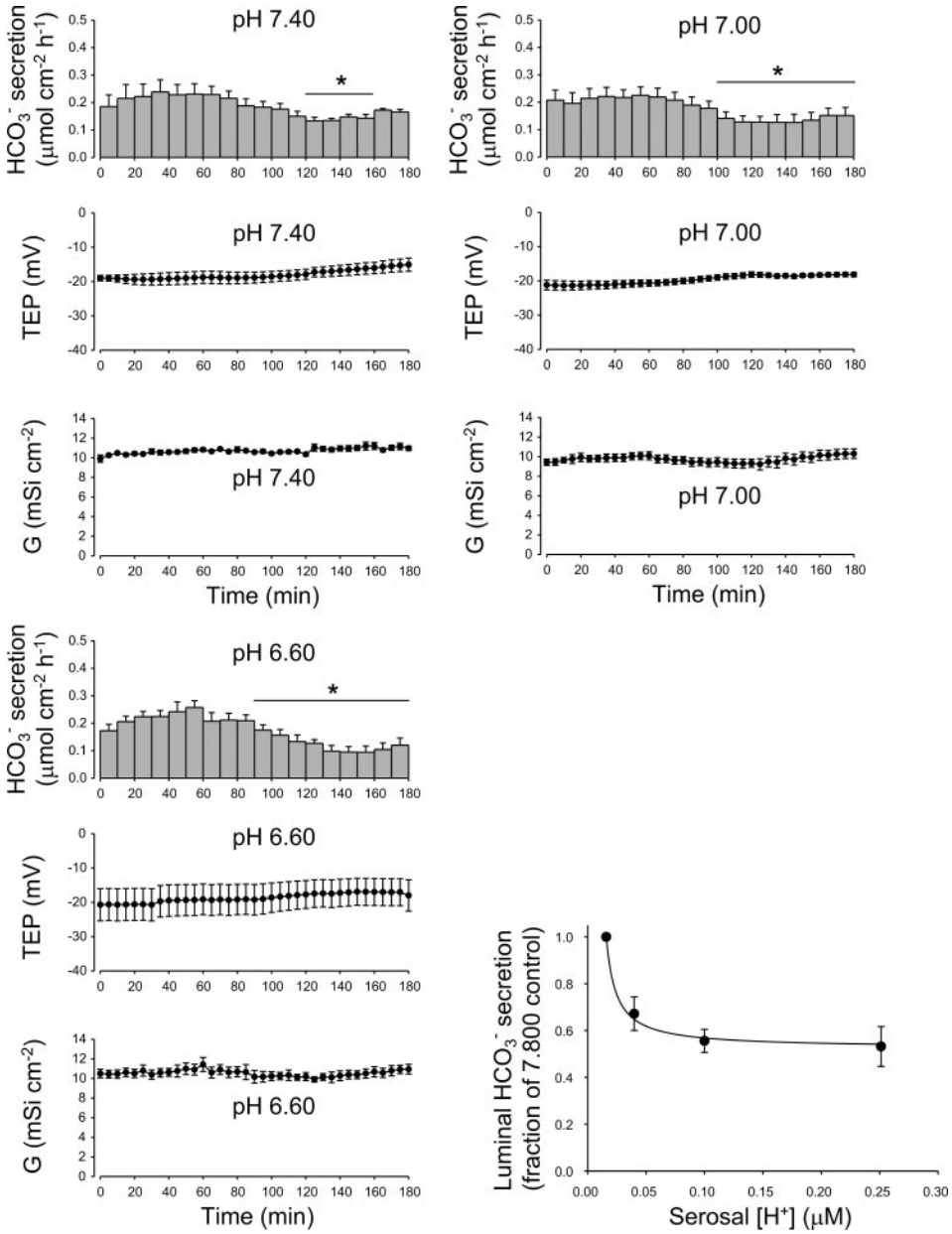

Fig. 6. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under control conditions (serosal pH 7.800) and serosal pH 7.400, 7.000, and 6.600. *Bottom right*: fractional maximal inhibition of mucosal HCO_3^- secretion as a function of serosal pH. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.23 ± 0.01 μmol·cm^{-2}·h^{-1}, −19.0 ± 0.2 mV, and 10.7 ± 0.1 mS/cm^2 for pH 7.4, 0 0.22 ± 0.01 μmol·cm^{-2}·h^{-1}, −20.9 ± 0.2 mV, and 10.0 ± 0.1 mS/cm^2 for pH 7.0, and 0.24 ± 0.01 μmol·cm^{-2}·h^{-1}, −19.4 ± 0.2 mV, and 10.9 ± 0.3 mS/cm^2 for pH 6.6. Values are means ± SE (n = 5–8). *Significantly different from control values (see Fig. 2 legend).

hydration and, thus, depletes the cytosolic HCO_3^- available for apical anion exchange. Direct evidence for a full polarization of HCO_3^- and H^+ secretion come from measurements of H^+ secretion to serosal fluids, which match those of the luminal HCO_3^- secretion. The basolateral H^+ secretion is important for the secondary active apical HCO_3^- secretion and must be carrier mediated, because it occurs against an electrochemical gradient. Under serosal HCO_3^--free conditions, the involve-

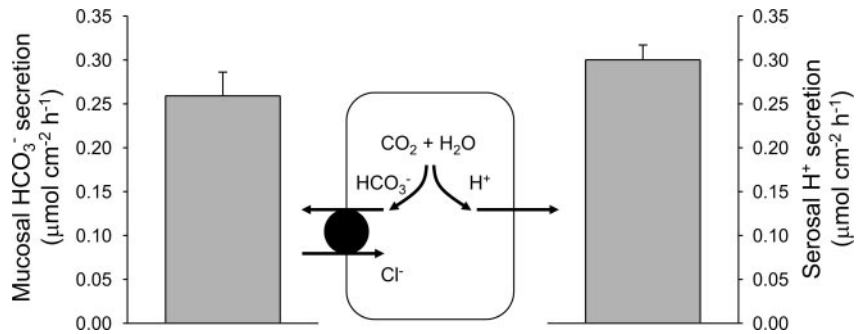

Fig. 7. Mucosal HCO_3^- secretion (n = 42) and serosal H^+ extrusion (n = 6) of isolated anterior toadfish intestine at luminal and serosal pH 7.800. Values are means ± SE.

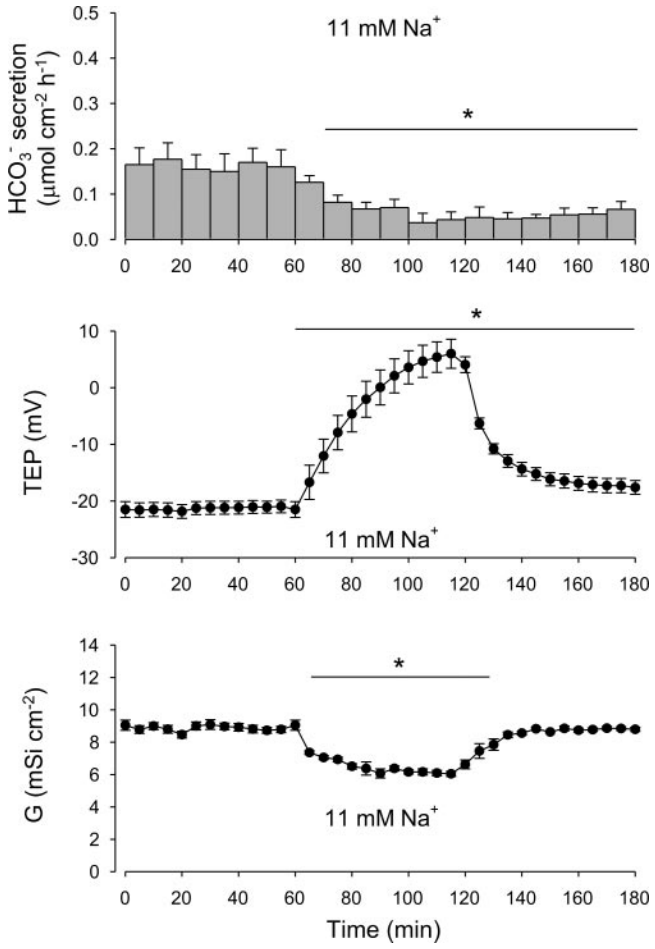

Fig. 8. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under control (162 mM serosal Na^+) and low-Na^+ (11 mM) conditions. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.16 ± 0.01 μmol·cm^{-2}·h^{-1}, −21.2 ± 0.2 mV, and 8.9 ± 0.1 mS/cm^2. Values are means ± SE (n = 7). *Significantly different from control values (see Fig. 2 legend).

ment of a basolateral Na^+-dependent Cl^-/HCO_3^- exchanger (5) in H^+ extrusion can be excluded, leaving NHE and H^+-ATPase as two possible mechanisms of basolateral H^+ excretion. The marked reduction in apical HCO_3^- secretion in response to lowered serosal Na^+ concentrations clearly indicates the involvement of an Na^+-dependent H^+ extrusion mechanism in HCO_3^- secretion by the marine teleost intestine and is consistent with transport mechanisms involved in rat pancreatic duct HCO_3^- secretion (29, 38). However, although the dependence of luminal HCO_3^- secretion on serosal Na^+ strongly suggests the involvement of a basolateral NHE-like protein, this could not be confirmed pharmacologically, because neither serosal amiloride nor EIPA at 10^{-3} M influenced luminal HCO_3^- secretion. The inhibitor concentrations should be sufficient to inhibit H^+ extrusion via EIPA-sensitive NHE isoforms (4), but the lack of amiloride and EIPA effects does not necessarily exclude the involvement of NHE, inasmuch as certain NHE isoforms or spliced variants of NHE isoforms are EIPA insensitive (2, 47).

Serosal addition of the Na^+-K^+-ATPase inhibitor ouabain (10^{-3} M) results in an ~70% inhibition of luminal HCO_3^- secretion after 120 min. The reduced HCO_3^- secretion is likely related to reduced basolateral H^+ extrusion via NHE resulting from the ouabain-induced reduction in basolateral electrochemical Na^+ gradient.

The basolateral H^+ extrusion is necessary for apical HCO_3^- secretion, as discussed above, and this basolateral H^+ extrusion seems to occur via an EIPA-insensitive NHE mechanism. The basolateral NHE is driven by the electrochemical Na^+ gradient established by the basolateral Na^+-K^+-ATPase, which thereby fuels the apical, secondary active HCO_3^- secretion (Fig. 11).

Although luminal HCO_3^- secretion is reduced by reduced serosal pH, it persists, although at lower rates, even at serosal pH 6.6, and the reduction observed at serosal pH 7.0 and 6.6 is not much different. These observations may indicate the recruitment of additional H^+ extrusion mechanisms in response to reduced extracellular pH.

The substrate for luminal HCO_3^- secretion by the gulf toadfish intestine is concluded to be a combination of endogenous epithelial CO_2 hydration and serosal CO_2 and/or HCO_3^-, with endogenous CO_2 hydration accounting for ~50% of the

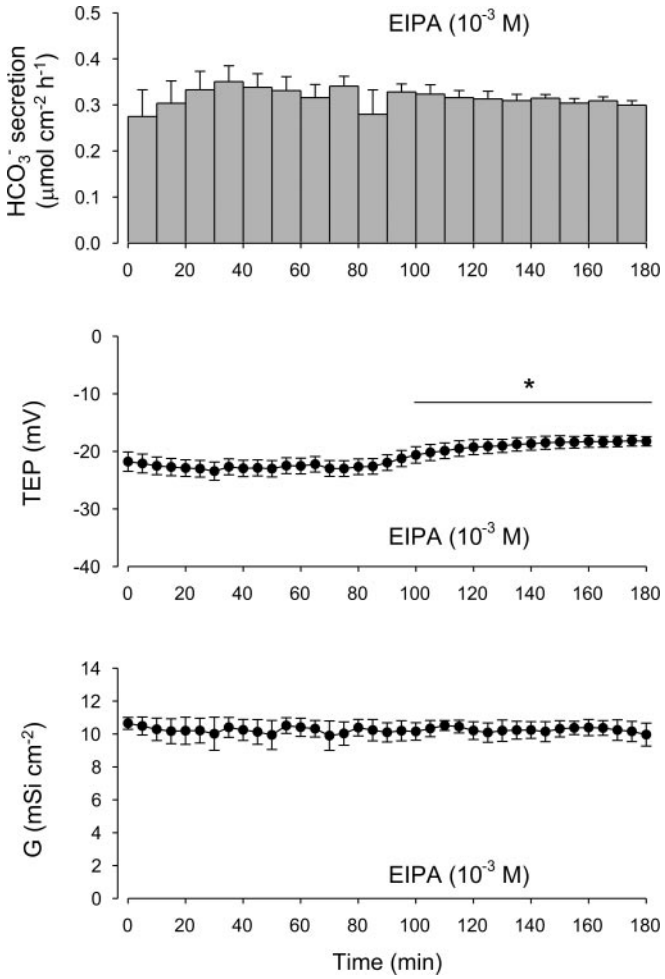

Fig. 9. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under control conditions and in the presence of the Na^+/H^+ exchange inhibitor ethylisopropylamiloride (EIPA) in serosal saline. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.35 ± 0.01 μmol·cm^{-2}·h^{-1}, −22.8 ± 0.2 mV, and 10.3 ± 0.2 mS/cm^2. Values are means ± SE (n = 6). *Significantly different from control values (see Fig. 2 legend).

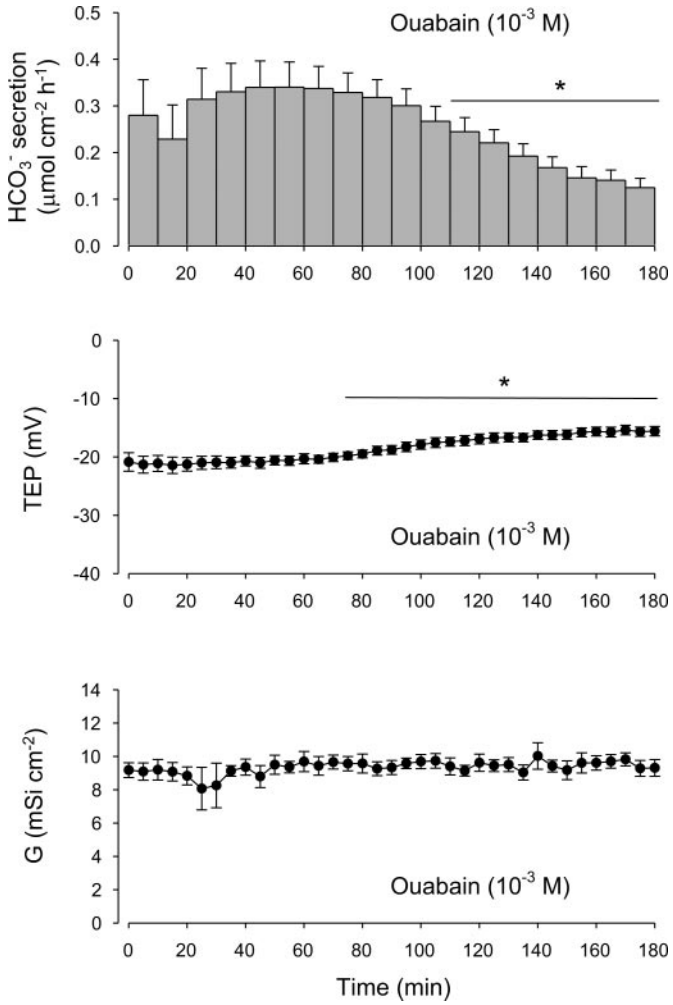

Fig. 10. HCO_3^- secretion, TEP, and conductance of isolated anterior toadfish intestine under control conditions and in the presence of the Na^+-K^+-ATPase inhibitor ouabain in the serosal saline. Average values for the last 30 min of the initial control period for HCO_3^- secretion, TEP, and conductance were 0.35 ± 0.01 μmol·cm^{-2}·h^{-1}, −20.7 ± 0.2 mV, and 9.3 ± 0.3 mS/cm^2, respectively. Values are means ± SE (n = 6). *Significantly different from control values (see Fig. 2 legend).

total HCO_3^- secretion rates under resting, in vivo-like conditions. The present study demonstrates that the polarized extrusion of H^+ and HCO_3^- (arising from cellular CO_2 hydration) accounts for 50% of the net epithelial secretion of HCO_3^-. The nature of the remaining 50% of the overall HCO_3^- secretion, which relies on serosal HCO_3^- and, possibly, CO_2, offers an interesting area for further studies. An Na^+-HCO_3^- cotransporter is a likely candidate for basolateral HCO_3^- uptake by intestinal epithelial cells (Fig. 11) and could provide for apical anion exchange, as in the case of the rat, pig, and guinea pig pancreatic ducts (38), but this possibility remains to be investigated.

High rates of intestinal anion exchange represent a significant contribution to marine teleost osmoregulation and, thus, can be expected to be regulated, depending on salt and water balance. In addition, intestinal HCO_3^- secretion in fish likely serves the same functions as in mammals, where duodenal HCO_3^- secretion protects against acidic gastric effluent (6) and can be potently stimulated by multiple factors. The characteristics of the gulf toadfish intestinal HCO_3^- secretion described in the present study apply to unstimulated, resting epithelia, and HCO_3^- secretion by stimulated epithelia may display different characteristics. The involvement of an H^+-ATPase in H^+ extrusion and a more significant contribution of transepithelial HCO_3^- transport to overall luminal HCO_3^- secretion may apply to stimulated intestinal epithelia.

In a recent study, it was suggested that basolateral H^+ extrusion from marine teleost intestine may account for a substantial fraction of intestinal H^+ absorption and that the H^+ secretion from the epithelium across the basolateral membrane could be as high as 0.3 μmol·cm^{-2}·h^{-1} (15). In the present study, this suggestion was verified by direct measurement of basolateral H^+ extrusion at ~0.3 μmol·cm^{-2}·h^{-1}, which, combined with direct measurements of water absorption, yields a theoretical H^+ concentration of 76.5 mM in the absorbed fluid. From this calculation and previous reports (15), it is clear that intestinal fluid absorption by marine teleosts is accompanied by acid absorption (equivalent to a theoretical pH of 1.1 in the absorbed fluid with the assumption of no buffer capacity) and that maintenance of systemic acid-base balance requires compensation for the acid load associated with vital intestinal water absorption.

Interestingly, elevated extraintestinal excretion of acidic equivalents when intestinal base secretion is stimulated has been reported recently (45), illustrating this intimate link between osmoregulation and acid-base balance.

Perspectives

The marine teleost intestine, which is capable of secondary active HCO_3^- secretion, resulting in luminal concentrations >100 mM in some cases, displays polarized apical HCO_3^- and basolateral H^+ secretion arising from endogenous CO_2 hydration. CA-catalyzed CO_2 hydration provides significantly for the apical anion exchange, whereas the electrochemical Na^+ gradient fuels basolateral H^+ extrusion via an EIPA-insensitive NHE mechanism. Basolateral H^+ extrusion is required for continued secondary active HCO_3^- secretion, and the energy

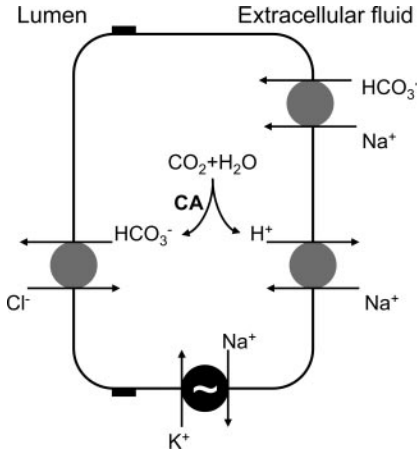

Fig. 11. Schematic transport model of HCO_3^- secretion by the gulf toadfish intestinal epithelium. Model includes polarized apical Cl^-/HCO_3^- exchange and basolateral EIPA-insensitive Na^+/H^+ exchange, which rely on the Na^+ gradient established by Na^+-K^+-ATPase. Hydration of endogenous CO_2, partly catalyzed by carbonic anhydrase (CA), supplies the majority of the cellular substrate for apical anion exchange. Basolateral HCO_3^- uptake, possibly via Na^+-HCO_3^- cotransport, may be involved in apical HCO_3^- secretion.

for this transport process is ultimately supplied from the basolateral Na^+-K^+-ATPase, as illustrated by ouabain-sensitive HCO_3^- secretion. Under nonstimulated conditions, transepithelial HCO_3^- transport, possibly via Na^+-HCO_3^- cotransporter-like proteins, appears to contribute ~50% to overall secretion rates. The intestinal apical anion exchange contributes significantly to Cl^- and, thereby, water absorption across marine teleost intestine, and a consequence of the CO_2 hydration required for this transport pathway is a highly acidic absorbate. If we consider that the immense diversity among marine teleosts and possible diversity in epithelial transport mechanisms, it is likely that the macroscopic intestinal epithelium may provide useful models for mammalian HCO_3^- secreting and/or fluid-absorbing epithelia.

GRANTS

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