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

Grosell, M.1* & Genz, J.1

University of Miami, Rosenstiel School of Marine and Atmospheric Sciences
4600 Rickenbacker Causeway
33149-1098 Miami
Florida, USA

* Corresponding author
Martin Grosell
Phone: 1 305 421 4623
Fax: 1 305 421 4001
Email: mgrosell@rsmas.miami.edu
Abstract

The gulf toadfish (*Opsanus beta*) intestine secretes base mainly in 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 ºC under *in vivo* like conditions secretion rates average 0.45 μmol cm$^{-2}$ h$^{-1}$, of which 0.25 μmol cm$^{-2}$ 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 reducing serosal pH reduced luminal HCO$_3^-$ secretion results. Basolateral H$^+$ secretion occurs in exchange for Na$^+$ via a 5-(N-ethyl-N-isopropyl) amiloride (EIPA) -insensitive mechanism and is ultimately fueled by the activity of the basolateral Na$^+$:K$^+$-ATPase. The fluid absorbed by the toadfish intestine to combat diffusive water loss to the concentrated marine environment is accompanied by a substantial basolateral H$^+$ extrusion, intimately linking osmoregulation and acid-base balance.

**Keywords:** HCO$_3^-$ transport, Cl$^-$ absorption, epithelial water transport, seawater ingestion, pH-stat titrations
**Introduction**

Osmoregulating marine fish maintain extracellular fluid osmolality at 300-350 mOsm/kg and as a consequence experience continuous diffusive water loss to the hypertonic surrounding seawater (~1000 mOsm/kg). To combat this osmoregulatory challenge fish drink seawater in amounts equivalent to approximately 5% of their body mass per day (25). The ingested fluid is modified along the gastro-intestinal 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 both 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 co-transport systems: Na\(^+\)::Cl\(^-\) (NC) and Na\(^+\)::K\(^+\)::2Cl\(^-\) (NKCC) co-transporters (9; 10; 16; 28), both of which rely on the electrochemical Na\(^+\) gradient established by the basolateral Na\(^+\)::K\(^+\)-ATPase (NKA) (42). However, in addition to these co-transport systems, apical anion exchangers have recently been associated with both 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 \(\frac{3}{4}\) 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 a trait common to fish inhabiting marine environments. Experiments on isolated intestinal preparations demonstrate that the source of luminal total CO₂ is HCO₃⁻ 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 HCO₃⁻, which accounts for the high luminal total CO₂ 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 directly measuring HCO₃⁻ secretion using a pH-stat approach at 15, 25 and 35 ºC. Furthermore, the requirement of aerobic energy metabolism and thus cellular ATP for the putative active HCO₃⁻ transport were examined. Studies to date on the source of HCO₃⁻ secreted by the intestinal epithelium range from demonstrating that extracellular HCO₃⁻ transported across the intestinal epithelium provides the majority of HCO₃⁻ secreted into the intestinal lumen (1), to showing that endogenous epithelial CO₂ constitutes the main source of luminal HCO₃⁻ (15; 45). Therefore, one goal of the present study was to determine the contribution of endogenous metabolic CO₂ and extracellular HCO₃⁻ to the overall apical HCO₃⁻ secretion by the intestine of a marine teleost, the gulf toadfish. To the extent that endogenous CO₂ provides for apical HCO₃⁻ secretion, cytosolic CO₂ hydration must occur. This prompted the examination of the potential involvement of carbonic anhydrase in intestinal HCO₃⁻ secretion. Our findings demonstrate that hydration of endogenous CO₂ provides for apical secretion of HCO₃⁻, which lead to investigations of the fate of H⁺ arising from CO₂ hydration. The prediction was that H⁺ from CO₂ hydration would be eliminated from the
epithelial cells to maintain cellular pH and to prevent reversal of the hydration reaction. Further, since the epithelium exhibits net base secretion it was predicted that this H⁺ extrusion 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 as in the rat pancreatic ducts (38) was examined by characterizing the dependence of apical HCO₃⁻ secretion on serosal Na⁺ and by pharmacological manipulations of Na⁺ gradients. Finally, considering the high apical HCO₃⁻ secretion rates and resulting need to eliminate H⁺ produced from the CO₂ 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 while the gill extrudes salt gained 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 proton 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

Biscayne Bay, Florida gulf toadfish (*Opsanus beta*) were obtained from shrimp fishermen during the spring and early summer 2005 and transferred to holding facilities at the University of Miami Rosenstiel School of Marine and Atmospheric Sciences. Upon arrival fish received a prophylactic treatment against ectoparasites as previously described (27). Fish were provided short lengths of PVC tubing as shelters in 80 l glass aquaria receiving a continuous flow of filtered seawater (salinity 27-30 ppt, temperature 22-26ºC Source: Bear Cut, Florida). Fish were fed frozen squid twice weekly but food was withheld 48 hours prior to experimentation.

pH-stat experiments - general experimental protocol

Segments of the anterior toadfish intestine (fish size: 25-40 grams) were obtained by dissection, cut open and placed in tissue holders exposing 0.7 cm² surface area with the serosal muscle layer intact. The true exposed surface area of the intestinal epithelium clearly exceed the 0.7 cm² due to 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 (P2300 Physiological Instruments, CA) containing 1.6 ml of appropriate, pre-gassed saline in each half-chamber (see Table 1 for chemical composition of salines). Mixing was achieved by gassing with O₂ through airlifts unless otherwise stated. Pre-gassing of the luminal saline and continued gassing with CO₂-free gas in the luminal half-chamber ensured stable titration curves throughout 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 (VCC600, Physiological Instruments, CA) recorded the trans-epithelial potential differences (TEP) under current clamp conditions at 0 µAmp. Once per 60 seconds, 50 µAmp pulses, 3 seconds in duration were passed from the mucosal to the serosal side. The TEP measurements were logged on a PC 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 (PHC4000.8 Radiometer) and a microburette tip both of which were connected to a pH-stat titration system (Radiometer, TIM 854 or 856). The pH stat titration system was grounded to the VCC600 amplifier to allow for pH readings during current pulsing. The pH-stat titrations were performed on luminal salines at a set-point of 7.800 throughout all experiments with pH values and rate of acid addition logged on PCs using Titramaster software (versions 1.3 and 2.1). Luminal pH was generally maintained within ± 0.003 pH units around the set-point throughout 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 approximately 60 min with stable TEP and base secretion rates prior to the manipulations outlined below.

*Nature of titrated base secretion*

To test the assumption that measured base secretion consisted of HCO₃⁻ a separate set of experiments were performed. In these experiments, 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, containing HCO$_3^-$ free saline was
gassed with O$_2$. Note that 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 hours without
pH-stat titration, after which total HCO$_3^-$ and CO$_3^{2-}$ concentrations in the luminal saline
were determined using double endpoint titrations that determine the titratable alkalinity
of the saline. This method is described in detail elsewhere (12). In brief, the sample is
gassed with CO$_2$-free gas for 30 minutes to rid gaseous CO$_2$ from the solution; pH is
titrated to 3.800 with HCl using Gilson microburettes, recording the HCl volume used. At
this low pH all HCO$_3^-$ and CO$_3^{2-}$ will convert to gaseous CO$_2$ which is removed from the
sample by continuous gassing for a minimum of 15 minutes. Thereafter pH is titrated
back to the initial value while still continuously gassing and carefully recording 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) is
determined from the difference in amounts of H$^+$ ions needed to bring the solution to pH
3.800 and the amount of OH$^-$ ions needed to return to the starting pH value after HCO$_3^-$
and CO$_3^{2-}$ are 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 three hour experimental period, the epithelial surface area
and the elapsed time. Since these experiments revealed that the majority of base secreted
was HCO$_3^-$, base secretion will be referred to as HCO$_3^-$ secretion in the following.

*Examining the longevity of the 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 minute flux periods separated by changing the serosal saline were performed. In the second series of control experiments, an initial 60 minute control period was followed by two subsequent 120 minute flux periods. The initial 60 minute flux period and the two 120 minute flux periods were again separated by serosal saline changes. The serosal saline in the first of the two 120 minute flux periods contained 0.1% dimethylsulfoxide (DMSO) which served as a vehicle control for some of the pharmacology experiments described below.

*Is aerobic energy metabolism required for HCO$_3^-$ secretion?*

The intestinal HCO$_3^-$ secretion in marine teleosts appear to be of a secondary active nature (15) and can therefore be expected to rely on aerobic energy metabolism and thus cellular ATP. Suppression of cellular ATP levels was performed by gassing both mucosal and serosal salines with N$_2$. Also for these experiments, the serosal HCO$_3^-$-free saline was replaced after 60 and 120 minutes, with salined pre-equilibrated for more than 90 minutes with N$_2$ or O$_2$, respectively. From 60 to 120 minutes, gassing of mucosal salines 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 due to the secondary active transport characteristics of luminal alkalination. To test this
hypothesis, measurements were performed, after an initial 60 minute control period at 25 ºC, for 120 minutes at 15 or 35 ºC after which temperature was returned to 25 ºC for an additional 120 minute recovery period. Exposure temperatures were verified by measurements of saline temperature in the Ussing chambers which revealed that complete change in temperature from 25 to either 15 or 35 degrees took up to 30 minutes.

**Determining the source of luminal HCO₃⁻ secretion**

To assess whether secreted HCO₃⁻ is derived from endogenous epithelial CO₂ or is dependent on serosal CO₂ and/or HCO₃⁻, experiments were performed to compare secretion rates obtained with serosal HEPES saline gassed with O₂ to those obtained with serosal salines containing 5 mM HCO₃⁻ and gassed with 0.3% CO₂ in O₂. In these experiments an initial 60 minute control period with a HEPES/O₂ serosal saline was followed by a 60 minute period with the above mentioned serosal HCO₃⁻/CO₂ containing saline before a final 60 minute control period with HEPES/O₂ serosal saline completed the experiment. The HCO₃⁻ concentrations and CO₂ levels employed results in pH and CO₂ partial pressure typical of the extracellular fluids of toadfish.

**Carbonic anhydrase mediated CO₂ hydration?**

Having established that hydration of endogenous CO₂ is the main source of luminal HCO₃⁻ secretion, the potential involvement of carbonic anhydrase (CA) was examined by applying the lipophilic carbonic anhydrase inhibitor, etoxzolamide (10⁻³ M in 0.1 % DMSO) to the luminal saline. Addition of the CA inhibitor occurred after a 60 minute
control period and measurements were continued for 120 minutes post addition. These experiments were performed with HEPES/O2 serosal saline.

*Involvement of Serosal Proton secretion*

To test the hypothesis of serosal H⁺ export being important for luminal HCO₃⁻ secretion, luminal HCO₃⁻ secretion was measured while manipulating serosal pH and thereby H⁺ gradients across the basolateral membrane, with the prediction that reduced serosal pH would inhibit luminal HCO₃⁻ secretion. In these experiments, which were performed with HEPES/O₂ serosal saline, an initial 60 minute control period at serosal pH 7.800 was followed by a 60 minute period with reduced serosal pH and finally with a 60 minute recovery period at serosal pH 7.800. A total of three series were performed on three sets of preparations with serosal pH reduced to either 7.4, 7.0 and 6.6.

*Rate of basolateral H⁺ extrusion*

Considering that CO₂ hydration within the intestinal epithelium provides the main source for luminal HCO₃⁻ secretion and that basolateral proton extrusion is critical for normal rates of apical HCO₃⁻ secretion, predictions of high basolateral H⁺ secretion rates across the basolateral membrane were tested. In these experiments, pH-stat titrations were performed with the electrode and burette tip in the serosal half-chamber rather than in the mucosal half-chamber and 0.0005 N NaOH was employed as titrant rather than HCl. The serosal saline for these experiments contained neither HEPES nor HCO₃⁻ (but were osmotically compensated with mannitol; Table 1) and was gassed with O₂. These titrations were also in this case performed with a titration set-point of 7.800 (normal
teleost fish blood plasma pH). Experiments were continued until stable titration and electrophysiological values were obtained for a minimum of 30 minutes.

**Na⁺ dependence of serosal H⁺ secretion – the involvement of Na⁺/H⁺ exchange**

The possibility that secretion of H⁺ across the basolateral membrane occurred via a Na⁺/H⁺ antiport was examined by measuring luminal HCO₃⁻ secretion under conditions with no serosal HCO₃⁻ and reduced serosal Na⁺. In these experiments, an initial control period of 60 minutes was followed by a 60 minute period in which the serosal saline [Na⁺] was reduced to 11 mM and by a final 60 minute recovery period under control conditions. In the low Na⁺ saline, the majority of NaCl was replaced with choline-Cl. The pH and osmolality was adjusted to match that of the serosal control saline. Subsequently experiments with the Na⁺/H⁺ antiport inhibitor 5-(N-ethyl-N-isopropyl)amiloride (EIPA) were performed. In these experiments, the initial 60 minute control period was followed by a 120 minute period in which the serosal saline contained 10⁻³ M EIPA in a final concentration of 0.1% DMSO.

**Na⁺/K⁺-ATPase inhibitor studies**

To further examine if serosal H⁺ extrusion and thereby luminal HCO₃⁻ secretion rely on Na⁺ gradients, experiments with the Na⁺/K⁺-ATPase inhibitor ouabain were performed. For these experiments a 60 minute control period was followed by a 120 minute 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

To measure rates of fluid absorption in the anterior intestine, 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 (PE) 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 (see Table 1 for composition). The proximal end of the approximately 30 mm long segments was subsequently closed with double silk ligatures. These intestinal sacs were then filled with gut saline containing 14C 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 PE catheter into a syringe and flushed back and forth three times before a sample was obtained. This procedure ensured complete mixing and that this initial sample of gut saline was truly representative of the content of the sac preparation. The catheter was thereafter sealed and the preparations were rinsed in serosal saline before being submerged individually in 20 ml of serosal saline in glass vials. The serosal saline for these studies contained HCO₃⁻ and was gassed with the 0.3 % CO₂ in O₂ gas mix for 90 minutes prior to and during experimentation. At the beginning and end of a three hour flux period, samples were taken from the serosal saline. Finally, at the end of the flux, a sample of the 14C-PEG labeled mucosal saline was obtained, the intestinal sac was cut open and the gross surface area of the exposed epithelial surface was determined by tracing its outline onto graph paper. This type of preparation has been employed in the past and exhibits stable transport characteristics for at least 6 hours (15). Water absorption was determined from the increase in the 14C
radioactivity of the luminal saline during the three hours of experimentation. Measurements of $^{14}$C radioactivity in the serosal salines confirmed that all $^{14}$C labeled PEG was retained in the mucosal saline despite considerable rates of fluid absorption.

**Data presentation and statistical analysis**

Data are presented as mean ± SEM throughout with number of observations ranging from 5-8 (exact number given in figure legends or table captions). Statistical evaluation revealed that data was normally distributed and was performed by paired $t$-tests with Bonferroni multi-sample comparison correction evaluating difference between individual time points post treatment to an average control value (34). This average control value was based on the last 30 minutes of the corresponding initial 60 minute control flux. Samples were considered statistically significantly different at $P<0.05$.

**Results**

Based on relatively stable HCO$_3^-$ secretion rates, TEP and conductance, the anterior toadfish intestine appears viable and stable for >5 hours under the conditions employed in the present study and is not influenced by 0.1% DMSO (Fig. 1). Control experiments of 3 hours of duration with saline changes every 60 minutes also revealed stable transport rates and electrophysiological parameters (data not shown). Rates of HCO$_3^-$ secretion in these initial experiments in the presence of serosal HCO$_3^-$ ranged from 0.3-0.5 $\mu$mol cm$^{-2}$ h$^{-1}$ (Fig. 1) while TEP and conductance consistently were ~ -20 mV and 10 mSi cm$^{-2}$ for all control experiments. Compared to an overall mean control base secretion rate of ~ 0.25 $\mu$mol cm$^{-2}$ h$^{-1}$ (n=42) in the absence of serosal HCO$_3^-$, double endpoint titration
determination of \( \text{HCO}_3^- \) equivalents building up in the mucosal saline in absence of titration revealed a \( \text{HCO}_3^- \) secretion rate of 0.15 \( \mu \text{mol cm}^{-2} \text{ h}^{-1} \) (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 vs Fig. 1).

As predicted, intestinal \( \text{HCO}_3^- \) secretion was greatly reduced by \( \text{O}_2 \) deprivation (Fig. 2). A trend towards recovery in secretion rates upon return to aerobic conditions was observed suggesting reversibility of the anaerobically induced inhibition, although full return to control values was not observed in the 60 minute recovery period. The 60 minutes of anaerobic conditions did not significantly influence TEP or conductance.

Intestinal \( \text{HCO}_3^- \) secretion rates displayed marked temperature dependence, especially when temperature was reduced from 25 to 15 \( ^\circ \text{C} \) (Fig. 3). Elevation of bath temperature from 25 to 35 \( ^\circ \text{C} \) appeared less potent and caused a transient and modest elevation of \( \text{HCO}_3^- \) secretion rates. In the experiments with reduced temperature \( \text{HCO}_3^- \) 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\( ^\circ \text{C} \) and elevated at 35\( ^\circ \text{C} \) (Fig. 3).

Both \( \text{HCO}_3^- \) secretion and conductance displayed reversibility of the temperature induced changes but this was not the case for TEP. The temperature increase from 25 to 35 \( ^\circ \text{C} \) caused a gradual decline in TEP and an increased variation among preparations. In contrast, a trend toward increased TEP at the end of the 120 minutes at 15\( ^\circ \text{C} \) was observed also with clear increase in variation.

Compared to \( \text{HCO}_3^- \)- and \( \text{CO}_2 \)-free conditions, the presence of serosal \( \text{HCO}_3^- \) and \( \text{CO}_2 \) caused an approximately two-fold reversible increase in luminal \( \text{HCO}_3^- \) secretion without
any change in conductance or TEP (Fig. 4). However, control HCO\textsubscript{3}\textsuperscript{-} secretion rates in absence of serosal HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2} were substantial (Fig. 4).

Luminal HCO\textsubscript{3}\textsuperscript{-} secretion rates in absence of serosal HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2} were greatly reduced upon addition of the lipophilic carbonic anhydrase inhibitor to the luminal saline (Fig. 5). This effect was accompanied by a significant reduction of TEP. Interestingly, HCO\textsubscript{3}\textsuperscript{-} 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 minutes post addition, at which time pH-stat titrations resumed. The etoxzolamide treatment resulted in elevated conductance for some individual time points toward the end of the 120 minutes of exposure (Fig. 5).

As hypothesized, reducing serosal pH in absence of HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2}, caused a reversible and H\textsuperscript{+} concentration dependent inhibition of luminal HCO\textsubscript{3}\textsuperscript{-} secretion (Fig. 6). Serosal pH of 7.40 resulted in maximal inhibition of 33 % after 60 minutes, while 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\textsuperscript{+} secretion across the basolateral membrane was measured directly by pH-stat titration. The H\textsuperscript{+} secretion rates measured in 6 individual preparations were in good agreement with the overall mean control HCO\textsubscript{3}\textsuperscript{-} 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 mSi cm\textsuperscript{-2}, respectively.
Having established that serosal H⁺ secretion is important for luminal HCO₃⁻ secretion and that the apical HCO₃⁻ 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 mM to 11 mM in the absence of serosal HCO₃⁻ caused a marked reduction in luminal HCO₃⁻ secretion (Fig. 8). Reduced serosal Na⁺ caused a much reduced and even inversed TEP and as could be expected a reduced conductance. While HCO₃⁻ secretion only showed a slight trend towards recovery after return to control conditions, TEP and conductance displayed almost full recovery (Fig. 8).

Surprisingly, considering the apparent Na⁺ dependence of basolateral H⁺ extrusion, EIPA (10⁻³ M) did not influence luminal HCO₃⁻ secretion or conductance while TEP was reduced after 40 minutes of exposure (Fig. 9). These results are similar to results 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 HCO₃⁻ 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 HCO₃⁻ transport and electrophysiological characteristics over at least 5 hours 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 to rates obtained by direct double endpoint titration measurements of 
$\text{HCO}_3^-$ and $\text{CO}_3^{2-}$ equivalents revealed that the majority of intestinal base secretion occurs 
in the form of $\text{HCO}_3^-$. These measurements permit using the term “$\text{HCO}_3^-$ secretion” 
rather than the term “base secretion”. 
As predicted, luminal $\text{HCO}_3^-$ secretion appears to rely on aerobic energy metabolism and 
thus ATP. This observation is in agreement with previous observations of reduced $\text{H}^+$ 
secretion which fuels $\text{Cl}^-$ uptake (via anion exchange) across anuran skin under anaerobic 
conditions (21-23) and with findings of $\text{HCO}_3^-$ 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 more than 1.5 fold per 10 ºC increase, are ascribed to 
biological rather than physiochemical processes (19). A 10 ºC reduction in temperature 
resulted in a 1.8-3.0-fold reduction in luminal $\text{HCO}_3^-$ secretion, clearly indicating that 
intestinal $\text{HCO}_3^-$ secretion in the gulf toadfish is biologically mediated. Experiments with 
increased temperature revealed a lower $\text{HCO}_3^-$ secretion response and perhaps a general 
deterioration as indicated by substantial non-recoverable reduction in TEP. This latter 
response may reflect that 35ºC is approaching the thermal maximal tolerance for the gulf 
toadfish which experience temperatures ranging from 13 to 35 ºC in its natural 
environment (3). In any case, the apparent dependence of $\text{HCO}_3^-$ secretion on aerobic 
estrogen metabolism and thus ATP and the strong reduction in secretion rates observed 
with reduced temperature support previous conclusions that intestinal $\text{HCO}_3^-$ secretion in 
marine teleosts is of secondary active nature (15).
The continued, although reduced, HCO$_3^-$ secretion in absence of serosal CO$_2$ and HCO$_3^-$ demonstrate that endogenous CO$_2$ provides a significant source (~50%) 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). It should be noted, however, that the studies on the Japanese eel employed hyper-physiological serosal HCO$_3^-$ levels (25 mM as opposed to the 5-8 mM normally present in teleost fish extracellular fluids (25)). With this in mind it seems that endogenous epithelial CO$_2$ generally contributes significantly to intestinal HCO$_3^-$ secretion in marine teleosts, at least under resting, non-stimulated conditions. The remainder of the studies presented here were designed to investigate the transport processes associated with the secretion of HCO$_3^-$ derived 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 in the order of 0.5 $\mu$mol cm$^{-2}$ h$^{-1}$ under resting conditions in the presence of serosal HCO$_3^-$ . These intestinal HCO$_3^-$ secretion rates from marine teleost fish are approximately 10-fold higher than the only other resting HCO$_3^-$ secretion rate reported from an exothermic vertebrate, the bull frog (11), and are comparable to the range (0.5-1 $\mu$mol cm$^{-2}$ 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 seen in the marine teleost, often at temperatures much below 37°C,
and significant contribution of endogenous CO₂ to luminal alkalinization led to the hypothesis that carbonic anhydrase might be involved in accelerating CO₂ hydration to fuel the apical anion exchange. The lipophilic carbonic anhydrase inhibitor, etoxzolamide, clearly inhibited luminal HCO₃⁻ secretion although a gradual recovery occurred to control secretion rates despite the continued presence of the inhibitor. We interpret this as an immediate depletion of cytosolic HCO₃⁻ caused by carbonic anhydrase inhibition followed by a gradual recovery mediated by non-catalyzed cellular CO₂ hydration perhaps fueled by an increased cellular partial pressure of CO₂. An increase in partial pressure of CO₂ might be expected in a situation with sustained metabolic CO₂ production and absence of carbonic anhydrase activity. What appears to be a complete inhibition of HCO₃⁻ secretion in the first 10 minutes 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 minutes of drug addition at which time a 50% reduction in luminal HCO₃⁻ secretion rates persisted. This observation is in agreement with previous reports of reduced, but not fully inhibited, intestinal HCO₃⁻ secretion by marine teleosts in presence of carbonic anhydrase inhibitors (8; 44) and with the strong physical and functional association between carbonic anhydrase and anion exchangers seen in mammals (35-37).

The H⁺ arising from hydration of CO₂ must be extruded from the epithelial cells to maintain intracellular pH and further, while HCO₃⁻ is excreted across the apical membrane, the H⁺ must be extruded preferentially across the basolateral membrane since the intestinal epithelium exhibits strong net base secretion. This polarization of HCO₃⁻
and H⁺ secretion is demonstrated for the toadfish intestinal epithelium by the reduced HCO₃⁻ 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 HCO₃⁻ and H⁺ secretion is characteristic of the pancreatic duct which secretes HCO₃⁻ to reach concentrations of 70-140 mM (38), values similar in magnitude to HCO₃⁻ concentrations in marine teleost intestinal fluids (14; 25; 26; 46). Manipulation of the H⁺ gradient across the basolateral membrane by reducing serosal pH revealed a concentration-dependent inhibition of luminal HCO₃⁻ secretion which appeared to be at least partly reversible within 60 minutes 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 CO₂ hydration and thus depletes cytosolic HCO₃⁻ available for apical anion exchange. Direct evidence for a full polarization of HCO₃⁻ and H⁺ secretion come from measurements of H⁺ secretion to serosal fluids which match the magnitude of the luminal HCO₃⁻ secretion. The basolateral H⁺ secretion is important for the secondary active apical HCO₃⁻ secretion and must be carrier mediated since it occurs against an electrochemical gradient. Under serosal HCO₃⁻-free conditions, the involvement of a basolateral Na⁺ dependent Cl⁻/HCO₃⁻ exchanger (5) in H⁺ extrusion can be excluded leaving Na⁺/H⁺ exchange and H⁺-ATPase as two possible mechanisms of basolateral H⁺ excretion. The marked reduction in apical HCO₃⁻ secretion in response to lowered serosal Na⁺ concentrations clearly indicate the involvement of a Na⁺-dependent H⁺ extrusion mechanism in HCO₃⁻ secretion by the marine teleost intestine and is
consistent with transport mechanisms involved in rat pancreatic duct HCO₃⁻ secretion (29; 38). However, although the dependence of luminal HCO₃⁻ secretion on serosal Na⁺ strongly suggest the involvement of a basolateral Na⁺/H⁺ exchanger (NHE) like protein, this could not be confirmed pharmacologically since neither serosal amiloride or EIPA at 10⁻³ M influenced luminal HCO₃⁻ secretion. The inhibitor concentrations employed should be sufficient to inhibit H⁺ extrusion via EIPA sensitive NHE isoforms (4) but the lack of amiloride and EIPA effects do not necessarily exclude the involvement of NHE as certain NHE isoforms or spliced variants of NHE isoforms are EIPA insensitive (2; 47).

Serosal addition of the Na⁺/K⁺-ATPase inhibitor ouabain (10⁻³M) results in an approximately 70% inhibition of luminal HCO₃⁻ secretion after 120 minutes. The reduced HCO₃⁻ secretion is likely related to reduced basolateral H⁺ extrusion via Na⁺/H⁺ exchange resulting from the ouabain induced reduction in basolateral electrochemical Na⁺ gradient. The basolateral H⁺ extrusion is necessary for apical HCO₃⁻ secretion as discussed above and this basolateral H⁺ extrusion seems to occur via an EIPA-insensitive Na⁺/H⁺ exchange mechanism. The basolateral Na⁺/H⁺ exchange is driven by the electrochemical Na⁺ gradient established by the basolateral NKA which thereby fuels the apical, secondary active HCO₃⁻ secretion as illustrated in Fig. 11.

Although luminal HCO₃⁻ 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 are not much different. These observations may indicate the recruitment of additional H⁺ extrusion mechanisms in response to reduced extracellular pH.
The substrate for luminal HCO₃⁻ secretion by the gulf toadfish intestine is concluded to be a combination of endogenous epithelial CO₂ hydration and serosal CO₂ and/or HCO₃⁻ with endogenous CO₂ hydration accounting for ~ 50 % of the total HCO₃⁻ secretion rates under resting, *in vivo*-like conditions. The present study demonstrates that the polarized extrusion of H⁺ and HCO₃⁻ (arising from cellular CO₂ hydration) accounts for 50% of the net epithelial secretion of HCO₃⁻. The nature of the remaining 50% of the overall HCO₃⁻ secretion which relies on serosal HCO₃⁻ and possibly CO₂ offers an interesting area for further studies. A Na⁺:HCO₃⁻ cotransporter (NBC) is a likely candidate for basolateral HCO₃⁻ uptake by intestinal epithelial cells (Fig. 11) and could be providing for apical anion exchange as is the case for rat, pig, and guinea pig pancreatic ducts (38) but this possibility remains to be investigated.

High rates of intestinal anion exchange form a significant contribution to marine teleost osmoregulation and can thus be expected to be regulated depending on salt and water balance. In addition, intestinal bicarbonate secretion in fish likely serves the same functions as in mammals where duodenal HCO₃⁻ secretion protects against acidic gastric effluent (6) and can be potently stimulated by multiple factors. The characteristics of the gulf toadfish intestinal HCO₃⁻ secretion described in the present study apply to unstimulated, resting epithelia and HCO₃⁻ secretion by stimulated epithelia may display different characteristics. The involvement of an H⁺-ATPase in H⁺ extrusion and a more significant contribution of transepithelial HCO₃⁻ transport to overall luminal HCO₃⁻ 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 cation 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 approximately 0.3 μmol cm⁻² h⁻¹ which combines with direct measurements of water absorption to yield 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 assuming no buffer capacity) and that the acid-load associated with vital intestinal water absorption must be compensated for to maintain systemic acid-base balance. Interestingly, elevated extra-intestinal 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**

In conclusion, the marine teleost intestine, which is capable of secondary active HCO₃⁻ secretion resulting in luminal concentrations exceeding 100 mM in some cases, displays polarized apical HCO₃⁻ and basolateral H⁺ secretion arising from endogenous CO₂ hydration. Carbonic anhydrase catalyzed CO₂ hydration provides significantly for the apical anion exchange while the electrochemical Na⁺ gradient fuels basolateral H⁺ extrusion via an EIPA-insensitive Na⁺/H⁺ exchange mechanism. Basolateral H⁺ extrusion is required for continued secondary active HCO₃⁻ secretion and the energy for this
transport process is ultimately supplied from the basolateral Na\(^+/K^+\)-ATPase as illustrated by ouabain sensitive HCO\(_3^-\) secretion. Under non-stimulated conditions, transepithelial HCO\(_3^-\) transport possibly via NBC like proteins appears to contribute \(\sim 50\%\) 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. Considering 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\(_3^-\) secretion and/or fluid absorbing epithelia.

**Grants**

This work was supported by a National Science Foundation Grant (0416440) to M. Grosell.
Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Serosal HCO\textsubscript{3}\textsuperscript{-} saline</th>
<th>Serosal HCO\textsubscript{3}\textsuperscript{-}-free saline</th>
<th>Serosal Low Na\textsuperscript{+}</th>
<th>Serosal buffer-free saline</th>
<th>Mucosal saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>151.00</td>
<td>151.00</td>
<td>0.00</td>
<td>151.00</td>
<td>69.00</td>
</tr>
<tr>
<td>N-methyl-D-glucamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>5.00</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO\textsubscript{4}</td>
<td>0.88</td>
<td>0.88</td>
<td>0.88</td>
<td>0.88</td>
<td>77.50</td>
</tr>
<tr>
<td>Na\textsubscript{2}HPO\textsubscript{4}</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>CaCl\textsubscript{2}</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>5.00</td>
</tr>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>5.00</td>
<td>5.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES (free acid)</td>
<td>11.00</td>
<td>11.00</td>
<td>11.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES (Na-salt)</td>
<td>11.00</td>
<td>11.00</td>
<td>11.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>4.50</td>
<td>4.50</td>
<td>4.50</td>
<td>4.50</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Osmolality\textsuperscript{1}</td>
<td>~330</td>
<td>~330</td>
<td>~330</td>
<td>~330</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.80</td>
<td>6.60, 7.00\textsuperscript{2}</td>
<td>7.80\textsuperscript{2}</td>
<td>7.80\textsuperscript{2}</td>
<td>7.80\textsuperscript{2}</td>
</tr>
<tr>
<td>Gassing</td>
<td>0.3%CO\textsubscript{2} in O\textsubscript{2}</td>
<td>0.3%CO\textsubscript{2} in O\textsubscript{2}</td>
<td>0.3%CO\textsubscript{2} in O\textsubscript{2}</td>
<td>O\textsubscript{2}</td>
<td>O\textsubscript{2}</td>
</tr>
</tbody>
</table>

Saline composition employed in pH-stat experiments (in Mm). The composition of the mucosal saline is based in measured composition of intestinal fluids obtained from unfed toadfish (39).

\textsuperscript{1} (in mosm/l) Adjusted with mannitol to ensure transepithelial iso-omotic conditions in all experiments.

\textsuperscript{2} A pH of 7.800 was maintained by pH-stat titration.

\textsuperscript{3} The pH was adjusted with HCl under continuous O\textsubscript{2} or N\textsubscript{2} gassing.
Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Anterior toadfish intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO₃⁻ secretion (μmol·cm⁻²·h⁻¹)</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>TEP (mV)</td>
<td>-21.6 ± 2.3</td>
</tr>
<tr>
<td>G (mS·cm⁻²)</td>
<td>8.4 ± 0.4</td>
</tr>
</tbody>
</table>

Mean (± SEM, n=8) HCO₃⁻ secretion, transepithelial potential (TEP) and conductance (G) of isolated toadfish intestinal epithelium mounted in Ussing chambers. The HCO₃⁻ secretion rates were determined from the increase in titratable alkalinity, measuring HCO₃⁻ and CO₃²⁻ equivalents, of the mucosal saline during a three hour period.
Figure legends

**Fig. 1.** Bicarbonate secretion (µmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mS cm⁻²) of isolated anterior toadfish intestine under asymmetrical conditions mimicking the *in vivo* intestinal fluid chemistry. Serosal salines were replaced after 60 and 180 minutes and contained 0.1 % DMSO during the time from 60 to 180 minutes. Based on secretion rates and electrophysiological parameters the preparation appears stable for > 5 hours. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.37 ± 0.01, -18.1 ± 0.1 and 11.6 ± 0.1, respectively. Mean ± SEM (n=6).

**Fig. 2.** Bicarbonate secretion (µmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mS cm⁻²) of isolated anterior toadfish intestine under normoxic and anoxic conditions. Both serosal and mucosal salines were gassed with O₂ from 0 to 60 min and from 120 to 180 minutes and with nitrogen from 60 to 120 minutes. Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student *t*-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.35 ± 0.01, -19.1 ± 0.1 and 10.0 ± 0.2, respectively. Mean ± SEM (n=5).

**Fig. 3.** Bicarbonate secretion (µmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mS cm⁻²) of isolated anterior toadfish intestine at 15 and 35°C. Left panels: an initial 60 minute period at 25°C was followed by 120 minutes of gradual temperature decrease to 15°C and a final 120 minute period of return to 25°C (n=7). Right
panels: an initial 60 minute period at 25°C was followed by 120 minutes of gradual temperature increase to 35°C and a final 120 minute period of return to 25°C (n=6). Bars or symbols under or over horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student \( t \)-test. Average values for last 30 minutes of initial control period for the 15 ºC series for bicarbonate secretion, TEP and G were 0.31 ± 0.01, -23.0 ± 0.1 and 11.6 ± 0.1, respectively and 0.31 ± 0.01, -19.5 ± 0.3 and 11.1 ± 0.1 for the 35 ºC series. Mean ± SEM (n=5).

Fig. 4. Bicarbonate secretion (µmol cm\(^{-2}\) h\(^{-1}\), top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm\(^{-2}\)) of isolated anterior toadfish intestine in presence and absence of serosal HCO\(_3\)\(^-\) and CO\(_2\). During the initial 60 minutes and the last 60 minutes from 120 to 180 minutes, serosal salines contained no HCO\(_3\)\(^-\) and were gassed with pure O\(_2\). During the 60 minutes from 60 to 120 minutes, the serosal saline contained 5 mM HCO\(_3\)\(^-\) and was gassed with 0.3% CO\(_2\) in O\(_2\). Both serosal salines contained HEPES and were adjusted to pH 7.800. Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student \( t \)-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.18 ± 0.01, -17.3 ± 0.1 and 6.3 ± 0.1, respectively. Mean ± SEM (n=7).

Fig. 5. Bicarbonate secretion (µmol cm\(^{-2}\) h\(^{-1}\), top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm\(^{-2}\)) of isolated anterior toadfish intestine under control
conditions and in the presence of the carbonic anhydrase inhibitor etoxzolamide (10^{-3}\, \text{M}) in the luminal saline. Addition of etoxzolamide caused a slight reduction in luminal pH (~0.2 pH units) and HCO_3^- secretion therefore could not be measured during the first 10-15 minutes until luminal pH recovered to 7.8. Thus, no HCO_3^- secretion data is reported for the first two time intervals. Bars under horizontal line and individual time points marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student \(t\)-test. Mean ± SEM (\(n=7\)). Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.21 ± 0.01, -17.3 ± 0.1 and 6.3 ± 0.1, respectively. Mean ± SEM (\(n=6\)).

**Fig. 6.** Bicarbonate secretion (\(\mu\text{mol cm}^{-2} \text{ h}^{-1}\), top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm^{-2}) of isolated anterior toadfish intestine under control conditions (serosal pH 7.800) and serosal pH 7.400 (top left panels), pH 7.000 (top right panels), pH 6.600 (bottom left panels). Fractional maximal inhibition of mucosal bicarbonate secretion as a function of serosal pH is displayed in the bottom right panel. Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student \(t\)-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G for the pH 7.4 series were 0.23 ± 0.01, -19.0 ± 0.2 and 10.7 ± 0.1, respectively, 0.22 ± 0.01, -20.9 ± 0.2 and 10.0 ± 0.1 for the pH 7.0 series and 0.24 ± 0.01, -19.4 ± 0.2 and 10.9 ± 0.3 for the pH 6.6 series. Mean ± SEM (\(n=5-8\)).
**Fig. 7.** Mucosal bicarbonate secretion (μmol cm⁻² h⁻¹; left panel, n=42) and serosal H⁺ extrusion (μmol cm⁻² h⁻¹; right panel, n=6) of isolated anterior toadfish intestine at luminal and serosal pH of 7.800. Mean ± SEM.

**Fig. 8.** Bicarbonate secretion (μmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm⁻²) of isolated anterior toadfish intestine under control conditions (serosal [Na⁺] = 162 mM) and low Na⁺ conditions (11 mM). Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student t-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.16 ± 0.01, -21.2 ± 0.2 and 8.9 ± 0.1, respectively. Mean ± SEM (n=7).

**Fig. 9.** Bicarbonate secretion (μmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm⁻²) of isolated anterior toadfish intestine under control conditions and in the presence of the Na⁺:H⁺ exchange inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA;10⁻³ M) in the serosal saline. Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student t-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.35 ± 0.01, -22.8 ± 0.2 and 10.3 ± 0.2, respectively. Mean ± SEM (n=6).

**Fig. 10.** Bicarbonate secretion (μmol cm⁻² h⁻¹, top panel), transepithelial potential (TEP, mV) and conductance (G, mSi cm⁻²) of isolated anterior toadfish intestine under control
conditions and in the presence of the Na\(^+\):K\(^+\)-ATPase (NKA) inhibitor ouabain (10\(^{-3}\) M) in the serosal saline. Bars under horizontal line marked by “*” are significantly different from control values (average of the last 30 minutes of initial control period), paired student \(t\)-test. Average values for last 30 minutes of initial control period for bicarbonate secretion, TEP and G were 0.35 ± 0.01, -20.7 ± 0.2 and 9.3 ± 0.3, respectively. Mean ± SEM (n=6).

**Fig. 11.**

Schematic transport model of HCO\(_3\)^\(-\) secretion by the Gulf toadfish intestinal epithelium. The model includes polarized apical Cl\(^-\)/ HCO\(_3\)^\(-\) exchange and basolateral EIPA-insensitive Na\(^+\)/H\(^+\) exchange which rely on the Na\(^+\) gradient established by the Na\(^+\):K\(^+\) ATPase. Hydration of endogenous CO\(_2\), partly catalyzed by carbonic anhydrase, supplies the majority of the cellular substrate for apical anion exchange. In addition, basolateral HCO\(_3\)^\(-\) uptake possible via Na\(^+\): HCO\(_3\)^\(-\) co-transport maybe involved in apical HCO\(_3\)^\(-\) secretion.


Fig. 1

HCO$_3^-$ secretion (µmol cm$^{-2}$ h$^{-1}$)

TEP (mV)

G (mSi cm$^2$)

Time (min)
Fig. 3.
Fig. 4.

- Top graph: Histograms showing HCO$_3^-$ secretion (µmol cm$^{-2}$ h$^{-1}$) over time (min).
- Middle graph: Graph showing TEP (mV) over time (min).
- Bottom graph: Graph showing G (mS cm$^{-2}$) over time (min).
Fig. 5.

- HCO₃⁻ secretion (µmol cm⁻² h⁻¹)
  - Etoxolamide (10⁻³ M)

- TEP (mV)
  - Etoxolamide (10⁻³ M)

- G (mSi cm⁻²)
  - Etoxolamide (10⁻³ M)
Fig. 6.
Fig. 7.

[Diagram showing the biochemical reactions involving CO₂, H₂O, HCO₃⁻, H⁺, and Cl⁻.]

Mucosal HCO₃⁻ secretion (µmol cm⁻² h⁻¹)

Serosal H⁺ secretion (µmol cm⁻² h⁻¹)
Fig. 8.

**HCO₃⁻ secretion (µmol cm⁻² h⁻¹)**

- **11 mM Na⁺**

**TEP (mV)**

- **11 mM Na⁺**

**G (mS/cm²)**

- **11 mM Na⁺**
Fig. 9.

- HCO$_3^-$ secretion (µmol cm$^{-2}$ h$^{-1}$)

- TEP (mV)

- G (mS/cm$^2$)

All graphs show the effect of EIPA ($10^{-3}$ M) on the indicated parameters over time (min) from 0 to 180 minutes.
Fig. 10.

Top graph: HCO$_3^-$ secretion (µmol cm$^{-2}$ h$^{-1}$) vs. Time (min) with Ouabain (10$^{-3}$ M) treatment.

Middle graph: TEP (mV) vs. Time (min) with Ouabain (10$^{-3}$ M) treatment.

Bottom graph: G (mS/cm$^2$) vs. Time (min) with Ouabain (10$^{-3}$ M) treatment.
Fig. 11.

Lumen  Extracellular fluid

HCO$_3^-$  Na$^+$

CO$_2$+H$_2$O

CA

HCO$_3^-$  H$^+$

Cl$^-$  Na$^+$

Na$^+$  K$^+$

Lumen  Extracellular fluid

HCO$_3^-$  Na$^+$