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Ionic transport by the opercular epithelia of freshwater acclimated tilapia (*Oreochromis niloticus*) and killifish (*Fundulus heteroclitus*)

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Abstract

Opercular epithelia from freshwater (FW)-acclimated *Oreochromis niloticus* and *Fundulus heteroclitus* were mounted in vitro with natural FW bathing the mucosal surface and saline bathing the serosal surface. Opercular epithelia from *Oreochromis*, a true FW fish, exhibited an inside-positive transepithelial potential (V_t) of +8 mV and non-diffusive uptake of both Cl^- and Na^+, as well as Ca^{2+}, from the mucosal FW media. When bathed with saline on the mucosal surface, active Na^+ and Cl^- uptake did not occur. To test whether exposure of the mucosal surface to saline during the dissection procedure was a problem, FW osmotically compensated with mannitol was used instead as the dissection medium. However ion transport rates were not altered. Opercular epithelia from FW-acclimated *Fundulus*, which is more normally an estuarine rather than a FW fish, exhibited an inside-negative V_t of −44 mV, 4–10-fold larger absolute flux rates of Na^+ and Cl^-, and a 40-fold greater density of mitochondrial-rich cells than the *Oreochromis* preparation. However, only non-diffusive uptake of Cl^- from the mucosal FW occurred, while Na^+ moved passively. Opercular epithelia from *Fundulus* acclimated to 10% seawater (SW) actively extruded Cl^- when bathed with either 10% SW or saline on the mucosal side; Na^+ moved passively under both experimental conditions. The FW *Oreochromis* opercular skin is the only teleost flat epithelial preparation known to actively take up all three ions from FW in vitro and may be a good model system for studying the mechanisms of Cl^-, Na^+ and Ca^{2+} transport in FW fish gills.

Keywords: Tilapia; Killifish; Opercular epithelia; Mitochondria-rich cell; Ussing chamber; Ion transport; Transepithelial potential; Freshwater gill model

1. Introduction

There is now a generally accepted model for the mechanisms of ion transport in the gills of seawater teleosts (see [5,21,25] for recent reviews). Historically, the genesis of this model was largely due to flat epithelial preparations which allowed mechanistic study of transport details in vitro. These in vitro studies used opercular epithelia from the killifish *Fundulus heteroclitus* [1–4,11–13,41,42] and the Mozambique tilapia *Sarotherodon* (= *Oreochromis*) *mossambicus* [6–8,33], and the jaw skin epithelium of the goby *Gillichthys mirabilis* [18,19].

In contrast, our understanding of the mechanisms of ion transport in the gills of freshwater teleosts remains controversial and incomplete (see [5,16,29] for recent reviews). While it is clear that freshwater fish can take up Na^+ and Cl^- actively and independently from the dilute external environment, there are competing theories as to the mechanisms and sites of Na^+ uptake, a complete lack of knowledge on the energetic basis of Cl^- uptake, and considerable uncertainty as to the mechanisms by which Na^+ and Cl^- fluxes are linked to acidic and basic equivalent fluxes respectively [9,28,29,32,38]. To a large extent, this deficit of under-

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standing can be linked to the absence of suitable flat epithelial models for in vitro study of the freshwater gill.

To date, the search for such freshwater model preparations has been disappointing. Foskett et al. [6] found no apparent active Cl^- uptake across the opercular epithelium of freshwater-adapted *O. mossambicus*, and Marshall et al. [20] found no apparent active Na^+ or Cl^- uptake across the cleithral epithelium of freshwater-adapted rainbow trout *Oncorhynchus mykiss*, despite the fact that both preparations exhibited populations of mitochondrial-rich (MR) cells. Recently, Marshall et al. [23] studied the opercular epithelium of freshwater-adapted *F. heteroclitus*, which has a much higher density of MR cells than the other two preparations. When both mucosal and serosal surfaces were bathed with isotonic saline, active uptake of both Na^+ and Cl^- occurred. However when the mucosal surface was bathed with freshwater, only the active uptake of Cl^- could be documented. Interestingly, active Ca^{2+} uptake from freshwater has been reported in skin preparations from trout [20], killifish [22] and Nile tilapia *Oreochromis niloticus* [24].

The present study extends the search for a suitable freshwater model by investigating the properties of the opercular epithelium of *O. niloticus*, and re-investigating the opercular epithelium of the freshwater-adapted *F. heteroclitus* originally studied by Marshall et al. [23]. With *O. niloticus*, our primary objective was to test whether Na^+ and/or Cl^- uptake could be detected across the opercular epithelium of freshwater-adapted animals. Inasmuch as the Nile tilapia is native to freshwater, unlike the killifish which typically lives and reproduces in estuarine brackish waters [34], its epithelium might be a more promising candidate to exhibit active uptake of both Na^+ and Cl^-. The second, related objective was to confirm the occurrence of active Ca^{2+} uptake across the opercular epithelium of *O. niloticus* [24]. A third objective was to test whether avoiding exposure to high external NaCl concentrations (i.e. isotonic saline) during dissection and preparation would promote Na^+ and Cl^- transport in the tilapia epithelium, because Karnaky [14] has suggested that the morphology and physiology of the epithelium, particularly the role of the tight junctions, may change rapidly in response to external salinity.

With *F. heteroclitus*, our first goal was to re-evaluate the conclusions of Marshall et al. [23] using a different batch of killifish and different freshwater quality (a natural freshwater with lower Na^+ and Cl^- but higher Ca^{2+} concentrations than the synthetic freshwater used in [23]). The second was to evaluate whether acclimation and testing in a more moderate salinity (10% seawater) would promote active Na^+ and Cl^- uptake in the preparation, because the killifish is more commonly an estuarine rather than a freshwater inhabitant [34].

2. Materials and methods

2.1. Animals

Adult killifish (*F. heteroclitus*, 3–10 g) were obtained from the Antigonish estuary (Antigonish, N.S.), transferred directly to holding tanks that were 10% seawater (3.0–3.2 ppt), and then air-freighted to Hamilton, Ont. In Hamilton, the fish were maintained in 10% synthetic seawater (Marinemix, Baltimore, MD) in a 500-l aerated, charcoal-filtered system, but transferred to freshwater at least 2 weeks prior to use. During freshwater acclimation, the killifish were held under 14 h light/10 h dark at 20 ± 1°C in dechlorinated Hamilton tapwater with an average composition (in mmol l^{-1}) of Na^+, 0.6; Cl^-, 0.7; Ca^{2+}, 1.05; and pH 7.5–8.0. The 60-l acclimation tanks were well aerated and were operated as charcoal-filtered recirculation systems, renewed weekly. Killifish were fed with commercial food (Wardley, Secaucus, NJ) at a rate of 1% of their body mass per day.

Nile tilapia (*O. niloticus*, 10–15 g) were obtained from Northern Tilapia, Lindsay, Ont., and were kept at 30 ± 1°C in a 500-l tank supplied at a rate of 50 ml min^{-1} with a flow-through of dechlorinated Hamilton tapwater, charcoal filtration, aeration, and 14 h light/10 h dark photoperiod. The tilapia were fed commercial trout pellets (Zeigler, Hazelton, PA) at a rate of 1% of their body mass per day.

2.2. Bathing solutions

A modified Cortland saline solution [37] used for dissection and for the basolateral bathing solution was composed of (in mmol l^{-1}) NaCl, 129.9; KCl, 2.55; $CaCl_2.H_2O$, 1.56; $MgSO_4.7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4.H_2O$, 2.97; glucose, 5.55; and NH_4Cl, 0.30 (all salts from Sigma). To substitute for plasma protein, the saline was supplemented with 20 mg ml^{-1} bovine serum albumin. The saline was equilibrated with a 0.3% CO_2, balance O_2, gas mixture and had a measured pH of 7.8–7.9.

In open-circuit experiments using asymmetrical bathing solutions, the solution for the mucosal surface of the membrane was taken from either the freshwater entering the holding tank or directly from the 10% seawater in the holding tank, and was equilibrated with 100% O_2 before use in the Ussing chamber. Prior to the start of the experiment and at the end of each 60 min flux period (see below), the mucosal compartment was rinsed extensively to maintain the original ion composition of the freshwater (Na^+, 0.7; Cl^-, 0.6; and Ca^{2+}, 1.05 mmol l^{-1}) or 10% seawater (Na^+, 47.0; Cl^-, 54.8; and Ca^{2+}, 1.02 mmol l^{-1}).

2.3. Isolated opercular epithelium preparation

The fish were killed by pithing and both opercular membranes were immediately dissected from their underlying opercular bone, teasing off the epithelia in a ventral–anterior to dorsal–posterior direction. Prior to dissection, mucus was carefully removed using a fine forceps. Each opercular epithelium yielded an approximate area of 1–2 cm^2. Throughout the dissection, the preparation was kept moist by the addition of saline.

The Ussing membrane apertures (0.125 cm^2) were prepared with stopcock grease (Dow Corning, Midland, MI) and a thin vinyl mesh was placed over each aperture to support the tissue. The opercular epithelium was carefully pinned down to the aperture mucosal side up, and then the second half of the aperture was used to sandwich the epithelium. After mounting, the mucosal surfaces were thoroughly rinsed with fresh water to remove the saline and any mucus that had accumulated during the dissection. The aperture sandwich was then placed between the two hemi-chambers and tightened into place. The hemi-chambers were filled with the appropriate solutions: freshwater or saline or 10% seawater for the mucosal side, and saline for the serosal side. Each hemi-chamber was filled at an equal rate to ensure there was no 'bagging' of the epithelium. The time between the start of dissection and completion of mounting was 12–15 min, and the membranes were then given a 30 min period to adjust to the in vitro conditions.

2.4. Mannitol dissection procedure

In one set of experiments on *Oreochromis* opercular epithelia, 250 mM mannitol (Sigma) was added to freshwater to serve as the dissection medium in place of saline. The goal here was to maintain the correct freshwater NaCl concentration yet at the same time provide a normal extracellular fluid osmotic pressure which would avoid osmotic stress to the basolateral surface.

2.5. Electrophysiology

Polyethylene 4% agar–saline bridges were used to measure transepithelial potential (V_t, mucosal side grounded) and membrane conductance (G_t). Each bridge was connected to the current–voltage clamp (WP Instruments, New Haven, CT, DVC-1000) by Hg/HgCl calomel half-cells. Membrane conductance was corrected for solution resistances. In asymmetrical solutions, corrections for liquid junction potentials were accomplished by measurements against a free flowing 3 M KCl half-cell. The Ussing chambers were water-jacketed to the killifish and tilapia acclimation temperatures at 20 ± 1 and 30 ± 1°C, respectively. In some flux experiments, the preparation was short-circuited (with saline bathing the mucosal surface) to negate any effect of V_t on ion transport. G_t was determined by clamping the membrane to a set voltage every 10 min, and V_t was recorded at the beginning and end of each 60 min short-circuited flux period.

2.6. Experimental protocol

One chamber was set up for each opercular epithelium for pair-wise measurements of influx and efflux. Each fish therefore yielded a mucosal to serosal unidirectional influx (J^{ms}) measurement and a serosal to mucosal unidirectional efflux (J^{sm}) measurement. After the initial 30 min adjustment period, the mucosal hemichamber volume was gently rinsed again with freshwater (20 times the chamber volume) and isotope was added to the appropriate side. In all cases, the preparations were set up so that radioisotope was added to the hemichamber with the larger volume (4.5 ml), and unidirectional flux was measured by monitoring the appearance in the hemi-chamber of smaller volume (3 ml).

Unidirectional fluxes were calculated from the measured specific activity on the labelled side and the measured appearance of isotope on the unlabelled side. Dual flux experiments were performed with ^{36}Cl ($Na^{36}Cl$ from I.C.N. Radiochemicals, Irvine, CA) and ^{22}Na ($^{22}NaCl$ from NEN-Dupont, Boston, MA) which were added to either the mucosal side (final specific activity of at least 300000 cpm μmol^{-1}) for the unidirectional influx or serosal side (at least 8000 cpm μmol^{-1}) to monitor the unidirectional efflux. In Ca^{2+} flux experiments, the final specific activity of ^{45}Ca ($^{45}CaCl_2$ from NEN-Dupont, Boston, MA) was 10–100-fold higher. Upon first addition of the radioisotope(s), a 45 min equilibration period was employed. Each experiment consisted of three 60 min periods, at the start and end of which samples were taken from the labelled side to determine the specific activity and ionic concentrations. Within each period, samples were taken from the unlabelled bath every 20 min for radioactivity analyses. Flux values for each 20 min segment were averaged to produce a final flux rate for each 60 min experimental period. Between periods, the chambers were thoroughly flushed, and new solutions with isotope were added and equilibrated for at least 45 min.

The observed flux ratio (J^{ms}/J^{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation [36]. Disagreement between the observed and predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was as follows:

$$\frac{J_i^{ms}}{J_i^{sm}} = \left(\frac{a_i^m}{a_i^s}\right) e^{(z_i F V_t / RT)}$$

.The activities of ion i are a_i^s and a_i^m on the serosal and mucosal sides respectively; valency of the ion is z_i, V_t is the transepithelial potential and F, R and T have their usual thermodynamic definitions. The ionic activities of Na^+ (109.5 mmol l^{-1}) and Ca^{2+} (0.79 mmol l^{-1}) in Cortland saline and Na^+ (42.2 mmol l^{-1}) in 10% seawater were taken from measurements with microelectrodes filled with the appropriate ionophore [35], while Cl^- was predicted to have the same relative activity (on a percent basis) as Na^+ from the theory for solutions of these ionic strengths [15]. The presence of 20 mg ml^{-1} of bovine serum albumin lowered the activity of Ca^{2+} in the saline, but Na^+ and Cl^- activity were unaffected. The ionic activities of Na^+, Cl^- and Ca^{2+} in freshwater were taken as equal to their measured concentrations.

2.7. Fluorescence microscopy

Opercular epithelia from the Ussing chamber were incubated for 30 min in oxygenated saline with 10 μmol l^{-1} DASPEI (2-(4-dimethylaminostyrl)-*N*-ethylpyridium iodide; I.C.N Biomedicals, Costa Mesa, CA) (a mitochondrial fluorophore) to determine the density of mitochondrial-rich (MR) cells. The membranes were then viewed as wet mounts on an epifluorescence microscope (Zeiss, Germany) with an excitation wavelength of 450–490 nm. Cell counts were made in the membrane area within the Ussing chamber aperture over four randomly chosen fields at a magnification of 160 (1.7 mm^2 field of view) and the quadruplicate values were then averaged to yield a density of MR cells mm^{-2}.

2.8. Analytical techniques

Saline Cl^- concentrations were measured by coulometric titration (model CMT10, Radiometer, Copenhagen) and freshwater Cl^- concentrations were determined by colorimetric assay [43]. Na^+ and Ca^{2+} concentrations in both media were analyzed by atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia). Samples of 40 μl from the labelled side and 250 μl from the unlabelled side were added to 4.0 ml Readysafe fluor (Beckman, Fullerton, CA). Radioactivities of ^{36}Cl and ^{45}Ca were determined by counting on a Rackbeta 1217 liquid scintillation counter (LKB-Wallac, Turku, Finland), and ^{22}Na radioactivity was counted on Minaxi Autogamma 5000 counter (Packard Instrument, Downers Grove, IL). ^{22}Na emits both γ and β radiation, therefore scintillation counts collected from a ^{22}Na and ^{36}Cl dual flux experiment were from both ^{22}Na and ^{36}Cl. ^{36}Cl counts were determined by a count subtraction procedure. This was accomplished by measuring the cpm of a known concentration of ^{22}Na in both the scintillation and γ counters and then determining the relative efficiency of the two counters for detecting ^{22}Na. The cpm from the γ counter was then multiplied by this ratio and subtracted from the cpm of the scintillation counter to yield the β emission of ^{36}Cl only. Each sample was counted to an accuracy of 1 S.D., rather than for a fixed time period, so in instances where flux rates were low, counting periods were several hours.

Data are presented as means $\pm$ 1 S.E. unless otherwise indicated. Comparisons within and between treatments were analyzed by paired or un-paired t-tests (two-tailed; $P < 0.05$), as appropriate. In cases where the F-test indicated significant differences in variance, the data were transformed (log or arc-sine) to correct this problem prior to test.

3. Results

3.1. Oreochromis opercular epithelium

The freshwater-acclimated *Oreochromis* opercular epithelium, bathed on the mucosal surface with freshwater, exhibited an inside-positive transepithelial potential (V_t) of about $+8$ mV, and a transepithelial conductance (G_t) of approximately 2 $mS_i\ cm^{-2}$ (Table 1). Over time, the unidirectional Cl^- influx (J^{ms}) was more or less stable, while the Na^+ influx (J^{ms}) showed a steady significant decrease. Notably, the observed flux ratios for both Na^+ and Cl^- were significantly greater ($P < 0.05$) than the flux ratios predicted on the basis of passive diffusion by the Ussing flux ratio equation (Fig. 1A), thus indicating active absorption of both ions from the dilute mucosal freshwater. However, the net balance for both ions remained highly negative throughout the experimental period as unidirectional effluxes (J^{sm}) exceeded influxes (J^{ms}) by a considerable margin.

Net uptake of Ca^{2+} occurred in each of the three experimental periods. Ca^{2+} influx was against both concentration and electrical gradients and was almost twice the magnitude of Ca^{2+} efflux (Table 1). The much greater observed flux ratio of Ca^{2+} (Fig. 1A) than predicted by the Ussing equation indicated active transport of this ion.

When saline was placed on both surfaces, the G_t of the *Oreochromis* opercular membrane increased approximately 8-fold, while V_t was reduced to approximately zero; the short-circuit current was less than 1 $\mu A_i\ cm^{-2}$ (Table 1). There was no net movement of either Na^+ or Cl^- that was significant in any direction and the flux ratios for Cl^- and Na^+ were not significantly different from the predicted flux ratios of unity, suggesting that both ions were passively distributed under these conditions.

Table 1
Unidirectional efflux (J^{sm}) and influx (J^{ms}) of Cl^-, Na^+ and Ca^{2+} in opercular epithelia of fresh water *O. niloticus* ($n = 6$) bathed on the mucosal side with fresh water. Open circuit conditions, except as noted.

Bathing solutions (serosa/mucosa)	V_t (mV)[a]	G_t (mS cm^{-2})[b]	Hour 1[c]		Hour 2[c]		Hour 3[c]	
			J^{sm}	J^{ms}	J^{sm}	J^{ms}	J^{sm}	J^{ms}
Freshwater *Oreochromis* (saline/freshwater)								
Cl^-	$+8.0 \pm 0.6$	1.78 ± 0.19	752 ± 122	30.4 ± 6.0	626 ± 158	23.5 ± 2.6	727 ± 147	27 ± 4.0
Na^+			520 ± 54	22.3 ± 2.6	552 ± 72	13.1 ± 4.0	587 ± 91	7.6 ± 3.0
Ca^{2+}			4.4 ± 0.6	7.1 ± 0.4	4.7 ± 0.6	7.6 ± 0.8	4.5 ± 0.8	8.4 ± 1.0
250 mM mannitol dissection media (saline/freshwater)								
Cl^-	$+7.9 \pm 0.3$	0.95 ± 0.33	1294 ± 313	48 ± 7	1230 ± 325	47 ± 12	1296 ± 197	39 ± 16
Na^+			798 ± 208	21 ± 7	960 ± 203	21 ± 4	887 ± 115	16 ± 4
Saline/saline (short circuit conditions)								
Cl^-	0 ± 0	14.48 ± 1.72	12047 ± 3162*	11267 ± 1494*	13801 ± 4849*	8971 ± 1798*	7281 ± 2137*	8082 ± 2051*
Na^+			9044 ± 1820*	7413 ± 999*	10607 ± 3316*	6410 ± 1171*	4720 ± 1675*	5948 ± 1430*

[a] Transepithelial potential (mucosal ground) corrected for junction potentials ($n = 24$).
[b] Tissue conductance corrected for solution resistance ($n = 24$).
[c] Units are in nmol cm^{-2} h^{-1}.
* Un-paired t-test, two-tailed. Comparison of mean flux values relative to the control saline/freshwater preparation; * $P < 0.05$.

To evaluate whether exposure to high NaCl levels on the mucosal surface during dissection was a problem, freshwater was osmotically compensated with mannitol and then used in place of saline in the *Oreochromis* dissection procedure. Unidirectional influx and effluxes of Na^+ and Cl^- were not significantly affected. The observed Na^+ and Cl^- flux ratios were again significantly different from their respective predicted ratios (Fig. 1B), and the magnitude of the observed ratios were almost identical to those seen during the normal saline dissection procedure (Fig. 1A).

3.2. Fundulus opercular epithelium

Opercular epithelia from freshwater-acclimated *Fundulus*, bathed on the mucosal side with freshwater, exhibited a highly negative V_t at open-circuit of about -44 mV, very different from the positive value of $+8$ mV in the *Oreochromis* preparation, but a very similar G_t of approximately 2 mS$_i$ cm^{-2} (Table 2). The unidirectional influx values for Cl^- generally decreased over time, however the difference was not significant from period 1 to period 3 ($P > 0.05$). The efflux was relatively constant and about 10-fold greater than the unidirectional influx; thus there was also a net loss of Cl^- over each of the three 1 h flux periods. There was a substantial difference between the observed and predicted flux ratios for Cl^- ($P < 0.05$) averaged over the three periods (Fig. 2A), thereby indicating active chloride absorption by the tissue according to the Ussing flux ratio criterion.

The unidirectional influx and efflux values for Na^+ were comparable to those for Cl^- (Table 2); thus the epithelium was also losing Na^+ over each of the three 1 h periods. The observed and predicted flux ratios for Na^+ were not significantly different, indicating passive movement of this ion (Fig. 2A).

Opercular epithelia of *Fundulus* acclimated to 10% seawater (with 10% seawater bathing the mucosal surface and the preparation at open circuit) exhibited a slightly positive V_t of about $+3$ mV in contrast to the very negative V_t seen with freshwater membranes (Table 2). However the G_t (approximately 2 mS$_i$ cm^{-2}) was not different from freshwater membranes. This preparation displayed significant net extrusion of Cl^- into the mucosal 10% seawater, with a similar, but non-significant trend for net extrusion of Na^+. Flux ratio analysis indicated passive diffusion of Na^+ and active extrusion of Cl^- (Fig. 2B). This conclusion was confirmed by placing the epithelia from 10% seawater acclimated *Fundulus* ($n = 6$) under short-circuit conditions with saline bathing both surfaces. V_t increased to $+9.5 \pm 1.9$ mV (prior to short-circuit), G_t was significantly greater at 5.97 ± 0.89 mS$_i$ cm^{-2}, and the short-circuit current was 34.08 ± 2.47 μA$_i$ cm^{-2} (not shown). Under these conditions, the epithelia exhibited higher unidirectional fluxes for both Na^+ and Cl^-, a clear net extrusion of Cl^- into the mucosal saline, but no significant net flux of Na^+ (see hour 3 in the bottom part of Table 2). The observed flux ratio for Cl^- ($J^{ms}/J^{sm} \simeq 3$) was significantly different from the predicted ratio of unity, confirming the active secretion of Cl^-, whereas the observed flux ratio for Na^+ was not significantly different from unity, confirming passive distribution of this cation (not shown).

3.3. MR cells

The freshwater *Oreochromis* epithelium contained a MR cell density of 46 ± 3 mm^{-2} ($n = 6$) in the area of the Ussing chamber aperture. The freshwater-acclimated *Fundulus* opercular epithelium exhibited an MR cell density which was almost 40-fold greater at 1868 ± 87 cells mm^{-2} in the same region ($n = 6$).

4. Discussion

4.1. Ussing flux ratio analysis, diffusion potentials, and active transport

A potential limitation of an analysis, such as the present one, based solely on the Ussing flux ratio criterion, is that it may be overly conservative [36]. The ratio must be exceeded to prove that the transport

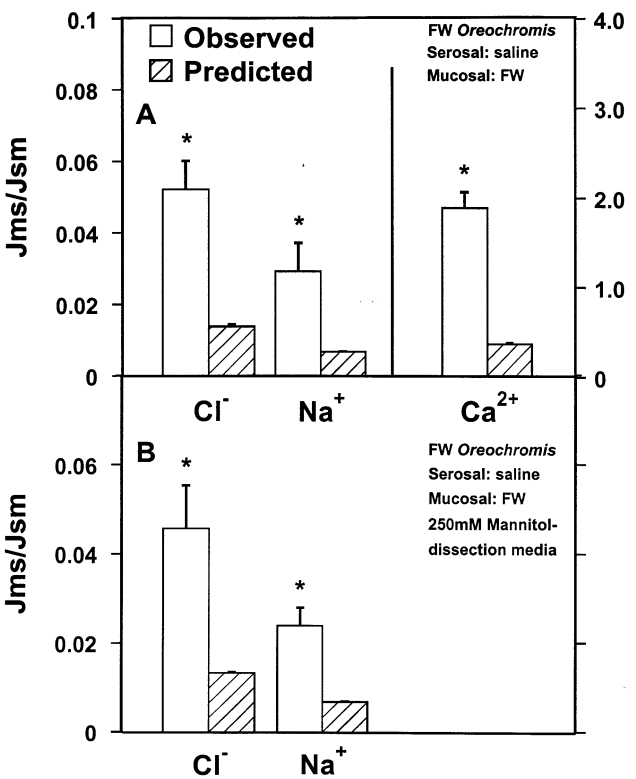

Fig. 1. (A) Observed and predicted flux ratios for the unidirectional movements of Cl^-, Na^+ and Ca^{2+} in isolated opercular epithelium of freshwater *O. niloticus* ($n = 6$). Values were averaged for all three periods for each experiment, then averaged for all six experiments. The open bars represent the observed flux ratios for Cl^-, Na^+ and Ca^{2+}, the hatched bars represent the predicted flux ratios for Cl^-, Na^+ and Ca^{2+} based on the Ussing flux ratio equation. (B) Observed and predicted Cl^- and Na^+ flux ratios for the isolated opercular epithelium of *Oreochromis* using freshwater osmotically compensated with 250 mM mannitol, instead of saline during the dissection ($n = 6$). Other methods the same as in (A). The asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), implying non-diffusive transport.

situation cannot be explained solely by passive diffusion. It is possible that the existence of active transport may be masked by large diffusion potentials, which must be taken into account in the calculation. This is especially likely in situations such as the present study when the epithelia separates solutions of vastly different ionic compositions (saline versus freshwater]. Nevertheless, this is exactly the situation applying in vivo. Accepting this limitation is an unavoidable cost of duplicating in vivo conditions; one way around the problem is to employ metabolic inhibitors to reveal active transport processes, but non-specific effects of such drugs are often a complicating factor. Diffusion potentials in freshwater gills [31] and opercular epithelia [23] are generally believed to be responsible for most of the transepithelial potential (V_t), and are thought to be due to the differential passive permeability of the preparation to Na^+ versus Cl^-. As seen in the present study, they may be quite large and differ between species (*Oreochromis*, $+8$ mV; *Fundulus*, -44 mV). We are aware of no in vivo measurements of V_t in freshwater-adapted killifish, but in freshwater-adapted tilapia, in vivo V_t's in the closely related *O. mossambicus* [40] were close to those measured across the isolated opercular epithelium ([24], and the present study).

Previous in vitro studies have used a variety of other isolated epithelial preparations as possible model systems for the transport mechanisms of Na^+, Cl^-, and Ca^{2+} in the freshwater teleost gill. Using the same Ussing flux ratio criterion analysis as in the present study, the cleithrum skin of the rainbow trout actively transported only Ca^{2+} while both Na^+ and Cl^- were passively distributed [20]. Isolated skin from *G. mirabilis* adapted to 5% seawater demonstrated active transport of Cl^- from serosa to mucosa [17], rather similar to the 10% seawater acclimated *Fundulus* preparations of the present study. Cultured branchial epithelia from rainbow trout exposed to apical freshwater were found to actively transport Cl^- against an electrochemical gradient, but again Na^+ was passively distributed [39]. Thus to date, no single preparation has exhibited active uptake of all three ions from mucosal freshwater.

4.2. Oreochromis opercular epithelium

In the present study with the isolated opercular epithelium of freshwater *O. niloticus*, observed flux ratios for both Na^+, Cl^-, and Ca^{2+} were all significantly greater than predicted by the Ussing flux ratio criterion (Fig. 2A). To our knowledge, the freshwater *Oreochromis* opercular epithelium is therefore the only isolated epithelial preparation from a teleost fish which exhibits in vitro the non-diffusive uptake of both Na^+ and Cl^- (as well as Ca^{2+}) from a dilute mucosal bathing medium. The preparation therefore holds con-

Table 2
Unidirectional efflux (J^{sm}) and influx (J^{ms}) of Cl^- and Na^+ in opercular epithelia of freshwater and 10% seawater adapted *F. heteroclitus* ($n = 6$) bathed on the mucosal side with freshwater and 10% seawater, respectively. Open circuit conditions, except as noted

Bathing solutions (serosa/mucosa)	V_t (mV)[a]	G_t (mS cm^{-2})[b]	Hour 1[c]		Hour 2[c]		Hour 3[c]	
			J^{sm}	J^{ms}	J^{sm}	J^{ms}	J^{sm}	J^{ms}
Freshwater adapted *Fundulus* (saline/freshwater)								
Cl^-	-43.9 ± 2.4	1.94 ± 0.34	1110 ± 256	125 ± 42.5	962 ± 265	116 ± 29	1082 ± 255	74 ± 22
Na^+			1217 ± 215	163 ± 47	1387 ± 286	121 ± 31	1504 ± 365	126 ± 31
10% Seawater adapted *Fundulus* (saline/10% seawater)								
Cl^-	$+3.3 \pm 0.5$	2.32 ± 0.43	1624 ± 427	664 ± 153*	1998 ± 407	879 ± 214*	7861 ± 949[d]*	2880 ± 391[d]*
Na^+			1868 ± 405	1310 ± 297*	1404 ± 96	944 ± 265*	2810 ± 242[d]*	2784 ± 277[d]*

[a] Transepithelial potential (mucosal ground) corrected for junction potentials ($n = 12$).
[b] Tissue conductance corrected for solution resistance ($n = 12$).
[c] Units are in nmol cm^{-2} h^{-1}.
[d] Preparation under saline/saline short-circuit conditions, $V_t = 0$ mV.
* Un-paired t-test, two-tailed. Comparison of mean flux values (J^{ms} and J^{sm}) between the two preparations; * $P < 0.05$.

siderable promise as a model system for studying the ionic transport mechanisms of the freshwater gill, but a great deal more work, such as pharmacological analysis (see below) and in vivo versus in vitro validation will be required to realize this promise. One potential problem is the low absolute rates of unidirectional Na^+, Cl^-, and Ca^{2+} fluxes in the freshwater *Oreochromis* preparation, 4–10-fold less than in the *Fundulus* preparation. This likely reflects the relatively low density of chloride cells in the *Oreochromis* opercular epithelium, approximately 40-fold less than in *Fundulus*.

The active uptake of Ca^{2+} against electrical and concentration gradients (Fig. 1A and Table 1) confirms an earlier finding [24] on this same tilapia species. In the present study, the mean net absorption rate of Ca^{2+} (3.1 nmol cm^{-2} h^{-1}) was markedly higher than the net flux (0.31 nmol cm^{-2} h^{-1}) reported by McCormick et al. [24]. However both the mucosal freshwater and the serosal saline had different compositions in the two studies, such that the concentration gradient opposing transport was almost 7-fold greater in the earlier investigation.

The *Oreochromis* opercular epithelium had a V_t of $+8$ mV compared to the -44 mV found for the *Fundulus* preparation (see also [23]). Marshall et al. [23] reasoned the highly negative V_t of *F. heteroclitus* was a diffusion potential reflecting the membrane's high selectivity to cations. The V_t of intact freshwater fish can be slightly positive or slightly negative [31] and in this study the freshwater *Oreochromis* membrane's V_t (8 mV) was very comparable to the 10 mV reported for the skin of the same species [24]. The closely related *O. mossambicus* in freshwater had a whole-animal V_t of -1 to $+10$ mV [40]. In this study the positive V_t of the *Oreochromis* membrane is likely a diffusion potential reflecting selectivity of anion movement out to the mucosal freshwater bath (Table 1).

Once the freshwater *Oreochromis* epithelium was bathed in saline on the mucosal surface, the V_t was reduced to near zero, the I_{sc} became very small (~ 1 μA cm^{-2}), and both Na^+ and Cl^- were passively distributed (Table 1). The isolated opercular membrane of a closely related species, the freshwater adapted *O. mossambicus*, also had a small I_{sc} (~ 1 μA cm^{-2}) and also did not actively transport Cl^- when bathed with mucosal saline [6]. Thus, in *Oreochromis*, the Na^+ and Cl^- uptake mechanisms appear to be shut down by elevated mucosal salinity. This contrasts with the freshwater-adapted *Fundulus* preparation which exhibits a net uptake of both Na^+ and Cl^- when bathed in mucosal saline [23], and in which the directionality of Cl^- transport seems to be controlled by serosal rather than mucosal osmolality [42].

Karnaky [14] proposed that the morphology and physiology of the epithelia may change rapidly in response to external salinity. Based on this suggestion, and our finding that the uptake mechanism(s) were shut down by mucosal saline, we hypothesized that mucosal exposure to high levels of NaCl during the dissection procedure might have impaired the transport properties of the freshwater *Oreochromis* preparation. However, when freshwater osmotically compensated with mannitol was substituted for saline during dissection, the uptake properties were not significantly altered. This finding does not rule out the possibility that mucosal osmolality, rather than salinity itself, may be a problem. Ideally, in future it would be desirable to develop a dissection procedure in which only freshwater would moisten the mucosal surface and only saline would contact the serosal surface.

4.3. Fundulus opercular epithelium

The freshwater acclimated *Fundulus* opercular epithelium actively transported Cl^- from the mucosal freshwater to the serosal saline according to the Ussing flux ratio equation (Fig. 2A), against a large concentration gradient (1:194) and a large electrical gradient (-44 mV). However, the movement of Na^+ across the freshwater *Fundulus* opercular epithelium was passive (Fig. 2A). These results in natural freshwater confirm those of Marshall et al. [23] who studied *Fundulus* opercular epithelia acclimated to and bathed mucosally in a synthetic freshwater with a 1.7-fold higher NaCl and a 10-fold lower Ca^{2+} level. Furthermore, unidirectional Na^+ and Cl^- flux rates and values of G_t were very similar in the two studies. Interestingly, V_t was less negative (-44 versus -64 mV) than in Marshall et al. [23], probably reflecting the well known effect of $[Ca^{2+}]$ in modulating the diffusion potential across epithelia of freshwater teleosts [31].

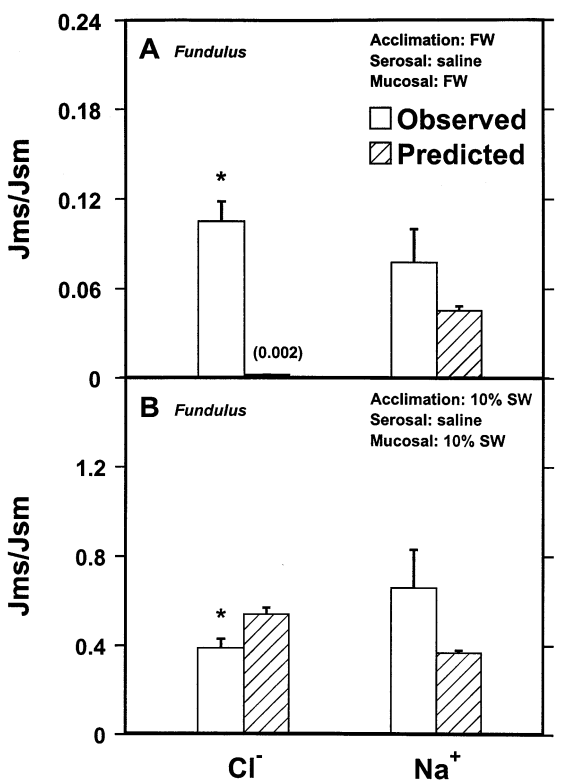

Fig. 2. (A) Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in isolated opercular epithelium of freshwater acclimated *F. heteroclitus* with freshwater in the mucosal bath ($n = 6$). Values were averaged for all three periods for each experiment, then averaged for all six experiments. The open bars represent the observed flux ratios for Cl^- and Na^+, the hatched bars represent the predicted flux ratios for Cl^- and Na^+ based on the Ussing flux ratio equation. (B) Observed and predicted ratios for Cl^- and Na^+ for the 10% seawater acclimated *Fundulus* opercular epithelium with 10% seawater in the mucosal bath ($n = 6$). The asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), implying non-diffusive transport.

To determine if active uptake of Na^+ and Cl^- could be facilitated, *Fundulus* were acclimated to a more normal medium for them, 10% seawater. Surprisingly, when their opercular epithelia were set up with 10% seawater on the mucosal surface, the preparations exhibited transport properties very similar to those of a seawater fish, not a freshwater fish. The V_t was small but positive and the epithelia demonstrated active extrusion of Cl^- and passive movement of Na^+ into the mucosal bath (Fig. 2B). Under short-circuit (I_{sc}) symmetrical saline conditions, Na^+ movement was passive and net extrusion of Cl^- was 4.8 $\mu mol_i\ cm_i^{-2}\ h^{-1}$ compared to 6.1 $\mu mol_i\ cm_i^{-2}\ h^{-1}$ of a seawater operculum [1]. The I_{sc} of this preparation (34 $\mu A\ cm^{-2}$) was intermediate between values previously found for freshwater adapted (~ 9–12 $\mu A\ cm^{-2}$; [23]) and seawater adapted *Fundulus* (136 $\mu A\ cm^{-2}$; [1]). These comparisons indicate that the 'seawater-adaptive' Cl^- secretion was partially activated.

It seems curious that the opercular epithelia would actively excrete salt when the fish is living in 10% seawater, because the tonicity of this media is only about one third of the blood levels. This situation would lead to salt depletion unless compensated for in some way. It is possible that the in vitro preparation does not properly mimic the in vivo condition due to the lack of inhibitory hormones or neurotransmitters. Nevertheless, the result is in accord with structural studies. *Fundulus* adapted to 10% seawater are reported to have MR cells that are identical in ultrastructure to a 100 and 200% seawater adapted *Fundulus* [10]. The appearance of freshwater-like MR cells has been found only upon acclimation to very low salinities (1/16 seawater; [30]). An alternate or additional explanation may relate to the recent findings by Zadunaisky et al. [42] that only small increases in serosal osmolality are needed to activate Cl^- secretion in the *Fundulus* preparation. If the serosal saline used in these experiments had a slightly higher osmolality than the blood plasma of the 10% seawater-acclimated fish from which the preparation originated, active salt excretion might be expected.

At present, it appears problematical whether the opercular epithelium of the freshwater-adapted *F. heteroclitus* will ever serve as a useful model for a 'typical' freshwater gill. The killifish is in fact a normally estuarine species that can readily adapt to a variety of salinities, but it does not normally live its entire life in freshwater [34]. A recent analysis of the ionic uptake mechanisms of freshwater-adapted killifish in vivo has identified a number of anomalies, most importantly a virtual absence of Cl^- uptake by the whole animal despite a vigorous Na^+ uptake [26]. In vitro, however, exactly the opposite situation occurs: the opercular preparation actively transports Cl^- but not Na^+ from freshwater (Fig. 2A, present study; [23]). In addition,

the application of SITS, a drug which blocks anion exchange and anion channels, had no effect on Cl^- influx in the freshwater adapted *Fundulus* preparation [23]. Amiloride, a well-known Na^+–H^+ exchange and Na^+ channel inhibitor, added to the mucosal freshwater, had no effect on Na^+ unidirectional influx [23]. According to these drug experiments, the transport properties of this freshwater in vitro preparation does not behave as a standard freshwater teleost is thought to function [16,29,32,38]. Nevertheless, the preparation does actively transport Ca^{2+} from freshwater [22], as in vivo [27].

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