THE JOURNAL OF EXPERIMENTAL ZOOLOGY 277:23–37 (1997)

NaCl Transport and Ultrastructure of Opercular Epithelium From a Freshwater-Adapted Euryhaline Teleost, *Fundulus heteroclitus*

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ABSTRACT We adapted killifish to defined freshwater (FW: 1.0 mM Na^+, 1.0 mM Cl^-, + 0.1 mM Ca^{2+}) and by fluorescence light microscopy and scanning and transmission electron microscopy found that the opercular epithelium retained mitochondria rich (MR) cells that were significantly larger but less numerous than in SW-acclimated tissues. Opercular epithelia mounted in vitro with FW bathing the mucosal surface take up Cl^- against a large negative inside transepithelial potential (V_t, grand mean –64.1 mV) and concentration gradient; the observed flux ratio was significantly different ($P < 0.001$) from that predicted for passive ion distribution but the net flux was consistently negative. The Na^+ flux ratio suggested that Na^+ was passively distributed. V_t was largely a Na^+ diffusion potential, based on unilateral manipulations of [Na^+]. Cl^- unidirectional uptake was unaffected by mucosally added SITS (0.1 mM) but was inhibited by SCN^- (1.0 mM) and by anaerobiosis. Killifish transferred from SW to FW for 48 h had reduced Cl^- secretion by the opercular epithelium compared to SW controls but had not yet developed Cl^- uptake, indicating a slow adaptive process for development of Cl^- absorptive transport. Opercular epithelia of FW adapted fish, if bathed with isotonic saline on both sides, has a modest net Na^+ and Cl^- uptake, unlike SW opercular epithelium that strongly secretes Cl^- under similar conditions. FW killifish opercular epithelium may provide a model to study ion regulation by euryhaline fish. *J. Exp. Zool. 277:23–37, 1997.*

Freshwater teleosts take up ions across the gill epithelium to help maintain ionic balance in this hypotonic medium. Until very recently, experiments investigating this ion transport were performed in vivo because there was no available epithelial preparation that could be used for this purpose. The skin and opercular epithelium of marine teleosts secrete NaCl via active Cl^- secretion in mitochondria rich chloride cells and these in vitro preparations continue to be very valuable in experiments to elucidate the mechanisms and regulation of NaCl secretion by marine teleosts. The need for a FW model of ion transport in fish has long been recognized: Karnaky and Kinter ('77), Marshall ('77), Evans et al. ('82), Zadunaisky ('84), and Péqueux et al. ('88) have all called for use of epithelial preparations from FW-adapted euryhaline teleosts as models of ion uptake by FW gill, but previous attempts have failed to establish such a transport system. When euryhaline teleosts are adapted to fresh water, in most cases the mitochondria rich cells of the opercular epithelium and skin largely disappear, for example in the tilapia *Oreochromis mossambicus* the density of mitochondria rich cells is half that of correspondent SW adapted animals, cell size is substantially reduced, and there is no apparent NaCl uptake (Foskett et al., '81). Among freshwater salmonids, brook trout (*Salvelinus fontinalis*) have no apparent mitochondria rich cells in the opercular and buccal skin (Marshall, '85) and rainbow trout, *Oncorhynchus mykiss,* have relatively few (< 200 mm^{-2}) small mitochondria rich cells and no apparent Na^+ or Cl^- uptake by the cleithrum epithelium (Marshall et al., '92). The marine goby (*Gillichthys mirabilis*) cannot adapt to salinities below 5% SW and at this salinity the skin contains chloride secreting cells, as evidenced by a

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Received 12 June 1996; Revision accepted 12 September 1996

serosa positive transepithelial potential and large short-circuit current that develops when these preparations are mounted with symmetrical Ringer's solutions (Marshall, '77). Initial work in adapting killifish (*Fundulus heteroclitus*) to hyposmotic salinities, 5–10% SW, did not produce an ion absorbing tissue because the opercular epithelium secreted ions when mounted in vitro in symmetrical Ringer's solutions (Degnan et al., '77; Karnaky, '86). An encouraging aspect of recent work is that Ca^{2+} seems to be actively transported across tilapia opercular epithelium (McCormick et al., '92), trout skin (Marshall et al., '92), and killifish opercular epithelium (Marshall et al., '95). As well, recent work with dissociated trout gill cells in confluent monolayer primary cultures have demonstrated epithelial characteristics similar in some respects to gill function (Pärt and Wood, '96).

The killifish is remarkably euryhaline and is able to adapt to any constant salinity from fresh water (0 ppt.) to strongly hypersaline conditions (120 ppt.; Griffith, '74). Secondary active chloride secretion by SW type MR cells and many aspects of its multihormonal control have been extensively reviewed (Zadunaisky, '84; Péqueux et al., '88; Wood and Marshall, '94). However, killifish are capable of acclimation to FW and in gill epithelium have numerous mitochondria rich cells when acclimated to "pond water" (Philpott and Copeland, '63). We reasoned that acclimation to such ion poor environments would stimulate production of the freshwater type of mitochondria rich cell in killifish opercular epithelium and the concomitant ability of epithelium to absorb NaCl.

The present work evaluates FW killifish opercular epithelium as a possible model for ion transport by MR cells of FW-adapted fish by demonstrating ion uptake capabilities using the Ussing flux ratio criterion, and the lack of ion secretion and sensitivity to transport inhibitors. We present some implications of the model and its potential importance as a means of studying hormonal control of FW to SW acclimation processes.

MATERIALS AND METHODS

Animals

Adult killifish of both sexes were obtained from the Antigonish estuary and were transferred to indoor holding facilities and were maintained in 10% SW (3.0–3.2 ppt.) at 20–25°C under artificial light operating on natural photoperiod. Animals were transferred directly to full strength SW (30–32 ppt.) or FW and were held for at least 10 days prior to flux experiments on FW acclimated fish. Composition (in $\mu mol.l^{-1}$) of the defined freshwater (FW) medium in which killifish were acclimated and tested was Na^+ 1,000, K^+ 20, Cl^- 1,000, SO_4^{2-} 140, Ca^{2+} 100, Mg^{2+} 60 with titration alkalinity (titration to pH = 4.0) of 280 μM and pH of 6.8–7.2 (other forms of dilute media are spelled out). Experiments were performed in the summer months of 1990–1995. The animals used in the flux experiments during salinity acclimation were transferred directly from SW to FW for 48 hours. Killifish readily survive such direct transfers to hypotonic medium if feeding is continued. Fish were killed by decapitation and opercular epithelia were removed and mounted in Ussing style membrane chambers. Ussing membrane chambers had an exposed membrane area of 0.125 cm^2 (for most pharmacological studies) and were water jacketed to control temperature at 22°C.

Tracer fluxes

Serosal side in vitro bathing solution was a modified Cortland saline with the composition (in mM) Na^+ 142, Cl^- 136, K^+ 2.6, HCO_3^- 9.7, Ca^{2+} 1.6, Mg^{2+} 0.9, PO_4^{2-} 3.0, SO_4^{2-} 0.9, and glucose 5.6 with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, grade III, fraction V) 20 $mg.ml^{-1}$ as a replacement for plasma protein; the solution, when bubbled with 0.3% CO_2/balance O_2 (or 0.3% CO_2/balance N_2 in the anaerobiosis experiment) had a pH of 7.8. Paired membranes were flushed mucosally (20 times the chamber volume) with FW on the mucosal side to remove saline. Unidirectional efflux from serosa to mucosa (J_{sm}), influx from mucosa to serosa (J_{ms}), and net flux ($J_{net} = J_{ms} - J_{sm}$) were determined as published previously (Marshall, '85). Briefly, addition of radioisotope was followed by an equilibration period of 1 hour, then by control and treatment periods, each comprising three 20 minute flux measurements. Because of the paired design, observed flux ratios could be calculated for each animal. Calculation of expected flux ratios used the Ussing flux ratio equation (Ussing, '49) and care was taken to adjust the concentration terms to reflect the activity of the ions in solution (see Marshall et al., '92). Transepithelial conductance (G_t) and transepithelial potential (V_t, mucosal side grounded and corrected for junction potentials) were monitored as described previously (Marshall, '85) using dual channel current-voltage clamps (D. Lee Co. Sunnyvale, CA, and World Precision Instruments, New Haven, CT, DVC 1000). In experiments where sa-

line bathed both sides of the epithelium, short-circuit current (I_{sc}) was also measured.

Pharmaceuticals

Competitive anion transport inhibitor SCN^- (as NaSCN dissolved in saline) was added at 1.0 mM on the mucosal side. Anion channel blocker diphenylamine-2-carboxylate (DPC), dissolved in ethanol, was added at 1.0 mM to the mucosal bath; final ethanol concentration was < 0.2% v/v and this ethanol vehicle has no effect on transport parameters. Disulfonic stilbene SITS (ICN Pharmaceuticals, Plainview, NY), which blocks anion exchange and anion channels of excitable cells but not CFTR-like channels, was dissolved in saline and added at 0.1 mM to the mucosal bath. Amiloride was dissolved in saline and added to the mucosal side to a final concentration of 0.1 mM. Amiloride blocks Na^+-H^+ exchange and amiloride sensitive Na^+ channels. Dibutyryl cyclic AMP (db-cAMP, 1.0 mM) and 3-isobutyl-1-methylxanthine (IB^-, 0.1 mM), in saline, were added to the serosal bath to augment chloride secretion rate.

Fluorescence microscopy

The fluorophore DASPEI (ICN Biomedicals, Costa Mesa, CA) is a mitochondrial vital stain (Bereiter-Hahn, '76) that has been used routinely to identify MR cells (e.g., Marshall and Nishioka, '80). DASPEI was dissolved in a stock solution of 0.2 $mg.ml^{-1}$ in distilled water. Opercular epithelia from the Ussing chambers were bathed in oxygenated Cortland's saline with 10 μM DASPEI for 30 minutes before viewing as a wet mount on a Zeiss (Germany) Photomicroscope III equipped with epifluorescence. Excitation wavelength was 485 nm (bandwidth 20 nm) and barrier filter was 540 nm (bandwidth 40 nm). Cell counts and cell diameter measurements were made at three or four randomly selected areas of membrane, each count comprising an area of 0.04 mm^2.

Electron microscopy

For transmission electron microscopy, opercular epithelia of animals adapted to FW were dissected, pinned out flat, and fixed with 2.5% ice-cold glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) for 3 hours followed by a buffer rinse with 0.1 M sodium cacodylate buffer (pH 7.4). Postfixation was with 1.0% ice cold osmium tetroxide buffered with 0.1 M sodium cacodylate (pH 7.4) for 1 hour followed by dehydration through a propanol series to 100% and embedding in Spurr's low viscosity resin. Thick sections for light microscopy were cut with glass knives on a Sorvall (Wilmington, DE) MT2C ultramicrotome and stained with Richardson's stain (Richardson et al., '60). Thin sections with silver-gold interference color were cut with a diamond knife (Diatome, Switzerland) and picked up on naked 150 mesh copper grids. Sections were stained sequentially with saturated uranyl acetate (10 minutes) followed by lead citrate (Reynolds, '63) for 5 minutes and examined in a Philips (Netherlands) 300 electron microscope.

For scanning electron microscopy, methods for fixation of the opercular epithelia closely followed those used for fish gills in previous studies (Perry and Laurent, '89). Opercular bone with attached epithelia was excised from each side of the fish. At the time of sampling, killifish were killed with an overdose of MS 222 anaesthetic (Syndel, Canada; 1.5 $g \cdot l^{-1}$ buffered to pH 7.8 with $NaHCO_3$). The operculum was then briefly rinsed in FW and fixed in ice-cold 2.5% glutaraldehyde solution (buffered with 0.15 M sodium cacodylate) for approximately 70–80 minutes. After fixation, opercula were washed three times with ice cold buffer and stored at 4°C overnight. The opercula were then partially dehydrated in an ethanol series (15 minutes in 30% and 50% EtOH, three exchanges in 70% EtOH, 10 minutes each) and stored at 4°C in 70% EtOH. Samples were then dehydrated to completion (10 minutes in 95% ethanol, 10 and 20 minutes in absolute ethanol) and taken through two successive (2 minutes each) baths of 1,1,1,3,3,3-hexamethyldisilazane (Aldrich Chemical Co., Milwaukee, WI) and air dried for approximately 1 hour. The opercula were then mounted, epithelium side up, on aluminum stubs with epoxy glue. To prevent rehydration, mounted opercula were held in a desiccator that was kept at 60°C for several hours. Immediately prior to viewing, samples were sputtered coated with a 10 μm layer of gold and subsequently viewed on a ISI-DS130 dual stage scanning electron microscope at 15 kV.

Data presentation

Data are expressed as the mean ± one standard error unless indicated otherwise. Generally, control and test conditions were analyzed by paired (or unpaired, as appropriate) *t*-tests. Regression statistics are given with 95% confidence limits on slope and intercept.

RESULTS

Microscopic structure

Opercular epithelium from FW acclimated killifish contains a high density of mitochondria-rich

cells, as detected by DASPEI fluorescence (Fig. 1a) and is comparable to the chloride cells of SW adapted killifish (Fig. 1b). MR cells of FW acclimated animals are significantly larger in diameter than those from SW acclimated killifish, 20.0 ± 0.3 vs. 13.0 ± 0.4 μm ($P < 0.01$; n = 10 membranes) and the density was lower, 2,044 ± 72 vs. 2,859 ± 269 DASPEI fluorescent cells per mm^2 ($P < 0.01$; n = 11 membranes). In both salinities there occurred closely apposed groups of MR cells as well as single cells. Intensity of fluorescence seemed more variable among cells in a single tissue than between the two salinities, hence the density of mitochondria in cells was not apparently different in the two salinities.

In FW adapted opercular epithelia, MR cells may be covered with pavement cells (Fig. 1c) or exposed (Fig. 2). Some MR cells formed thickenings or bulges of the epithelial surface (Fig. 1c), a feature also seen by scanning electron microscopy (Fig. 3a). MR cells that are exposed to the environment have potential to contribute to transmural ion transport, while those that are covered likely are not involved in ion transport in FW. A striking feature of opercular epithelia from both salinities is the presence of numerous granular cells (presumably granular lymphocytes; Fig. 1c) that in living preparations are highly motile and may be observed by phase microscopy moving among epithelial cells. These cells are DASPEI negative.

Most MR cells of FW acclimated killifish have apical membranes that are exposed to the environment (Fig. 2a,b). These cells have apical crypts where the apical membrane is folded apparently into microvilli (Fig. 2a,b), leading to an enhancement of exposed surface area. More than one MR cell often share a common crypt opening; Figure 2a shows three cells with varying degrees of apical membrane folding sharing one crypt that is apparently covered by a pavement cell. Because apical crypt openings are small relative to MR cells, even cells that have apical crypts will appear covered in many sections. Figure 2b is the apical portion of two MR cells sharing an apical crypt that is exposed to the surface. Again, there is great variation in the degree of apical membrane folding. There are well-developed tight junctions between pavement cells and MR cells, 1–2 μm in length (Fig. 2c), even though the pavement cells that overlie the MR cell are very thin (Fig. 2b,c). The junctional complexes are complete, in that they often have structural desmosomes immediately proximal to the tight junction (Fig. 2b). Between MR cells there are also well-developed apical tight junctions (Fig. 2b) that are more extensive than junctions between SW chloride cells and accessory cells (Fig. 3b). Chloride cells of SW adapted killifish have much less elaboration of apical membrane surface, and apical crypts are usually smooth in appearance (Fig. 3b). Tight junctions between SW MR cells and pavement cells are similar to those seen previously (Karnaky et al., '76a; Karnaky, '86) and lack desmosomes below the tight junctions as in the FW adapted tissues. Several MR cells can share an apical crypt, with less extensive tight junctions between chloride and accessory cells, as compared to those between chloride cells and pavement cells (Fig. 3b). The tubular system is more highly developed in SW MR cells than in FW cells (compare Fig. 2a,b with Fig. 3b) and the apical zone where there is no tubular system is noticeably thicker in MR cells of FW adapted fish (compare Figs. 2a,b and 3b). Clearly, structural changes in opercular epithelium have occurred during adaptation to FW that likely are connected to functional changes.

To determine if some MR cells in opercular epithelia of FW adapted fish were exposed to the environment while others were covered over by pavement cells, we compared transmission and scanning electron microscopic images of epithelium. The appearance of the apical crypts of MR cells by transmission electron microscopy (Fig. 2a,b) corresponds to that seen by scanning electron microscopy (Fig. 3a). In both cases, crypts are bordered by microridged pavement epithelial cells and the diameter of crypt openings by transmission electron microscopy, 1.7 ± 0.2 μm, corresponds well to the 1.8 ± 0.3 μm openings seen by scanning electron microscopy. Pores seen by scanning electron microscopy therefore are openings to crypts for one or more MR cells. Some MR cells, however, appear to be covered completely by pavement cells in the transmission electron microscopy sections (Fig. 1c). Further evidence of this is the appearance by scanning electron microscopy of hemispherical bulges of surface epithelial cells that

Abbreviations

MR,	mitochondria rich
FW	freshwater
SW	seawater
DASPEI	2-(4-dimethylaminostyryl)-N-ethylpyridinium iodide
SITS,	4-acetamino-4-isothiocyano stilbene-2,2´-disulfonic acid
db-cAMP	dibutyryl cyclic AMP
IB^-	3-isobutyl-1-methylxanthine
DPC	diphenylamine-2-carboxylate.

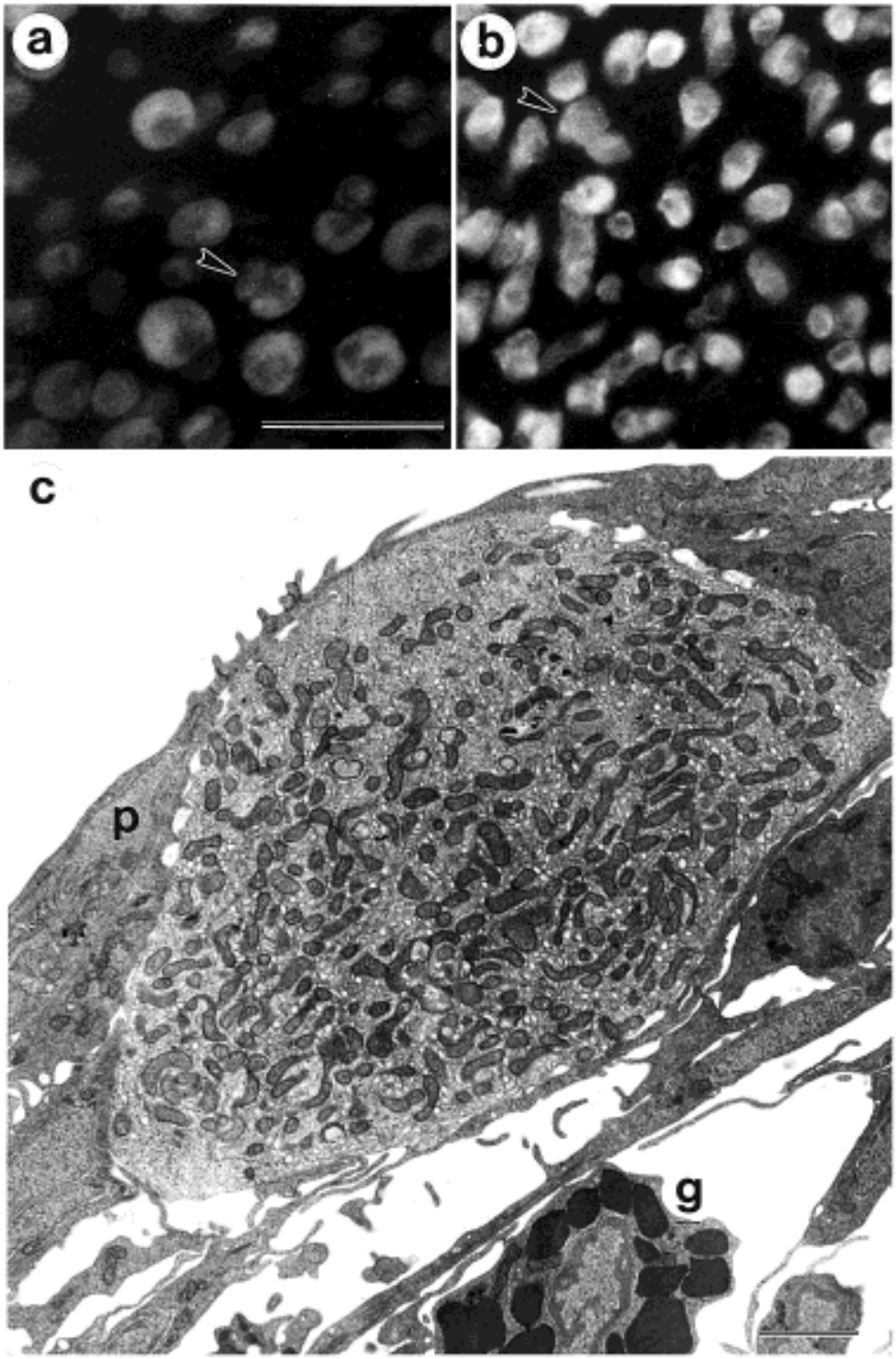

Fig. 1. Mitochondria rich cells in opercular epithelia of FW-acclimated **(a)** and SW-acclimated **(b)** killifish. Both membranes were exposed to 10 μM DASPEI for 30 minutes in oxygenated saline and are at the same magnification (bar = 100 μm). Note the larger, rounded MR cells in the FW preparation and the appearance of clusters of MR cells (arrowheads) in both salinities. In transverse thin section, FW MR cells **(c)** have typical high density of mitochondria and extensive tubular system. This cell, typical of covered MR cells, forms a thicker portion of epithelium such that overlying pavement cells (p) form a bulge. In both salinities numerous granular lymphocytes (g) are also present in epithelium. Bar = 2.0 μm.

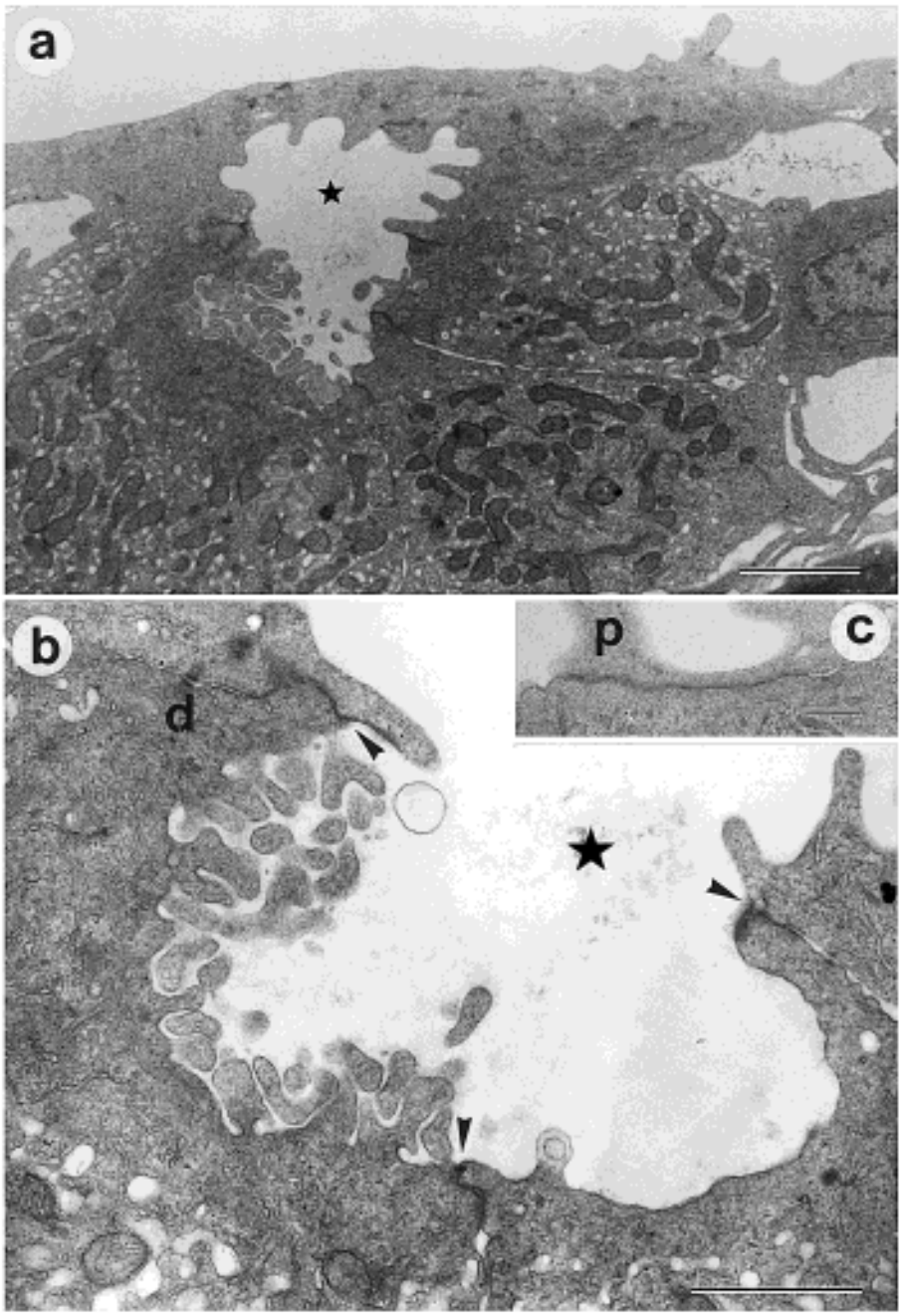

Fig. 2. Transmission electron microscopy of MR cells from FW-adapted opercular epithelia showing **(a)** several MR cells in a cluster around a single apical crypt (star). Note variation in degree of apical membrane folding. Bar = 2.0 μm. A higher magnification of two MR cells sharing an open apical crypt **(b)** shows one cell with extensive microvilli and the other with a smooth apical surface. Note the well-developed apical tight junctions (arrowheads) and supporting desmosomes (d) of the junctional complex. Bar = 1.0 μm. **c:** Detail of an apical tight junction between pavement (p) and MR cell. Bar = 0.2 μm.

correspond to those seen by transmission electron microscopy (Fig. 1c) but do not have the pore-like openings of apical crypts. As well, the size of the bulges are roughly correspondent (5–10 μm diameter) to bulges that do have apical pores. Hence it is clear that some but not all MR cells of FW opercular epithelium are exposed to the environment and could be involved in ion transport. With this knowledge, it became important to test whether these cells absorb Na^+, Cl^-, or other ions.

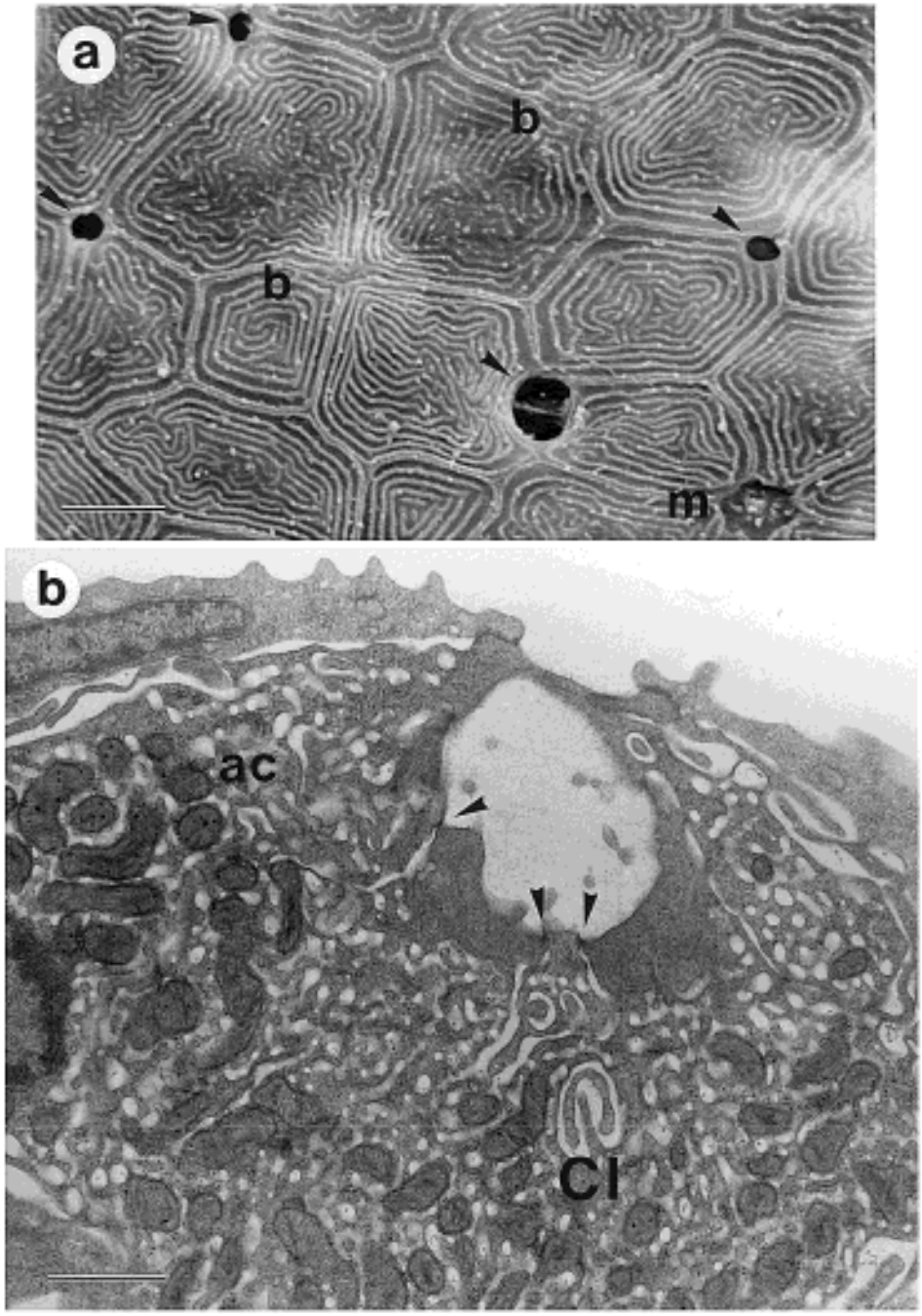

Fig. 3. A scanning EM image of a FW opercular epithelium **(a)** shows microridged pavement cells, openings of apical crypts (arrowheads), a mucous cell (m), and the bulges (b) formed by underlying MR cells. Bar = 5.0 μm. **b:** SW opercular epithelium with chloride (Cl) and accessory (ac) cells showing a more extensive tubular system than in FW, simple junctions between chloride and accessory cells (arrowheads) in contrast to the extensive tight junctions between MR cells in FW. Bar = 1.0 μm.

Ion fluxes

Opercular epithelia from FW adapted killifish (at least 10 days acclimation) were bathed in FW (same composition) on the mucosal surface and saline on the serosal surface in vitro while unidirectional and net ion fluxes were determined. Unidirectional fluxes of $^{36}Cl^-$ (Table 1) produced an observed flux ratio that was significantly greater than that predicted from the Ussing flux ratio equation (favoring uptake direction), suggesting

TABLE 1. Unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratio of $^{36}Cl^-$ in opercular epithelium of FW-adapted Fundulus heteroclitus *(N = 10 membrane pairs)*

Bathing solutions (serosa/mucosa)	V_t (mV)[1]	G_t (mS·cm^{-2})[2]	J_{sm} (nmol·cm^{-2}·h^{-1})	J_{ms} (nmol·cm^{-2}·h^{-1})	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	P*
Saline/FW	–65.4 ± 3.4	2.0 ± 0.5	658 ± 83	66 ± 25	27.2 ± 14.5	2,073 ± 196	<0.001
Saline/saline	+1.1 ± 1.0	6.6 ± 1.2	1,256 ± 195	1,637 ± 468	1.4 ± 0.5	0.9 ± 0.02	NS

[1]Transepithelial potential measured with respect to the mucosal bath and corrected for junction potentials; N = 20.
[2]Total tissue conductance corrected for solution resistance; N = 20.
*Paired *t*-test, two-tailed. NS = not significant.

that distribution of Cl^- was not passive. There was a large negative inside V_t in addition to a large concentration gradient against uptake of Cl^-, hence net flux was negative, apparently because of a leak component to Cl^- efflux. In a separate series, we measured mucosal to serosal unidirectional Cl^- flux in oxygenated solutions and in anaerobic solutions equilibrated with nitrogen. The control period Cl^- uptake was 159 ± 37 nmol.cm^{-2}.h^{-1} (n = 11) and this was significantly reduced in anaerobic conditions to 52 ± 15 nmol.cm^{-2}.h^{-1} ($P < 0.005$, paired *t*-test). In this series G_t and V_t were unaffected by the treatment: 1.69 ± 0.41 mS.cm^{-2} and –54.2 ± 7.9 mV in the control and 1.76 ± 0.36 mS.cm^{-2} and –55.2 ± 6.0 mV in anaerobic conditions (P = 0.73 and 0.72, respectively, paired *t*-tests, n = 11).

When saline was added on the mucosal side of the preparations (Table 1), V_t rose to approximately zero and the flux ratio for Cl^- was not significantly different from the expected ratio, suggesting that diffusional Cl^- fluxes were predominant. Because of the agreement between observed and predicted flux ratios and the fact that net flux was not significantly different from zero, there appeared to be no Cl^- secretion evoked by the introduction of saline to the mucosal side. In the third period with V_t clamped to zero, lack of net Cl^- flux and agreement between observed and predicted flux ratios were confirmed. SW type chloride cells under these conditions develop a net secretion of Cl^- that is equal to the I_{sc} (Degnan et al., '77; Marshall, '81) and very small I_{sc} seen here indicates the SW adaptive Cl^- secretion mechanism was not operating.

While full acclimation to FW produced MR cells that did not secrete Cl^-, we reasoned that chloride cells from animals that were instead partially adapted to FW may retain this capability. We transferred killifish directly from full-strength SW to FW for 48 hours and repeated the above experiment (Table 2). Survival of the direct salinity transfer was better than 90%. With FW on the mucosal side, partially acclimated FW opercular epithelia had a flux ratio that was significantly different from the predicted flux ratio, suggestive of some active Cl^- uptake, but the mucosal to serosal flux was much lower (30%) than that of fully acclimated fish. In addition, V_t was significantly smaller in partially acclimated fish. When these tissues were bathed in symmetrical saline there developed a large serosa positive V_t and a net flux of Cl^- in the secretory direction, and net flux under short-circuit conditions was not significantly different from I_{sc}.

Two inhibitors of anion transport were used in an attempt to partially characterize the Cl^- uptake mechanism. Anion SCN^- in equimolar concentration to Cl^- (both ions at 1.0 mM) decreased Cl^- unidirectional influx but increased unidirectional efflux and conductance (Table 3). If influx of Cl^- was diffusive, then Cl^- influx should have increased with increase in G_t; this was not the case. Hence, decrease in Cl^- influx while G_t was

TABLE 2. Unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratio of $^{36}Cl^-$ in opercular epithelium of Fundulus heteroclitus *transferred from SW to FW for 2 days (N = 7 membrane pairs)*

Bathing solutions (serosa/mucosa)	V_t (mV)[1]	G_t (mS·cm^{-2})[2]	J_{sm} (nmol·cm^{-2}·h^{-1})	J_{ms} (nmol·cm^{-2}·h^{-1})	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	P*
Saline/FW	–19.6 ± 3.4	1.8 ± 0.2	1,190 ± 150	20 ± 10	83.5 ± 16.1	235 ± 53	<0.05
Saline/saline	+7.1 ± 1.0	3.4 ± 0.3	1,890 ± 150	1,140 ± 190	2.0 ± 0.3	0.7 ± 0.1	<0.05
Saline/saline	0[3]	3.8 ± 0.3	2,280 ± 200	1,220 ± 140	2.0 ± 0.2	1.0	< 0.05

[1]Transepithelial potential measured as in Table 1; N = 14
[2]Total tissue conductance corrected for solution resistance; N = 14.
[3]Short-circuited.
*Paired *t*-test, two-tailed.

TABLE 3. Effect of SCN^- on Cl^- fluxes and amiloride on Na^+ fluxes in opercular epithelium of FW-adapted Fundulus heteroclitus *bathed in saline (serosal) and FW (mucosal) (N = 6 membrane pairs)*

Treatment	V_t (mV)[1]	G_t ($mS \cdot cm^{-2}$)[2]	J_{sm} ($nmol \cdot cm^{-2} \cdot h^{-1}$)	J_{ms} ($nmol \cdot cm^{-2} \cdot h^{-1}$)	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	*P**
Cl^- fluxes							
Control	–64.5 ± 21.5	3.6 ± 1.2	654 ± 62	88 ± 13	9.4 ± 2.4	1,488 ± 193	<0.001
SCN (1.0 mM, mucosal)	–61.0 ± 20.0	5.6 ± 2.0	905 ± 93	56 ± 10	27.5 ± 9.7	1,285 ± 190	<0.001
*P**	<0.05	<0.05	<0.01	<0.02	<0.05		
Na^+ fluxes							
Control	–62.6 ± 3.8	3.6 ± 1.5	1,301 ± 306	119 ± 26	12.6 ± 3.33	13.5 ± 1.8	NS
Amiloride (0.1 mM, mucosal)	–55.7 ± 4.2	2.9 ± 0.7	1,628 ± 331	76 ± 15	23.4 ± 5.44	17.5 ± 2.1	NS
*P**	<0.05	NS	NS	NS	<0.05		

[1]Transepithelial potential, as in Table 1; N = 12
[2]Total tissue conductance corrected for solution resistance; N = 12.
*Paired *t*-test, two-tailed. NS = not significant.

increasing supports the idea that most of the Cl^- influx is via active transport or ion exchange. There is further evidence that Cl^- influx was not a function of G_t and therefore is likely not conductive. In a pooled (across five different experiments) regression there was no significant relation between control period chloride influx and G_t (slope –0.0015 ± 0.0115 $mS.\mu mol^{-1}.cm^2.h$, intercept 0.040 ± 0.023 $\mu mol.cm^{-2}.h^{-1}$ r = 0.04, $P > 0.5$, n = 39). Increase in G_t with SCN^- was coincident with an increase in Cl^- efflux; these observations are consistent with an effect of SCN^- on opening a passive leak pathway (Table 3). The relationship between chloride efflux and G_t is also present in pooled regression of chloride efflux on G_t (slope 0.366 ± 0.134 $mS.\mu mol^{-1}.cm^2.h$, intercept 0.141 ± 0.166 $\mu mol.cm^{-2}.h^{-1}$, r = 0.673, $P < 0.001$, n = 39; Fig. 4), suggesting that most of the chloride efflux is conductive and passive. SITS (0.1 mM) had no effect on Cl^- influx (86 ± 25 vs. 88 ± 33 $nmol.cm^{-2}.h^{-1}$, n = 11) but increased G_t from 3.5 ± 0.75 to 5.5 ± 1.1 $mS.cm^{-2}$ ($P < 0.01$, n = 11) and augmented Cl^- efflux by 21% ($P < 0.01$, n = 11).

Sodium transport

Unidirectional fluxes of Na^+ produced an observed flux ratio that was not significantly different from that predicted by the flux ratio equation (Table 3). A repeat of this experiment produced similar results (J_{sm} = 953 ± 154 J_{ms} = 125 ± 23 $nmol.cm^{-2}.h^{-1}$, J_{sm}/J_{ms} = 7.15 ± 1.46 not different from the predicted flux ratio of 11.9 ± 1.23; n = 12). As with Cl^- fluxes, the tissue was in negative balance, consistently losing Na^+ into the mucosal FW bath. Na^+/H^+ exchange inhibitor amiloride, 1.0 mM on the mucosal side, had no significant effect on Na^+ unidirectional influx or efflux and no effect on G_t, although there was a small decrease in V_t (Table 3).

The negative inside V_t in FW-adapted fish has been postulated to be a Na^+ diffusion potential (Potts, '84). To test this hypothesis we changed the mucosal bath to isotonic mannitol with 1.0 mM NaCl and then sequentially increased the NaCl concentration while maintaining a constant osmolality in the mucosal bath. Initially, the change from FW to isotonic mannitol with 1.0 mM NaCl produced no change in V_t (–59.4 ±

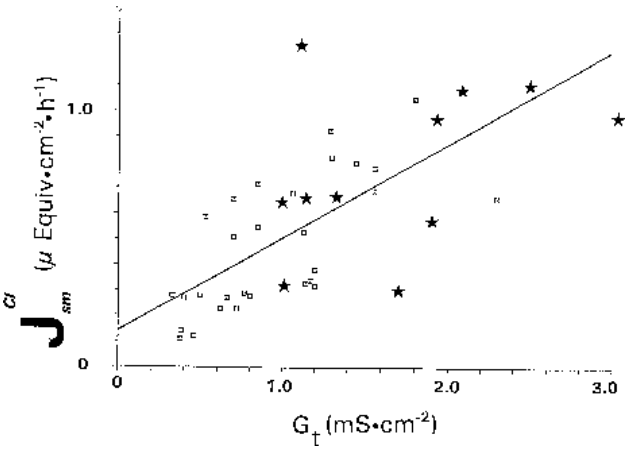

Fig. 4. Regression of unidirectional Cl^- efflux by FW opercular epithelium on total tissue conductance (G_t, $mS.cm^{-2}$). Open squares are spontaneous variation of control period fluxes while the stars are tissues that were treated with IB^- + db-cAMP. The significant ($P < 0.001$) regression suggests that Cl^- efflux is dominated by passive diffusion. There was no significant relation between Cl^- influx and G_t.

4.1 to –60.0 ± 4.2 mV, n = 7), indicating that there was no detectable solvent drag component to V_t. Addition of NaCl to 10.0, 32.0, and 100 mM NaCl depolarized epithelium markedly (Fig. 5). Between 10 and 100 mM the slope of V_t vs. log[NaCl] approached that predicted by the Nernst equation. There appears to be little contribution by Cl^- because V_t with serosal saline and mucosal 1.0 mM NaCl (–68.4 ± 3.8 mV, n = 7) is not significantly different from that observed with a Cl^--free gluconate replaced Cortland's saline on the serosal side (–67.6 ± 4.5 mV, n = 7). Hence V_t appears largely to be a Na^+ diffusion potential that could be explained by the presence of a Na^+ selective paracellular pathway.

Fluxes in symmetrical saline

In another series of experiments with saline on both sides of the epithelium we were able to detect net fluxes in uptake direction for Na^+ and Cl^- (Table 4). These preparations with saline on both sides had G_t that averaged 1.32 and 1.78 $mS.cm^{-2}$ and was significantly lower than previous values of 6.7 (Table 1) and 3.4 $mS.cm^{-2}$ (Table 2; $P < 0.005$, unpaired *t*-test). In short-circuited preparations, net Na^+ and Cl^- fluxes were not significantly different from each other ($P = 0.445$, unpaired *t*-test) and neither net flux was equivalent to I_{sc}, the latter averaging 328 and 430 $nmol.cm^{-2}.h^{-1}$ as (presumed) cation uptake. Stimulation of the tissue with db-cAMP and IB^-, a protocol that induces large increases in I_{sc} and Cl^- efflux in SW skin and opercular epithelia (Marshall and Bern, '79; May and Degnan, '85) appeared to have little effect on Cl^- fluxes but there was a trend toward an increase in Cl^- efflux (Table 4). In treatments following db-cAMP + IB^- on sodium fluxes, we applied 1.0 mM diphenylamine-2-carboxylate, an anion channel blocker. This drug significantly inhibited I_{sc} from 936 ± 121 to 497 ± 139 $nmol.cm^{-2}.h^{-1}$ ($P < 0.001$, paired *t*-test, two-tailed, n = 15). When the db-cAMP + IB^- treatment was used with Na^+ fluxes, conductance and I_{sc} increased by approximately twofold, accompanied by a fall in Na^+ influx and a drop in Na^+ net flux to levels not significantly different from zero.

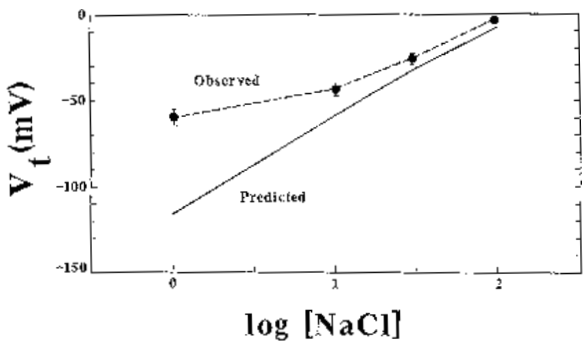

Fig. 5. Effect of raising NaCl concentration in the mucosal bath (supplemented with mannitol to maintain constant osmolality) on V_t, compared to the predicted Nernst equilibrium potential for Na^+. Subsequent complete substitution of NaCl with Na gluconate had no significant effect on V_t.

DISCUSSION

FW mitochondria rich cells

Killifish adapted to 10% SW (Karnaky et al., '76b) exhibited an almost identical ultrastructure of gill MR cells compared to 100 and 200% SW and all had marked binding of tritiated ouabain to the basolateral cell surface. It is clear from this and earlier studies that acclimation to very dilute FW was a necessary prerequisite to development of MR cells with potential for ion uptake. Acclimation of killifish to dilute pond water produced gill MR cells that had surface indentations but not apical crypts (Philpott and Copeland, '63) and the authors observed that the transition from the salt water appearance to that of fresh water occurred at very low salinities, < 1/16 SW. Hossler et al. ('85) examined MR cells in gills of killifish adapted to FW and SW and by scanning electron microscopy described microvilli on the apical surface of MR cells in FW as well as more shallow apical crypts compared to SW acclimated animals. For opercular epithelium, we confirm the presence of microvilli on the apical membrane of MR cells of FW-adapted animals seen previously by scanning electron microscopy in gill (Hossler et al., '85) and expand the picture to include transmission electron microscopy of FW MR cells. Elaboration of apical membrane by microvilli is likely to enhance available sites for transport.

Grouping of MR cells in FW-adapted fish (Fig. 2a) and the well-developed tight junctions between these cells (Fig. 2c) have been observed repeatedly (reviewed by Karnaky, '91). Karnaky ('91) also observed a reduction in the depth of tight junctions after opercular membranes had been dissected and mounted in vitro in symmetrical saline, compared to tissues taken directly from 1% SW adapted animals. It is possible that tight junctions respond rapidly to changes in bathing solutions. Deep tight junctions are important because they predict that the opercular epithelium of FW-adapted fish should have a low shunt permeability. Consistent with this, we observe lower conductance

TABLE 4. Na^+ and Cl^- fluxes across FW-adapted Fundulus heteroclitus *opercular epithelium bathed in symmetrical saline and shortcircuited (N = 6 for Cl; 8 for Na)*

Ion	Treatment	G_t ($mS \cdot cm^{-2}$)[1]	J_{sm}	J_{ms}	J_{net}	I_{sc}[2]
			($nmol \cdot cm^{-2} \cdot h^{-1}$)			
Cl^-	Control	1.32 ± 0.13	250 ± 62	1,408 ± 301	1,161 ± 357	430 ± 74
	IB^-/db–cAMP	2.65 ± 0.27	343 ± 114	1,167 ± 308	825 ± 416	915 ± 104
	*P**	<0.0001	NS	NS	NS	<0.0001
Na^+	Control	1.78 ± 0.38	1,435 ± 413	3,391 ± 701	1,956 ± 737	328 ± 103
	IB^-/db-cAMP	3.34 ± 0.69	2,405 ± 779	2,289 ± 497	–116 ± 822	699 ± 93
	*P**	<0.001	<0.05	<0.02	<0.01	<0.0001

[1]Total tissue conductance corrected for solution resistance; N = 12.
[2]Short-circuit current expressed as positive for cation uptake (or anion secretion).
*Paired *t*-test, two-tailed. NS = not significant.

in FW opercular epithelia (averaging 1.32 and 1.78 $mS.cm^{-2}$, Table 4) compared to SW opercular epithelia (averaging 6.5, 7.3, 6.8, and 7.8, Péqueux et al., '88) under similar conditions with saline on both sides of the epithelium. The paradigm of an available shunt pathway physiologically corresponding to single-stranded tight junctions between accessory cells and chloride cells (Sardet et al., '79; Hootman and Philpott, '80) therefore is limited to the SW condition. In FW this pathway is reduced by the greater development of tight junctions.

Grouping of MR cells in killifish opercular epithelium may simply be a function of the large numbers of cells. In the tilapia *Oreochromis mossambicus* the density of MR cells is about 250 $cells.mm^{-2}$ (Foskett et al., '81) and in trout skin it is about 140 $cells.mm^{-2}$ (Marshall et al., '92), compared to 2,044 ± 72 in killifish. Hence, crowding would result in some cell-to-cell interactions. Alternatively, it may reflect cells at different stages of development, as seen in SW accessory and chloride cells (Karnaky, '86) or different cell types, as in alpha and beta chloride cells of teleost gills (Pisam and Rambourg, '91). This may explain the varying degree of apical membrane folding of cells sharing a crypt (Fig. 2a).

The finding of larger diameter MR cells in FW-adapted fish by fluorescence microscopy (Fig. 1a) was unexpected, as hypertrophy of opercular epithelial MR cells has been associated with hypersaline conditions (Karnaky et al., '76b; Foskett et al., '81). The result supports the previous observation in gill epithelia, where MR cells increase in size with adaptation to fresh water (Laurent and Hebibi, '89).

It is curious that there are very few desmosome junctions in opercular epithelium; these junctions only appear proximal to the tight junction (Figs. 2, 3). This paucity of desmosome junctions has been observed repeatedly, but without comment (Karnaky et al., '76a,b, Hootman and Philpott, '79, '80; Pisam and Rambourg, '91). With few desmosome junctions, movement of MR cells and nearby pavement cells is clearly possible and consistent with some previously observed variations in ultrastructure. There are dynamic changes in the depth of the apical crypts of MR cells of killifish gills with changing salinity (Hossler et al., '85). Also, the observation of physical covering and uncovering of trout gill MR cells during acid/base disturbances demonstrates the plastic nature of the system (Goss et al., '92b). The fact that we observe some covered MR cells and others that are clearly exposed (Figs. 1c,2) suggests some regulation of transport rates based on how many MR cells are recruited.

Chloride transport

Evidence for active uptake of Cl^- across opercular epithelium of FW-adapted killifish includes (1) the disagreement of the observed flux ratio with that predicted by the Ussing equation, i.e., purely on the basis of diffusion (Tables 1, 3); (2) the partial inhibition of Cl^- uptake by anaerobiosis; and (3) the presence of net Cl^- flux in the uptake direction when epithelium is bathed in symmetrical saline and short-circuited (Table 4). Clearly SW transport functions of MR cells are absent because of the inability of epithelium to secrete Cl^- when bathed in symmetrical saline (Tables 1, 4). Development of the ion uptake functions by MR cells appears to be part of a slow acclimation process because animals transferred for 2 days to FW from SW have not yet fully developed the uptake (compare Tables 1, 2) and are still capable of secreting Cl^- (Table 2), albeit at a lower rate than in SW. In combination, these results demonstrate that development, under the influence of slow-acting hormones, of SW and FW types of MR cells

and/or conversion of existing MR cells to express the respective ion transport systems can now be studied using killifish opercular epithelium.

The mechanism of Cl^- uptake by MR cells in FW is not yet proven conclusively. Presumably, there should be anion exchangers connected with acid base balance, as is true from in vivo studies with trout (e.g., Goss et al., '92a). The present results demonstrate that Cl^- influx likely is nonconductive, inasmuch as changes in G_t have no effect on Cl^- influx (Table 4), while the efflux increases with increasing G_t (Fig. 4). However, evidence for Cl^-/HCO_3^- exchange involvement in Cl^- uptake is not compelling, because the anion exchange inhibitor SITS was ineffective and our preliminary experiments with 0.1 mM DIDS (mucosal side, data not shown) also suggest no effect.

Sodium transport and shunt permeability

The current model for Na^+ uptake (Avella and Bornancin, '89; Lin and Randall, '91), is comparable to models of frog skin in FW (Ehrenfeld et al., '85). In this model, an active electrogenic extrusion of H^+ ions across the apical membrane by H^+-ATPase creates an electrochemical gradient for the entry of Na^+ through separate Na^+-selective channels. This provides a coupling of Na^+ uptake to acid excretion and Cl^- uptake to base excretion. Unfortunately, Na^+ transport across FW opercular epithelium (with FW on the mucosal surface) appears to be dominated by passive movement through a cation selective shunt pathway. Na^+ fluxes with FW on the mucosal side agree with the Ussing flux ratio equation (Table 3); hence, most if not all of the Na^+ moves by passive diffusion. The cation exchange inhibitor amiloride had no significant effect on Na^+ movement; therefore, Na^+/H^+ exchange is not indicated, nor is the amiloride-sensitive Na^+ channel. If the Na^+ channel were present one would expect to observe development of electrogenic Na^+ uptake when mucosal bathing solution Na^+ activity is increased, as happens with amphibian skin (Kirschner, '83). However, in symmetrical saline FW killifish opercular epithelium develops only a small I_{sc} (much smaller than amphibian skin that has epithelial Na^+ channels) and apparently an electroneutral NaCl uptake (Table 4). The apparent lack of Na^+ transport in the FW opercular epithelium is consistent with recent work demonstrating Na^+/H^+ exchange in gill pavement cells in primary culture (Pärt and Wood, '96).

Given the relationship between chloride efflux and G_t and the overall negative balance of ions across opercular epithelium (Tables 1, 2), at least some of the chloride efflux could result from microscopic damage done during dissection and mounting of tissue in spite of normal precautions to minimize edge damage (Marshall, '85). In previous work with trout opercular epithelium edge damage is apparently small, as gauged by the low permeability of the nominally extracellular marker ^{14}C-mannitol (Marshall, '85) and our work (using the same equipment and procedures) with cleithral epithelium (Marshall et al., '92) and trout urinary bladder (Marshall, '86) has routinely produced high-resistance epithelial preparations. It seems that killifish, in adapting to FW, do not (or cannot) eliminate entirely the cation selective shunt pathway. This shunt pathway is well recognized in SW adapted opercular epithelium (reviewed by Zadunaisky, '84; Péqueux et al., '88) and serves (in SW) as the Na^+ secretion pathway. Na^+ apparently is driven through the paracellular path by gradient established by active Cl^- extrusion in the transcellular path. It is clear that the high density of MR cells in FW killifish opercular epithelium correlates with the (limited) persistence of the cation-selective shunt pathway but it is less clear what is the physiological advantage of such cation permeability. The present results are consistent with Karnaky (1991) who described tight junctions that were responsive to salinity change. Persistence of the shunt may be inherent to the in vitro system and may account for the negative ion balance of the preparation in FW but would not likely affect directly ion uptake mechanisms.

Transport in symmetrical saline

Response of the opercular epithelium to sudden introduction of a saline solution to the apical surface and the concomitant changes in transport rates is physiologically relevant, inasmuch as the animal in its estuarine habitat may encounter such rapid changes in environmental salinity. Fluxes performed in symmetrical saline are not easily compared to those in FW because of the well-documented changes in shunt permeability and possible initiation of transport systems that are inactive in FW (Kirschner, '83; Karnaky, '91). However, they provide a convenient experimental situation for comparisons with other epithelia. When this epithelium is bathed in symmetrical saline there develops a net Na^+ uptake of about 2.0 $\mu mol.cm^{-2}.h^{-1}$ which is not significantly different from the corresponding uptake of Cl^-, at about 1.2 $\mu mol.cm^{-2}.h^{-1}$ (Table 4). There is a small I_{sc} that could represent net cation uptake or anion

secretion. The latter is unlikely because net movement of the major anion, Cl^-, is in the uptake direction and the treatment with db-cAMP + IB^- that significantly stimulates I_{sc} (Table 4). It is not clear which transported ion accounts for the change in I_{sc}. It is possible that I_{sc} represents cation uptake and, consistent with this, net Na^+ flux is in the uptake direction. However, stimulation of epithelium with db-cAMP + IB^- increased I_{sc} while net Na^+ flux instead decreased markedly. As well, there is no significant correlation between Na^+ influx with I_{sc} ($P > 0.5$). There was a (nonsignificant) trend to an increase in Cl^- efflux, hence the current change may represent an initiation of SW-like Cl^- secretion, as is suggested by inhibition of the current by a mucosally added anion channel blocker diphenylamine-2-carboxylate. This could be addressed more directly, perhaps using dual isotope techniques.

Transepithelial potential in FW

FW acclimated fish generally exhibit a transbody potential, measured in vivo on free swimming or anaesthetized animals, of 0 to –30 mV relative to the external medium and this apparently is a diffusion potential caused by the greater permeability of paracellular pathways to Na^+ than to Cl^-. It has been shown previously that negative transbody potential in fresh water is related to low environmental calcium activity (Potts, '84). Ca^{2+} appears to play a critical role in stabilizing the tight junctions and in controlling their conductance. In three studies of teleost skin in vitro, with low calcium bathing solutions (100 μM) on the mucosal side, we find that only epithelia that had large numbers of MR cells (i.e., *Fundulus*) also had the large negative inside V_t and associated large ionic conductance. Opercular epithelia of the tilapia, *O. mossambicus,* (McCormick et al., '92) and rainbow trout (Marshall et al., '92) instead develop serosa-positive transepithelial potentials and have, by comparison with killifish, very few mitochondria rich cells. V_t across rainbow and brook trout skins bathed with FW on the mucosal side are approximately +2.5 and –8.5 mV, respectively (Marshall, '85; Marshall et al., '92). Because these measurements are within the range for in vivo measurements for the respective species, it is unlikely that dissection per se of killifish opercular epithelium produces a damage-associated shunt. Voltage is more negative than that seen in vivo for killifish larvae in 5% SW (–11 ± 5.0 mV, Guggino, '80), but there are no comparative measurements of transbody potential of adult killifish. In other work, we observed that mucosally added La^{3+} significantly reduced G_t and the negative inside V_t to about –10 mV (Marshall et al., '95), consistent with a recognized action of La^{3+} to displace Ca^{2+} from intercellular tight junctions and to close a paracellular shunt pathway. V_t reflects a high degree of selectivity of epithelium for cations, particularly Na^+, over anions.

Partial adaptation to FW

The experiment with partially acclimated animals, only 2 days after transfer to FW, illustrates several adaptive changes. Transepithelial conductance (in symmetrical saline) is 50–60% lower than for opercular epithelia from SW adapted animals (3.4 $mS.cm^{-2}$, Table 2, compared to 6.5 to 7.8 $mS.cm^{-2}$ for SW animals; Péqueux et al., '88) and V_t is also much lower than for fully adapted SW animals (V_t = 19.1 ± 1.9 mV [n = 10], Péqueux et al., '88). The net Cl^- transport rate has dropped 75% from about 4.0 $\mu mol.cm^{-2}.h^{-1}$ in short-circuited preparations from SW animals (Péqueux et al., '88) to approximately 1.0 $\mu mol.cm^{-2}.h^{-1}$ (Table 2). Whereas the SW ion secretion capability is substantially reduced, the FW ion uptake mechanism is not apparently developed fully. This transitional state presumably is evoked by pertinent osmoregulatory hormones (see Bern and Madsen, '92). For instance, over several days, prolactin causes a dedifferentiation of chloride cells (Foskett et al., '83) and a reduction in Cl^- secretion rate in tilapia opercular epithelia. Killifish opercular epithelium, because it demonstrates ion uptake in FW conditions, therefore may finally allow the study of transport by and hormonal control of MR cells, particularly the issue of de novo generation of FW MR cells vs. expression of FW ion uptake systems in existing MR cells.

ACKNOWLEDGMENTS

We thank A.L. MacDonald for animal care and M. Murphy for help in manuscript preparation. The work was supported by NSERC research grants to W.S.M. and C.M.W., an NSERC Undergraduate Student Research Award to C.W., and the W.F. James Chair in Pure and Applied Science awarded to C.M.W.

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