Paracellular pathway remodeling enhances sodium secretion by teleost fish in hypersaline environments

Regina R. F. Cozzi, George N. Robertson, Melanie Spieker, Lauren N. Claus, Gabriella M. M. Zaparilla, Kelly L. Garrow and William S. Marshall*

ABSTRACT
In vertebrate salt-secreting epithelia, Na⁺ moves passively down an electrochemical gradient via a paracellular pathway. We assessed how this pathway is modified to allow Na⁺ secretion in hypersaline environments. Mummichogs (Fundulus heteroclitus) acclimated to hypersaline (2× seawater (2SW), 64‰) for 30 days developed invasive projections of accessory cells with an increased area of tight junctions, detected by punctate distribution of CFTR (cystic fibrosis transmembrane conductance regulator) immunofluorescence and transmission electron microscopy of the opercular epithelia, which form a gill-like tissue rich in ionocytes. Distribution of CFTR was not explained by membrane raft organization, because chlorpromazine (50 µmol l⁻¹) and filipin (1.5 µmol l⁻¹) did not affect opercular epithelia electrophysiology. Isolated opercular epithelia bathed in SW and in 2SW had higher Vₑ of +40.1±0.9 mV (N=24), sufficient for passive Na⁺ secretion (Nernst equilibrium voltage: E_Na=+24.11 mV). Opercular epithelia from fish acclimated to 2SW and bathed in 2SW had higher Vₑ of +45.1±1.2 mV (N=24), sufficient for passive Na⁺ secretion (E_Na=+40.74 mV), but with diminished net driving force. Burnetanide block of Cl⁻ secretion reduced Vₑ by 45% and 29% in SW and 2SW, respectively, a decrease in the driving force for Na⁺ extrusion. Estimates of shunt conductance from epithelial conductance (Gₑ) versus short-circuit current (Iₛ) plots (extrapolation to zero Iₛ) suggested a reduction in total epithelial shunt conductance in 2SW-acclimated fish. In contrast, the morphological elaboration of tight junctions, leading to an increase in accessory-cell–ionocyte contact points, suggests an increase in local paracellular conductance, compensating for the diminished net driving force for Na⁺ and allowing salt secretion, even in extreme salinities.

KEY WORDS: Tight junction, CFTR, Immunocytochemistry, Electrophysiology, Fundulus heteroclitus, Euryhaline, Teleost fish, Gill epithelium, Ionocyte, Chloride cell

INTRODUCTION
The mummichog (or common killifish) Fundulus heteroclitus is well known for its euryhaline capabilities, readily adapting to environments ranging from freshwater to hypersaline conditions as high as 120‰, 3.75× seawater (SW) (Griffith, 1974). Thus F. heteroclitus has been and continues to be an important model organism for understanding mechanisms of teleost osmoregulation, as documented in two major reviews. Karnaky (1986) focused on chloride cell structure and function in the ionocyte-rich opercular epithelium, while Wood and Marshall (1994) compared in vitro and in vivo approaches to understanding euryhalinity in teleost fishes. Recently, the species has been recognized as a valuable genomic model in physiology (Burnett et al., 2007; Whitehead, 2010; Whitehead et al., 2011a,b) and toxicology (Whitehead et al., 2012) and sequencing of the genome is complete (www.fundulus.org). Current models for teleost ion transport and acid–base regulation in gills (Evans et al., 2005) include transcellular Cl⁻ secondary active transport in a two-step process in seawater-type NaCl-secreting ionocytes [Type IV ionocytes as per functional classification (Hiroi et al., 2005)]. The opercular epithelium, an epithelium rich in ionocytes, is a valuable model for the operation of the main ion-secreting organ (the gills) that has contributed greatly to the understanding of ionocyte function (Karnaky and Kinter, 1977; Karnaky, 1986; Wood and Marshall, 1994). By this model, accumulation of intracellular Cl⁻ is by transmembrane entry across the basolateral membrane via the Na,K,2Cl cotransporter (NKCC1) (Haas and Forbush, 2000), followed by Cl⁻ exit down its electrochemical gradient via the cystic fibrosis transmembrane conductance regulator (CFTR) anion channels localized in the apical membrane. CFTR has been identified in ionocytes of the opercular epithelium (Marshall et al., 1995), the CFTR gene cloned from mummichogs (Singer et al., 1998, 2008), and in seawater fish the channel is present in the apical membrane of ionocytes (Hiroi et al., 2005; Marshall et al., 2002; McCormick et al., 2003; Tang and Lee, 2007). Adaptation to seawater augments ion secretion in parallel with increased CFTR expression (Marshall et al., 1999) and there is mobilization of both the CFTR and NKCC1 cotransporter in ionocytes, placing more of the former in apical membrane and more of the latter in the basolateral membrane (Marshall, 2002; Marshall et al., 2002). Hence Cl⁻ secretion is transcellular and depends on intracellular Cl⁻ activity and a favorable transmembrane potential across the apical membrane to drive Cl⁻ into the environment (SW or hypersaline). However, the intracellular Cl⁻ activity and electrical driving force across the apical membrane have not yet been measured.

The secretion of Na⁺, the counter-ion to Cl⁻, is also thought to proceed by passive transport, aided by the positive transepithelial voltage that is generated at least in part by the exit of Cl⁻ at the apical membrane. The location of the Na⁺ leak pathway is between the ionocyte and accessory cells in the apical crypts where Cl⁻ activity and electrical potential may be at least partially responsible for Na⁺ efflux. In this arrangement, Na⁺ efflux generates a ‘loop current’ where Cl⁻ exit carries current (of negative charge) out of the gill, while Na⁺ paracellular exit carries the current back into the gill, completing the loop. To drive Na⁺ efflux efficiently by a given transepithelial voltage, close juxtaposition of the two pathways is important to minimize stray electrical resistance; by Ohm’s Law, excessive stray resistance in the circuit will otherwise limit the current flow, i.e. the rate of NaCl secretion.
NaCl secretion in seawater and hypersaline conditions

A morphologically-distinct cell type in eel gills, rich in mitochondria, was thought to be responsible for NaCl secretion by marine teleost gills and these cells were termed ‘chloride-secreting cells’ (Keys and Willmer, 1932). Philpott and Copeland (1963) recognized a field of these cells in the gills, skin and buccal epithelium of F. heteroclitus and described the curious ultrastructure, with elaborated basolateral membrane surface in serpentine tubules that formed a meshwork among the well-organized mitochondria. Na⁺,K⁺-ATPase, localized specifically on the basolateral membrane of these ‘chloride cells’ (Karnaky et al., 1976) displayed higher activity in the gills of F. heteroclitus adapted to seawater than in those adapted to freshwater (Epstein et al., 1967; Towle et al., 1977). Na⁺,K⁺-ATPase activity proved to be the indirect driving force for transcellular Cl⁻ exit from the animal into seawater (Silva et al., 1977a,b). Na⁺,K⁺-ATPase, which is responsible for producing the Na⁺ transmembrane gradient that drives NKCC operation, has been cloned from F. heteroclitus (Semple et al., 2002). There is complex regulation of Na⁺,K⁺-ATPase at the protein and mRNA expression levels associated with adaptation to seawater (Choe et al., 2006; Mancera and McCormick, 2000; McCormick et al., 2009; Scott and Schulte, 2005). Tilapia (Oreochromis mossambicus) in hypersaline conditions significantly shift their gill proteome, featuring increased expression of mitochondrial proteins and the stress protein NDRG1 (Kültz et al., 2013). The Salton Sea, an endorheic lake in California (current salinity 44‰) has endemic fish populations of strongly euryhaline fish (Riedel et al., 2002) and responses to hypersalinity involve ionoregulatory responses primarily to gill and posterior intestine epithelia (reviewed by Laverty and Skadhauge, 2012). Whereas strongly euryhaline fish can survive indefinitely in salinities well above full-strength seawater (32‰), hypersaline conditions above 65‰ cause significant increases in plasma ions (Na⁺ and Cl⁻), loss of tissue water content and increased apoptosis in gill ionocytes of the strongly euryhaline tilapia hybrid (Oreochromis mossambicus × O. urolepis honorum) (Sardella et al., 2004). Hypersaline conditions (2SW) evoke hypertrophy of ionocytes of pupfish gills, elaboration of the basolateral membrane surface area (Cyprinodon variegatus) and, in pupfish and mummichog gills, marked increases in basolateral Na⁺,K⁺-ATPase and enzyme activity (Karnaky et al., 1976). In sea bass (Dicentrarchus labrax), hypersalinity evokes ionocyte hypertrophy and increased accessory cell interdigitiation with ionocytes, forming complex morphologies attributed as ‘leaky junctions’ (Varsamos et al., 2002). However, research is lacking on the effects of hypersalinity on the electrophysiology of passive transport components, CFTR and the paracellular pathway.

Paracellular pathway and Na⁺ secretion

The tight junctions between ionocytes and accessory cells of seawater teleost fish gills are permeable to cations, even to La³⁺, and form a localized cation-permeable pathway (Sardet et al., 1979; Scheffey et al., 1983) in an epithelium of pavement cells that are connected by deep, relatively impermeable tight junctions (Monteiro et al., 2010; Sardet et al., 1979). These permeable junctions balance the anion current emanating from the ionocyte and together comprise the total measurable epithelial conductance, as detected by vibrating probe experiments (Foskett and Machen, 1985). The mechanism of maintaining high cation permeability in intercellular junctions between ionocytes and accessory cells is not known. One possibility is that a special claudin junctional protein in these junctions imparts cation selectivity. Claudins are a complex family of intermembrane proteins in a large gene family with more than 30 members (Chasiotis et al., 2012). In smolting of Atlantic salmon, claudin 10e may be important in seawater acclimation and tight junction structure in the gill epithelium (Tipsmark et al., 2008a,b). Acclimation of tilapia (O. mossambicus) to seawater decreases transcript abundance of claudin-3 and claudin-4 junctional proteins (Tipsmark et al., 2008a,b), suggesting that cation permeability could actually rely on the selective lack of claudins in Na⁺-permeable junctions rather than their extra presence. Acclimation of mummichogs to seawater and low salinity reveals lower transcript abundance of claudins 3 and 4 in gill epithelium in seawater (Whitehead et al., 2011a,b) and in the pufferfish Tetraodon nigroviridis, acclimation to seawater and hypersaline reduces gill transcript levels of claudin 3 (Bagherie-Lachidan et al., 2008). In cultured pavement cells from seawater pufferfish, claudin 6, claudin 10d and claudin 10e are absent, yet these are responsible to salinity change in whole gill epithelium where ionocytes are present (Bui et al., 2010). The cation permeability thus may reside in the protein make-up of the special junctions between ionocytes and accessory cells.

Transepithelial potentials measured in vivo in seawater teleost fish have long been recognized to approximate the Na⁺ diffusion potential (Nernst equilibrium potential) (Potts, 1984) for most marine species tested, including F. heteroclitus (+18 mV), but there are several teleost fish species for which the potential is much lower, and, in some species, the opposite polarity (e.g. Opsanus beta with a Vₑ of −8 mV). For the fish with positive Vₑ, this voltage was thought to be the driving force for Na⁺ secretion, such as in the model proposed (Silva et al., 1977a,b) for teleost fish gills and confirmed by vibrating probe studies of ionocytes (Foskett and Machen, 1985; Scheffey et al., 1983) and in ion flux experiments with voltage-clamped isolated opercular epithelia (Degnan and Zadunaisky, 1980). Thus for the symmetrical saline condition, Na⁺ certainly has sufficient driving force to produce Na⁺ secretion via the paracellular pathway. The potential measured was usually smaller than the calculated Nernst equilibrium potential for sodium (Eₙa, approximately +25 mV in seawater fish), which is probably a result of the actual Vₑ being shunted by leak pathways, such as leakage around the catheter or physiological leakage represented by large surface areas of respiratory epithelium. Importantly, measurements made in preparations with the same salt-secretion mechanism but with much less shunt area, such as the yolk sac larvae of F. heteroclitus that is capable of seawater ion regulation yet has no gill area, gave results that were significantly higher than Eₙa, thus demonstrating that in seawater there could be a voltage sufficient by itself to drive Na⁺ secretion. Specifically, Vₑ measured across the yolk sac of F. heteroclitus embryos in seawater was +50±1.7 mV and +40±1.3 mV for F. ber�idae, (Guggino, 1980), clearly higher than the Eₙa of approximately +25 mV in seawater. The remaining question is whether there is sufficient driving force to secrete Na⁺ in asymmetrical conditions, particularly in conditions of hypersaline exposure, conditions that are physiologically relevant to the fish in the environment.

In this study, we hypothesized that Vₑ should exceed Eₙa in seawater and in hypersaline conditions and that ultrastructure should indicate changes in shunt structure in hypersaline conditions. To test these hypotheses, we acclimated adult F. heteroclitus to SW and 2SW and tested isolated opercular epithelia in Ussing chambers electrophysiologically to compare Vₑ with calculated Eₙa, with SW or 2SW bathing the outside surface. We also found that there were clear differences in the ultrastructure and arrangement of intercellular junctions between accessory cells and ionocytes, featuring punctate CFTR distribution in ionocytes and multiple interdigitations of accessory cells with ionocytes. We estimated that...
the increased availability of junctions increases the efficiency (conductance) of the pathway such that, even with diminished voltage driving force in hypersaline conditions, Na\(^+\) secretion would continue. Paradoxically, the overall conductance of the paracellular pathway, extrapolated from the linear relationship between short circuit current (\(I_{sc}\)) and epithelial conductance (\(G_e\)), decreases in 2SW, implying that the majority of tight junctions become tighter while a minority of junctions, those involved in Na\(^+\) secretion, become more conductive.

**RESULTS**

**Immunocytochemistry**

CFTR was present in the opercular epithelia of killfish acclimated to SW and 2SW. Although all of the ionocytes showed immunofluorescence in the apical crypts, the distribution of the CFTR varied between the salinities. In epithelia of fish acclimated to SW, the majority of the apical crypts showed a continuous and diffuse distribution of CFTR, thus forming a fluorescent ring delineating each crypt (Fig. 1A–C). Although this type of distribution was also visible in epithelia of fish acclimated to 2SW, the majority of the apical crypts showed discontinuous staining along the crypt ring, indicating that CFTR formed punctate (bright fluorescent dots surrounded by lower fluorescence, Fig. 1D–F). In fact, 78% of SW ionocytes contained diffuse CFTR in their apical crypts, while the rest (22%) had punctate CFTR distribution. In 2SW-acclimated fish, the number of apical crypts with diffuse CFTR significantly decreased to 25%, while the number of crypts containing CFTR nodes significantly increased to 75% (Fig. 2A). Also, apical crypts were significantly deeper in the SW ionocytes displaying diffuse CFTR (4.14 \(\mu\)m deep) compared with all other groups (2.6–3.1 \(\mu\)m deep) (Fig. 2B). Furthermore, for those apical crypts with condensed nodes, the number of dots per apical crypt was significantly higher in the 2SW epithelia compared with the SW epithelia (6.58\(\pm\)0.079 dots per apical crypt, \(N=12\) animals, 106 crypts scored versus 5.08\(\pm\)0.152 dots per apical crypt, \(N=12\) animals, 32 crypts scored, respectively; \(P<0.0001\), unpaired \(t\)-test).

**Electron microscopy**

Transmission electron microscopy (TEM) revealed that the apical crypts of ionocytes of fish acclimated to 2SW had complex interdigitations of accessory cell processes and ionocyte processes alternating around the circumference of the apical crypt (Fig. 3), compared with the typical single projection of the accessory cell. In the figure, there are six patches of ionocyte membrane alternating with six sections of accessory cell in the apical portion of the ionocyte, the accessory cell projections are distinguishable because of their greater electron density. The junctions are simple and shallow (<0.5 \(\mu\)m), as is typical of accessory-cell–ionocyte junctions.

A diagrammatic rendering of the accessory-cell–ionocyte interaction (Fig. 4) indicates that processes from one accessory cell may interrupt CFTR distribution at multiple locations around the perimeter of the apical crypt. Our results did not show any evidence for multiple accessory cells (numerous nuclei adjacent to the ionocyte nucleus), but immunocytochemistry suggests the presence of only one accessory cell and the TEM data revealed multiple cell processes.

**Electrophysiology**

**Membrane raft disruptors**

Filipin, added at 1.53 \(\mu\)mol l\(^{-1}\) for 30 min had no effect on \(V_t\) (or the resting \(I_{sc}\), not shown) and had no effect on the response of the opercular epithelia (OE) to hypotonic shock; control and test sets of membranes had large reductions in \(I_{sc}\) that were more than fully reversed with the reintroduction of isotonic Cortland’s saline (Fig. 5A). Chlorpromazine, added at doses from 1.0 to 50 \(\mu\)mol l\(^{-1}\), a dose sufficient to disrupt membrane rafts, had no significant effect on the spontaneous \(V_t\) (or \(I_{sc}\), not shown) of OE that were mounted in vitro in symmetrical saline (Fig. 5B).

**Open-circuit voltages**

Opercular epithelia from 2SW-acclimated fish had significantly higher \(V_t\), +28% compared with SW-acclimated fish in symmetrical saline, which, in both cases, was substantially greater than \(E_{Na}\) (\(=0\) mV). Perfusion of SW on the mucosal side increased \(V_t\) by 78% for SW and 48% for 2SW fish (Fig. 6) and the voltages were significantly higher than the calculated Nernst Equilibrium potential, \(E_{Na}\), indicating that Na\(^+\) net driving force would be inside to outside. Increasing the external salinity to 2SW for OE from fish acclimated to SW increased \(V_t\) a further 12% and the net driving force for Na\(^+\) was still positive (Fig. 6). For fish acclimated to 2SW, the same manipulations resulted in a similar pattern.

![Fig. 1. CFTR immunofluorescence in ionocyte apical crypts of killifish.](image)
and accessory cell processes are shallow. Scale bars: 1 μm.

OE from 2SW-acclimated fish had the high NaCl Cortland’s saline to help mimic 2SW plasma. The calculated $E_{\text{Na}}$ was thus slightly lower, but physiologically relevant. Overall, the Na$^+$ net driving force was positive for Na$^+$ efflux into SW and into 2SW.

Electrogenic Cl$^-$ active transport was inhibited by two means: bumetanide, which blocks NKCC directly, and low K$^+$ Cortland’s saline, which blocks NKCC by depriving the transporter of K$^+$ while simultaneously blocking Na$^+$, K$^+$-ATPase (Fig. 7). When Cl$^-$ secretion was blocked by bumetanide, $V_t$ decreased by 80% ($\sim$19 mV) in symmetrical Cortland’s saline and with low-K$^+$ Cortland’s saline bathing both sides of the OE, the decrease was 64% ($\sim$16 mV), thus reducing the driving force for Na$^+$. Perfusion of the mucosal sides with SW and 2SW increased $V_t$ as expected, but it was 45% lower than the SW control $V_t$ and 29% lower than the 2SW control $V_t$. The measured $V_t$ in asymmetrical conditions was less than the calculated $E_{\text{Na}}$. In the case of low-K$^+$ Cortland’s with SW outside, there was a small (approx. +5 mV) $V_t$ in excess of $E_{\text{Na}}$.

Similar manipulations were also performed on pieces of buccal epithelium, a membrane that has very few ionocytes and more mucus cells, compared with the OE. In symmetrical saline, the transepithelial resistance $R_t$ was significantly higher (+280%) and this ratio constant with SW and with 2SW bathing the mucosal side; for both epithelia, higher salinity reduced $R_t$ (Fig. 8A). The $V_t$ measured across the buccal epithelium was significantly lower (63%) than the opercular epithelium in symmetrical saline, in SW (50%) and in 2SW (53%) of opercular epithelium $V_t$ (Fig. 8B). For the buccal tissue, the net driving force for Na$^+$ was negative (less than $E_{\text{Na}}$), implying that if Na$^+$ were permeable at these locations, then net Na$^+$ movement would be not contribute to Na$^+$ secretion, rather the reverse would be true. The calculated $I_{sc}$ for buccal epithelium in symmetrical saline was therefore much lower (22%) than that for the opercular epithelium, 45.4±11.0 $\mu$amp cm$^{-2}$ ($N=7$) versus 205±15.5 $\mu$amp cm$^{-2}$ ($N=13$), respectively.

**Leak conductance**

In the leak conductance estimate, initial $I_{sc}$ of SW epithelia was 122±13.5 $\mu$amp cm$^{-2}$ ($N=11$) and in 2SW epithelia was 137.8±14.1 ($N=12$), not significantly different ($P=0.430$, two-tailed t-test). $I_{sc}$ of SW epithelia after hypotonic shock was 31.0±4.7 $\mu$amp cm$^{-2}$ ($N=11$) and in 2SW epithelia was 39.9±6.3 $\mu$amp cm$^{-2}$ ($N=12$) and therefore not significantly different ($P=0.310$, two-tailed t-test). Individual plots of the hypotonic response as $G_{sc}$ versus $I_{sc}$ extrapolated to $I_{sc}=0$ (y intercept) yielded leak conductance ($G_{sl}$) estimates of 6.35±0.49 mS cm$^{-2}$ for SW-acclimated opercular epithelia ($N=11$) and a significantly lower value ($P=0.005$, two-tailed t-test) of 3.74±0.40 mS cm$^{-2}$ for 2SW-acclimated fish ($N=12$).

**Fig. 3. Junctional complexes between ionocytes and accessory cells.** Transmission electron micrograph in the plane parallel to the epithelial surface at the level of the apical crypt of an ionocyte (ic) with accessory cell (ac) in an OE from a 2SW-acclimated fish (A) and a SW-acclimated fish (B). The accessory cell processes (relatively electron-lucent) alternate with ionocyte apical membrane (relatively electron dense; arrowheads in A). Junctions between ionocyte and accessory cell processes are shallow. Scale bars: 1 μm.
Compared with controls in SW, acclimation to 2SW significantly increased plasma osmolality, from 356.5±2.11 to 378.7±4.27 mOsm kg\(^{-1}\) (\(P<0.001, N=13\)) and plasma Na\(^+\) from 170.4±2.42 to 178.8±3.13 mmol l\(^{-1}\) (\(P<0.05, N=13\)), while there was no change in plasma K\(^+\) in SW (5.747±0.23 mmol l\(^{-1}\)) or 2SW (5.790±0.18 mmol l\(^{-1}\); \(P=0.89, N=12\)).

**Plasma ions and osmolality**

Compared with controls in SW, acclimation to 2SW significantly increased plasma osmolality, from 356.5±2.11 to 378.7±4.27 mOsm kg\(^{-1}\) (\(P<0.001, N=13\)) and plasma Na\(^+\) from 170.4±2.42 to 178.8±3.13 mmol l\(^{-1}\) (\(P<0.05, N=13\)), while there was no change in plasma K\(^+\) in SW (5.747±0.23 mmol l\(^{-1}\)) or 2SW (5.790±0.18 mmol l\(^{-1}\); \(P=0.89, N=12\)).

**DISCUSSION**

The main focus of this paper is to demonstrate that elaboration of the special leaky intercellular junctions between ionocytes and accessory cells is a necessary process to maintain conditions favorable for Na\(^+\) secretion in a passive transport process. The elaboration is clear in that the distribution of CFTR immunofluorescence in the apical crypts of ionocytes is more often punctate in hypersaline conditions. The large number of loci for Na\(^+\) secretion are thus close (about 1 μm or less) to the CFTR-rich zones, where there is secretion of Cl\(^-\). The close juxtaposition of Na\(^+\) and Cl\(^-\) secretion areas will reduce stray electrical resistance and enhance the coupling between the two ion-transport systems.

**CFTR redistribution in hypersaline water**

Acclimation of mummichogs to hypersaline conditions significantly alters the distribution of CFTR in the apical membranes of ionocytes in the OE. The ionocytes develop punctate CFTR distribution, detected by immunocytochemistry, in a ring around the apical crypt. Thus the junctions between accessory cells and ionocytes were more numerous in 2SW. In SW, CFTR distribution was restricted to the apical crypt and CFTR occupied all the membrane surface, except where the accessory cell interacts with the ionocyte to form a leaky junctional pathway. This is true for the OE of mummichogs where CFTR immunofluorescence was found to redistribute from sub-apical zones into the apical membrane by 24–48 h after transfer of FW-acclimated fish to SW (Marshall et al., 2002). We observed an increase in the number and complexity of accessory-cell–ionocyte interactions that were significantly more elaborate in hypersaline conditions. By transmission electron microscopy, sections through the apical crypts of ionocytes from mummichogs acclimated to hypersaline conditions revealed multiple loci of ionocytes alternating with cellular processes of accessory cells and these were loosely connected by intercellular junctions. Punctate distribution of apical CFTR immunofluorescence was observed previously in seawater-adapted Hawaiian goby ionocytes in the gill interlamellar region (McCormick et al., 2003).

**Interdigitations with accessory cells**

Accessory cells are small Na\(^+\),K\(^-\)-ATPase-poor cells loosely attached to mitochondrion-rich chloride-secreting cells (Hootman and Philpott, 1980) and the junctions are permeable to cations...
including lanthanum (Sardet et al., 1979). There is evidence that ionocyte morphology can change quickly; transfer of larval Ayu (Plecoglossus altivelis) yielded interdigitation of ionocytes and accessory cells within 1–3 h of transfer, as detected by TEM (Hwang and Hirano, 1985). Accessory-cell–ionocyte junctions are dynamic, because OE from mummichogs acclimated to 1% SW rapidly (~45 min) respond in vitro to external isotonic saline by opening of the junctions to the SW-like conformation (Karnaky, 1991). Multicellular ionocyte–accessory-cell complexes, detected by TEM, are present in gill epithelium of Nile tilapia (O. niloticus) that were slowly acclimated to full-strength seawater (a salinity that is near their maximum) (Cioni et al., 1991) and similar multicellular alternating interdigitation structures were observed in SW-acclimated O. mossambicus gills (Shiraiishi et al., 1997) and in sea bass (Varsamos et al., 2002). In the yolk sac epithelium of tilapia (O. mossambicus) transferred to seawater, the CFTR apical distribution was punctate and had multiple (up to 9) intense immunopositive concentrations arranged in a ring at the apical membrane (Hiroi et al., 2005). The authors interpreted the punctate distribution as processes of the ionocytes and accessory cells arranged alternately and interdigitated; they speculated that these could be multiple loci for paracellular Na⁺ secretion. Transfer of FW-acclimated O. mossambicus to SW likewise redistributed CFTR into the apical membrane of type IV ionocytes, and some of the CFTR distribution was distinctly punctate, with 7–9 immunopositive dots surrounding the apical crypt wall (Hiroi et al., 2005). They speculated that the development and degeneration of the interdigitation between ionocytes and accessory cells occurs reversibly in parallel with the appearance and disappearance of CFTR at the apical membrane of ionocytes. Acclimation of hybrid salinity-tolerant tilapia to hypersaline conditions evoked more interdigitation of accessory cells and ionocytes in gill epithelium and apparent proliferation of accessory cells relative to ionocytes (Sardella et al., 2004) but at extremely high salinity (75% and above), many of the ionocytes become covered over by pavement cells, interpreted as an attempt to seal off permeable junctions against excessive salt gain. Our findings confirm morphologically the punctate distribution of CFTR in apical membranes of ionocytes of seawater teleost fish gills and the interdigitation of ionocytes and accessory cells. We offer a functional explanation that the close juxtaposition and increased number of Na⁺-permeable paracellular pathways offers a low-resistance pathway for Na⁺ secretion so that a modest transjunctional voltage gradient can drive Na⁺ efflux through these junctions.

**Functional interpretation**

To reveal the functional significance of the morphological change to punctate CFTR distribution, we measured the transepithelial voltage and conductance in vitro with SW and 2SW bathing the external surface and found significant increases in $V_t$ with SW and 2SW, averaging +45 mV. Previous investigations suggest that the interdigitations are associated with Na⁺ paracellular transport (Hiroi et al., 2005) and could comprise ‘leaky junctions’ (Varsamos et al., 2002). Assuming that each point of contact between the accessory cell and ionocyte has the same conductance, most cells in SW have only two such contacts, whereas the 2SW fish would have 14, representing a 7-fold higher local conductance. To secrete Na⁺, the measured voltage should exceed the Nernst equilibrium potential for Na⁺ ($E_{Na}$) in all conditions, even in hypersaline solutions. We found this to be true: for SW and 2SW bathing the outer surface the measured $V_t$ exceeded the calculated $E_{Na}$, demonstrating that the paracellular pathway Na⁺ secretion is feasible and likely, even in 2SW. The maximal $V_t$ we observed in 2SW was +52 mV and 25% (6 of 24 epithelia) generated $V_t$ above +50 mV. Indeed, in mummichog larvae

---

**Fig. 6.** Open-circuit $V_t$ (corrected for junction potentials) for opercular epithelia from SW- and 2SW-acclimated killifish. OE were bathed in symmetrical Cortland’s saline (Cortland’s) or asymmetrically with seawater (SW) or twice seawater (2SW) on the mucosal side and compared with the calculated Nernst equilibrium potential, $E_{Na}$, for each asymmetrical condition ($E_{Na}$, 0 for symmetrical conditions). The measured transepithelial potential, $V_t$, is higher than $E_{Na}$ in all cases but this difference, the net driving force for Na⁺ secretion, is markedly diminished in 2SW conditions. Values are means±s.e.m. Different letters represent P<0.001.

**Fig. 7.** Open-circuit $V_t$ measurements in opercular epithelia from SW-acclimated killfish treated with bumetanide or K⁺-free Cortland’s saline to inhibit electrogenic Cl⁻ secretion. Either (A) bumetanide (0.1 mmol l⁻¹, serosal side) or (B) K⁺-free Cortland’s saline (serosal side) was used to inhibit electrogenic Cl⁻ secretion. Both treatments significantly inhibited $V_t$ while subsequent addition of SW and 2SW to the mucosal side increased $V_t$ but not to the level of $E_{Na}$ (dotted lines) (except for SW condition with K⁺-free Cortland’s, where K⁺ reverse gradient would tend to increase $V_t$). Thus with electrogenic Cl⁻ secretion blocked, Na⁺ secretion would slow down or reverse. Values are means±s.e.m. Different letters represent P<0.001.
Opercular epithelium and a loss of responsiveness to hypotonic shock if endocytosis had where we would have expected increases in baseline NaCl secretion.

loss of sensitivity of the ion transport to osmotic (hypotonic) shock, endocytosis. In neither case did we detect changes in transport rates or regulation using these two raft inhibitors, which inhibit clathrin-coated (Ares and Ortiz, 2012). We tested for sensitivity of ion transport and its chlorpromazine and filipin inhibits the rate of NKCC2 endocytosis the steady-state levels of cell-surface CFTR (Bradbury et al., 1999). formation of clathrin-coated vesicles inhibits the internalization raft-disrupting agent filipin (Haggie et al., 2004). Disruption of mobile in the plasma membrane and this mobility is impaired by the

provides a high conductance paracellular pathway.

is close to the upper limit voltage and elaboration of tight junctions

maximum salinity for this type of salt secretion mechanism: +50 mV

V

Buccal epithelium had higher epithelial resistance Rt (A) and lower Vt (B) in symmetrical saline and significantly lower Vt than the calculated Ena of the measured Vt of opercular epithelium in SW and 2SW, suggesting that the buccal epithelium would not contribute to Na+ secretion in either SW or 2SW. Values are means±s.e.m. Different letters represent P<0.001.

in SW, the yolk sac membrane, rich in ionocytes and without the shunt formed by lamellae or edge damage, develops a Vt of +50±1.7 mV (Guggino, 1980), so this value may be close to the upper limit that these salt-secreting systems can develop. In extremely high salinities of 95%, Tilapia have elevated plasma Na+ to 260 mmol l−1 (Sardella et al., 2008); the calculated Ena of +44.3 mV suggests that fish are forced to adjust their plasma Na+ upward, so that the maximal Vt can still produce Na+ secretion via the paracellular pathway. In contrast, we found that the buccal epithelium, which has few ionocytes, develops much lower Vt and Iec, which confirms previous findings (Karnaky, 1980), but we predict that buccal epithelium would not contribute to Na+ secretion in SW or 2SW. In conclusion, 3SW appears to be the maximum salinity for this type of salt secretion mechanism: +50 mV is close to the upper limit voltage and elaboration of tight junctions provides a high conductance paracellular pathway.

Another possible explanation of punctate CFTR distribution could come from membrane raft organization. CFTR anion channels are mobile in the plasma membrane and this mobility is impaired by the raft-disrupting agent filipin (Haggie et al., 2004). Disruption of formation of clathrin-coated vesicles inhibits the internalization of CFTR from the plasma membrane, resulting in an increase in the steady-state levels of cell-surface CFTR (Bradbury et al., 1999). Also, treating the thick ascending loop of Henle renal cells with chlorpromazine and filipin inhibits the rate of NKCC2 endocytosis (Ares and Ortiz, 2012). We tested for sensitivity of ion transport and its regulation using these two raft inhibitors, which inhibit clathrin-coated endocytosis. In neither case did we detect changes in transport rates or loss of sensitivity of the ion transport to osmotic (hypotonic) shock, where we would have expected increases in baseline NaCl secretion and a loss of responsiveness to hypotonic shock if endocytosis had been effectively disrupted. Although high concentrations of the drugs could possibly produce greater effects, the doses used were higher than most mammalian membrane raft experiments to date. We see no evidence that membrane rafts are the main mechanism to maintain Na+ transport rate or CFTR distribution in hypersaline conditions. The source of structural stability of these interdigitations is unclear, but our unsuccessful preliminary experiments with disrupting agents suggest that membrane rafts are probably not the main stabilizing force. Traditional cytoskeletal-stabilizing structures are a possibility.

Vt compared with Ena

Trans-gill potentials (TEPs) measured in vivo with full-strength seawater generally are smaller than the Ena for most marine fish (Potts, 1984). For example, in F. heteroclitus, TEP was +18–23 mV, while the calculated Ena was +28 mV (Pic, 1978). It is well known that stress-generated catecholamines are potent inhibitors of active Cl− secretion in OE (Degnan et al., 1977; Foskett et al., 1982; Marshall and Bern, 1980) and this approach is a way of estimating the electrogenic component of the measured voltage, suggesting that 21 mV of the total TEP was electrogenic. Recently, confirming these estimates, TEP measured in SW for adult mummichogs was found to be +23 mV compared with the Nernst Ena of +29.1 mV (Wood and Grosell, 2008). The authors concluded that the TEP in SW was dependent on open paracellular pathways and they estimated that at least 1/3 of the TEP was electrogenic; however, this study did not examine hypersaline conditions.

The in vitro mummichog OE bathed in symmetrical saline develops a Vt of 15–30 mV, a transepithelial potential that is strongly inhibited by addition of the α-adrenergic agonist clonidine (Marshall et al., 1993) and by the loop diuretics furosemide and bumetamide (Degnan et al., 1977; Eriksson and Wistrand, 1986) and here Vt might be taken as being mostly electrogenic, in that full inhibition in symmetrical saline brings the Vt close to zero. In these conditions, Na+ influx and efflux respond to voltage clamping (+ and −25 mV) and conform to the predicted flux ratio equation, all indicating that Na+ moves through a single barrier to Na+ diffusion (Degnan and Zadunaisky, 1980). Furthermore, 1,3,5-triamino pyridine (TAP, 10 mmol l−1) inhibited Na+ and urea fluxes and decreased total epithelial conductance by 70–80% (Degnan and Zadunaisky, 1980), demonstrating that blockade of paracellular junctions by TAP blocks Na+ permeation. Conversely, low pH (pH 3.9–4.0, a mimic of acid rain exposure) increased both epithelial conductance and Na+ and mannitol fluxes, indicative of opening of a paracellular pathway to solute permeation in brook trout opercular epithelium (Marshall, 1985). There is general agreement that in symmetrical saline, Na+ is passively distributed across these epithelia, in which Na+ responds to changes in the voltage gradient in a manner predicted by the Ussing flux ratio equation that describes diffusion across a simple barrier. Structurally, there is also general agreement that Na+ movement is via a simple cation−anion mechanism that describes diffusion across a simple barrier. Structurally, there is also general agreement that Na+ movement is via a simple cation−anion mechanism that describes diffusion across a simple barrier.
Estimates of shunt conductance

One measure of shunt conductance is extrapolation of $I_c$ versus $G_i$ plots to zero $I_c$. Assuming that the complete removal of the conductance associated with the transcellular active transport (as $I_{ac}$) would leave the net conductance of other presumably passive pathways. Assuming also that in mounting epithelia in Ussing-style membrane apertures produces minimal ‘edge’ damage, the residual conductance would approximate the sum of all the remaining intercellular junctions. The fact that we measure a lower shunt conductance in 2SW acclimated fish, compared with SW-acclimated fish, suggests that most passive pathways in the epithelium have less-permeable junctions in 2SW. This result confirms previous findings in opercular epithelium of *O. mossambicus* (Kültz and Onken, 1993). Also, cultured pavement cells from SW-acclimated sea bass *Dicentrarchus labrax* have lower transepithelial conductance (Avella et al., 1999) than in similar pavement cell cultures from freshwater fish, such as rainbow trout (Wood et al., 1998), tilapia (Kelly and Wood, 2002) and goldfish (Chasiotis and Kelly, 2011). Finally, the gill and skin epithelia have different patterns of claudin 27a and claudin 27c mRNA abundance with salinity acclimation, suggesting differential junctional permeability between typical pavement cell junctions and the ionocyte-accessory-cell junctions (Bagherie-Lachidian et al., 2009). To reconcile the apparent paradox of observing more-leaky Na$^+$ junctions localized near ionocytes, yet lower overall shunt conductance for the whole system, we infer that while the Na$^+$-permeable pathways increase in their conductance and restructure themselves to be nearer the anion pathway, simultaneously, the other tight junctions across the general surface of the epithelium, namely between pavement cells, become much tighter, presumably to minimize ion permeation from hypersaline solutions into the animal. The prediction from vibrating probe experiments is that the ionocytes of opercular epithelium of SW-acclimated tilapia comprise all of the tissue conductance except for 0.5 mS cm$^{-2}$ and that most of that residual conductance may be an artifact of edge damage in Ussing chamber studies (Foskett and Macken, 1985) leaving a shunt (pavement cell junction) conductance near zero. More recently, the scanning ion selective electrode technique (SIET) has been applied to ionocytes in yolk sac membrane of seawater medaka embryos, and while ionocyte complexes (with accessory cells) had approximately equal Na$^+$ and Cl$^-$ currents, single ionocytes had a Cl$^-$ current but lacked a Na$^+$ current (Shen et al., 2011). Consistent with this interpretation, our results with the mummichog buccal epithelium, a structure similar to the OE but with fewer ionocyte and accessory cells (Karnaky, 1980), suggests that the Na$^+$ shunt conductance varies with the density of ionocyte complexes.

MATERIALS AND METHODS

**Animals**

Adult killifish (*Fundulus heteroclitus* Linnaeus 1766) of both sexes were trapped in Jintown estuary, Antigonish, NS, and transported in coolers containing estuarine water to the St Francis Xavier University Animal Care Facility. The fish were placed in full-strength seawater (32.0‰ salinity) in 450 l recirculating tanks at room temperature (20±1°C), adjusted to an ambient photoperiod under artificial lighting and were held for several weeks prior to experimentation. Approximately 40 fish were acclimated for at least 4 weeks to hypersaline conditions of 2SW (64% salinity), made hypersaline by addition of artificial sea salt (Instant Ocean, Blacksburg, VA, USA) to seawater. Fish were fed adjusted amounts of Nutrafin flakes (R.C. Hagen, Montreal, QC, Canada) twice daily to provide each fish with 1.0 g of food daily per 100 g of body weight. Fish were also fed meal-worms (*Tenebrio molitor*) 3 days a week. Fish were anesthetized in 1.0 g l$^{-1}$ tricaine methane sulfonate (buffered to neutral pH) and were killed by pithing. The tail region was dried and blood was collected from razor-severed caudal vessels in heparinized capillary tubes, centrifuged immediately at 2000 g for 2 min and plasma frozen in microcentrifuge tubes for ion composition and osmolality. Opercular epithelia were dissected and bathed in modified Cortland’s saline (composition in mmol l$^{-1}$: NaCl 159.9, KCl 2.55, CaCl$_2$ 1.56, MgSO$_4$ 0.93, NaH$_2$PO$_4$ 2.97, NaHCO$_3$ 17.85 and glucose 5.55, pH 7.7–7.8 when bubbled with 99%:1% O$_2$:CO$_2$ gas mixture, osmolality 317 mOsm kg$^{-1}$). For 2SW-acclimated fish, a high-NaCl Cortland’s saline was used: regular Cortland’s supplemented with 15.0 mmol l$^{-1}$ extra NaCl (osmolality 334 mOsm kg$^{-1}$). To inhibit NaCl secretion, a K$^+$-free version of Cortland’s saline was used: regular Cortland’s without KCl but with 2.55 mmol l$^{-1}$ NaCl extra (osmolality 316 mOsm kg$^{-1}$).

**Immunocytochemistry**

OE were dissected without the dermal chromatophore layer and pinned to modeler’s sheet wax. Preparations were rinsed three times in rinsing buffer comprising 0.1% bovine serum albumin (BSA) in 0.05% Tween 20 in phosphate-buffered saline (TPBS) (composition in mmol l$^{-1}$: NaCl 137, KCl 2.7, Na$_2$HPO$_4$ 4.3, and KH$_2$PO$_4$ 1.4 at pH 7.4). The membranes were fixed for 2 h at ~20°C in 80% methanol, 20% DMSO, then rinsed and immersed in a blocking solution with 5% normal goat serum (NGS), 0.1% BSA, 0.2% NaN$_3$ in TPBS, pH 7.4 for 30 min at room temperature in the dark. The membranes were then incubated in the primary anti-CFTR antibody (10 µg ml$^{-1}$ in blocking solution) at 4°C overnight. Membranes were then rinsed three times and exposed to the secondary antibody (8 µg ml$^{-1}$ in blocking solution) for 4 h at room temperature in the dark. After three final rinses, the membranes were mounted in mounting medium (Geltol; Immunon Thermo Shandon, Pittsburgh, PA, USA). Slides were viewed in single blind fashion and images were collected with a laser scanning confocal microscope (Olympus, Markham, ON, Canada; model FV300). In each OE, randomly selected Z-stack series were collected using a ×40 water objective (NA 1.15W), zoom of 3.0 and with optical sections of 0.50–0.02 µm. An average of 35 sections was collected for each image. Perspective 3D surface plots were obtained using the Fiji image processing program. Apical crypt measurements (depths, CFTR and node distributions) were calculated in single blind fashion; non-adjacent areas were selected at random and apical crypts visible within this area were scored, excluding those touching the top and right borders.

**Antibodies**

Primary antibody used for detection of killifish CFTR was mouse monoclonal anti-human CFTR (R&D Systems, Minneapolis, MN, USA) with the epitope at the C-terminus, a zone that is conserved in killifish to human (Singer et al., 2008) and therefore is selective for this protein (Marshall et al., 2002; Robertson and Hazel, 1999). The secondary antibody used for immunofluorescence microscopy was goat anti-mouse immunoglobulin (IgG) conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Negative controls were performed by omitting the primary antibody.

**Electron microscopy**

Dissected OE were pinned flat to a wax square and immersion fixed for 1 h in a solution of 2% formaldehyde, 2% glutaraldehyde in 0.1 mol l$^{-1}$ phosphate buffer, pH 7.4. The tissue was transferred to a glass vial, containing the same fixative, and stored overnight at 4°C. The fixative was rinsed out by two changes of phosphate buffer followed by one change of 0.1 mol l$^{-1}$ sodium cacodylate (10 min per change). This was followed by immersion in a solution of 1% osmium tetroxide and 0.0015% Ruthenium Red in distilled water for 1 h. Ruthenium Red (740 molecular mass) bears six positive charges and stains the cell surfaces but does not cross the plasma membrane to the cellular interior (Chambers, 1973). The tissue was then rinsed with distilled water (2×10 min per change) and kept at 4°C for 4 days, after which it was stained en bloc in saturated aqueous uranyl acetate for 1 hour then dehydrated through an ascending series of ethanol. The ethanol was replaced by two changes of propylene oxide (10 min each) and the tissue was then slowly infiltrated with epoxy resin (EMBed-812, EMS, 3260).
Hatfield, PA, USA), placed in flat rectangular wells, and cured at 60°C for 2 days.

Thick sections (1 µm) were cut, parallel to the apical face of the operculum, and stained with Toluidine Blue until tissue was encountered. The blocks were then thin sectioned (100 nm) on a Reichert microtome and the sections stained with uranyl acetate (1 h) and lead citrate (4 min) for viewing on a Philips 410 transmission electron microscope. Digital images were captured using a SIA L12C digital camera and adjusted for contrast and brightness using Adobe Photoshop.

Electrophysiology theory

The Nernst equation predicts for systems that are permeable to one ion:

\[ \Delta \psi = \frac{RT}{z_i F} \ln \left( \frac{[a_i C_i^o]}{[a_i C_{i}]} \right), \]

(1)

where the transmembrane voltage (\(\Delta \psi\)) is generated in asymmetrical conditions and \(C_i^o\) is the concentration on the outside and \(C_i\) is the concentration on the inside, \(R=8.3143\) Coul. \(\ V/\)mole, \(F=96,494\) Coul mol\(^{-1}\) equiv and \(z_i\) is the valence. Because salt solutions above 0.1 mol l\(^{-1}\) are non-ideal, we apply the empirically derived activity coefficient (\(a_i\)) to describe the degree of dissociation of the salt (NaCl principally) (Robinson and Stokes, 1959). Although no biological system is uniquely permeable to one ion species, this relationship for the Na\(^+\) gradient (\(E_{Na}\)) approximates the voltage against which marine fish must secrete Na\(^+\), assuming that Na\(^+\) moves via a simple barrier to diffusion.

Leak conductance (\(G_L\)) was calculated as an extrapolation of the epithelial conductance (\(G_i\)) versus \(I_{sc}\) adapted from a previous method (Költz and Onken, 1993), but instead of using the spontaneous variation of \(G_i\) and \(I_{sc}\), the epithelia in Ussing chambers were inhibited by application of hypotonic shock (60 mOsm kg\(^{-1}\) reduction of basolateral and apical bathing solutions), a treatment that rapidly inhibits the transcellular pathway of active ion secretion by dephosphorylation of NKCC and CFTR (Marshall et al., 2009, 2005).

Pharmaceuticals

To test for the presence of membrane rafts in the apical membrane of ionocytes, we added drugs to inhibit the operation of dynamin and caveolin, two major endo- and exocytosis effectors (Ares and Ortiz, 2012; Bajmoczi et al., 2009). Chlorpromazine, a dopamine 1 and dynamin 2 inhibitor (and Ca\(^{2+}\) calmodulin inhibitor) (Ares and Ortiz, 2012), was added stepwise from 1.0 to 50 µmol l\(^{-1}\) to the mucosal side of paired OE in symmetrical Cortland’s saline, one serving as a time control and the test side receiving the drug. Changes in \(I_{sc}\), \(V_O\) and \(R_L\) were measured for at least 30 min before the drug was rinsed off the OE. Similarly, filipin, a disruptor of caveolin-1 operation and a cholesterol sequestration drug, was added at 1.53 µmol l\(^{-1}\) to the apical side of the OE, incubated for up to 1 hour, when the OE were challenged with hypotonic shock (80% diluted Cortland’s saline, both sides) and the responses compared with paired time control OE.

Statistical analyses

Data are expressed as means±s.e.m. Significant difference for apical crypt depths was determined by a two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison \(a posteriori\) tests. Fisher’s exact test was performed to analyze nominal data of CFTR distribution in the apical crypts (condensed versus diffused). Node distribution differences and plasma osmolality and ions were compared using unpaired two-tailed \(t\)-tests. Significant difference was ascribed if \(P<0.05\).

Acknowledgements

Many thanks to SFX animal care facility for expert animal care and to the helpful suggestions of the reviewers.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

This work was supported by a Natural Sciences and Engineering Research Council Discovery grant [RGPIN3698-2009 to W.S.M.], a Chaisson Scholarship to L.N.C., an Irving Mentorship to K.L.G. and a University Council Research Grant 2013-47 to W.S.M.

References


