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REVIEW

Freshwater Fish Gill Ion Transport: August Krogh to morpholinos and microprobes

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Abstract

August Krogh proposed that freshwater fishes (and other freshwater animals) maintain body NaCl homoeostasis by extracting these ions from the environment via separate Na^+/NH_4^+ and Cl^-/HCO_3^- exchangers in the gill epithelium. Subsequent data from other laboratories suggested that Na^+ uptake was more probably coupled to H^+ secretion via a vesicular proton pump (V-ATPase) electrically coupled to a Na^+ channel. However, despite uncertainty about electrochemical gradients, evidence has accrued that epithelial Na^+/H^+ exchange indeed may be an alternative pathway for Na^+ uptake. The specific pathways for Na^+ uptake may be species and environment specific. An apical Cl^-/HCO_3^- exchanger is generally accepted for most species (some species do not extract Cl^- from freshwater), but the relative roles of anion exchanger-like (SLC4A1) vs. pendrin-like (SLC26Z4) exchangers are unknown, and also may be species specific. Most recently, data have supported the presence of an apical $Na^+ + Cl^-$ cotransporter (NCC-type), despite thermodynamic uncertainty. Ammonia extrusion may be via NH_3 diffusing through the paracellular junctions or NH_4^+ substitution on both basolateral and apical ionic exchangers ($Na^+ + K^+$-ATPase; $Na^+ + K^+ + Cl^-$ - cotransporter; and Na^+/H^+ exchanger), but recent evidence suggests that Rhesus-glycoproteins mediate both basolateral and apical movement of ammonia.
Keywords fish, gill, Krogh, NaCl uptake, transport.

Among his many scientific accomplishments, August Krogh performed a series of experiments in the late 1930s, which provided the foundation for the study of the mechanisms of ionic transport across the skin and gills of freshwater animals (including fishes). These animals live in a medium that is much more dilute than their body fluids (Na^+ and Cl^- ratios are nearly 200 : 1, plasma vs. freshwater). Because of earlier studies in other laboratories (e.g. Smith 1929), Krogh knew that the osmotic water uptake and diffusional ion loss, and the excretion of ammonia, probably took place across the gill epithelium of freshwater fishes. He, therefore, designed a series of experiments on a variety of species to investigate the mechanisms of the ion uptake that should be present to offset the diffusional loss into the surrounding medium.

Krogh's demonstration of uncoupled Na^+ vs. Cl^- uptake

Using various freshwater fish species, Krogh first showed that they could reduce the Cl^- content of very dilute solutions. Divided chamber experiments with the goldfish (*Carassius auratus*) demonstrated that this net uptake of Cl^- was from the front end of the fish, presumably the gills (Krogh 1937). He then measured the net, chemical uptake of Na^+ or Cl^- from solutions containing different anions (NaCl, NaBr, $NaHCO_3$ or $NaNO_3$) or cations (i.e. NaCl, KCl, NH_4Cl and $CaCl_2$) and found that the accompanying ion had no effect on either Na^+ or Cl^- uptake. Krogh concluded that the uptake of Na^+ and Cl^- was independent, and he proposed that they were in exchange for, respectively,

NH_4^+ and HCO_3^- (Krogh 1937, 1938, reviewed in Krogh 1939).

Early support for independent Na^+/NH_4^+ and Cl^-/HCO_3^- exchanges

Nearly 30 years passed before the advent of the use of radioisotopes allowed a more careful examination of the coupling of Na^+ influx with ammonia efflux and Cl^- influx with bicarbonate influx. Maetz and Garcia Romeu confirmed that the uptake of Na^+ vs. Cl^- was independent of each other (Garcia-Romeu & Maetz 1964) and found that the addition of $(NH_4)_2SO_4$ to the external medium inhibited both chemical and isotopic uptake of Na^+ by the goldfish, but injection stimulated Na^+ uptake. Addition of $KHCO_3$ to the medium inhibited both chemical and isotopic uptake of Cl^-, and injection of this salt stimulated Cl^- uptake (Maetz & Garcia-Romeu 1964). Thus, the idea of Na^+/NH_4^+ and Cl^-/HCO_3^- exchangers mediating the separate uptake of Na^+ vs. Cl^- across the freshwater fish gill epithelium seemed well established (Fig. 1).

Evidence that Na^+ uptake is associated with H^+ efflux

A more complete study of the relationship between Na^+ influx and ammonia efflux in the goldfish, however, demonstrated that the best correlation was between the Na^+ uptake and the sum of ammonia and acid efflux (Maetz 1973), and a subsequent study, using the rainbow trout (*Oncorhynchus mykiss*), found that Na^+ influx was correlated with proton excretion, not ammonia excretion (Avella & Bornancin 1989). The authors proposed that this correlation

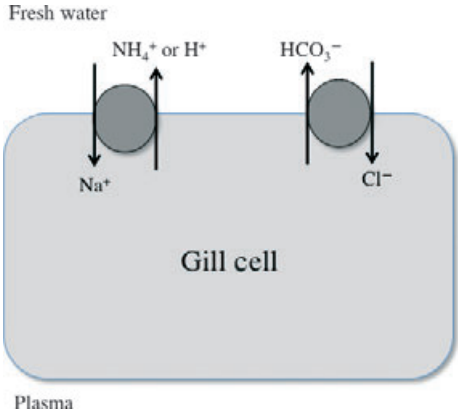

Figure 1 Working model for ionic exchange mechanisms mediating the independent uptake of Na^+ and Cl^- by the freshwater fish gill epithelium, approx. 1975. Apical uptake of Na^+ is via exchange for intracellular NH_4^+ or H^+, and apical uptake of Cl^- is via exchange for intracellular HCO_3^-. See text for supporting evidence. Redrawn from Evans (1975).

either could be the result of an apical Na^+/H^+ exchanger or an apical proton pump associated with an apical Na^+ channel. A Na^+/H^+ exchanger had been suggested by earlier studies of fish gill transport (e.g. Kerstetter *et al.* 1970, Cameron & Heisler 1983, Perry *et al.* 1985), as well as studies of Na^+ uptake by low-resistance (leaky) epithelial tissues, including rat small intestine (Mürer *et al.* 1976), rabbit gallbladder (Cremashi *et al.* 1979) and rabbit renal proximal tubule (Kinsella & Aronson 1980). However, the authors favoured the alternative model, an apical, electrogenic proton pump associated with the passive entry of Na^+ via a channel. This type of coupling had been described for high resistance (tight) epithelia that (like the freshwater fish gill) are able to extract Na^+ from very dilute solutions [e.g. toad (*Bufo marinus*) urinary bladder; Ludens & Fanestil 1972] and frog (*Rana esculenta*) skin (Ehrenfeld *et al.* 1985). In fact, earlier studies had demonstrated that the freshwater fish gill showed the morphological characteristics of 'tight' epithelia (Sardet 1980, Pisam *et al.* 1987). Moreover, thermodynamic considerations suggest that freshwater Na^+ concentrations are too low to drive an apical, passive Na^+/H^+ exchange (reviewed in Potts 1994, Kirschner 1995, 2004, Parks *et al.* 2008).

More recently, evidence has accumulated suggesting that, depending upon species and salinity, Na^+ uptake can be via either Na^+/H^+ exchange, a Na^+ channel associated with an apical, electrogenic H^+-pump, or $Na^+ + Cl^-$ cotransport in the fish gill epithelium (for reviews, see Hirose *et al.* 2003, Perry *et al.* 2003b, Evans *et al.* 2005, Marshall & Grosell 2006, Hwang & Lee 2007, Evans & Claiborne 2009). It should be noted that the complex morphology of the transporting cells (see below) and the difficulty in producing viable cell cultures, have precluded some of the single-cell techniques (measurement of trans-membrane potentials or patch clamping, for instance) that have allowed a more biophysical approach to revealing apical vs. basolateral transport steps in other epithelial cells, but molecular techniques have provided major advances in the past decade.

Physiological evidence for an apical H^+ pump associated with an apical Na^+ channel

The finding that bafilomycin (a specific inhibitor of vacuolar H^+-ATPase) (Yoshimori *et al.* 1991) inhibited Na^+ uptake in tilapia (*Orechromis mossambicus*), carp (*Cyprinus carpio*) (Fenwick *et al.* 1999) and zebrafish (*Danio rerio*) (Boisen *et al.* 2003) suggests that a proton pump does play a significant role in Na^+ uptake. In fact, this bafilomycin inhibition has been localized to one type (see below) of MRC isolated from the rainbow trout gill (Reid *et al.* 2003). Using a proton-sensitive ion probe, acid-secreting cells have

now been identified in the skin of the zebrafish embryo, and this acid efflux is inhibited by bafilomycin (Lin *et al.* 2006). Physiological data supporting the proposition of an apical Na^+ channel are relatively sparse, with three studies finding phenamil-sensitive, Na^+ uptake by the goldfish (Preest *et al.* 2005) and rainbow trout (Bury & Wood 1999, Grosell & Wood 2002), and two studies have demonstrated a phenamil-sensitive Na^+ uptake into the same isolated cell subtype that showed a bafilomycin-sensitive Na^+ uptake (Reid *et al.* 2003, Parks *et al.* 2007). Phenamil is considered a specific Na^+ channel blocker (Barbry *et al.* 1986, Garty & Palmer 1997).

Differentiation of gill epithelial transport cells

Further discussion of cellular and molecular data relevant to this review of gill transport pathways requires a better understanding of the differentiation of gill epithelial cells. The fish gill is divided into horizontal filaments on each gill arch, which are subdivided into numerous lamellae projecting vertically above and below each filament (see Evans *et al.* 2005). Putative, ion-transporting cells were first identified by Keys (Keys & Willmer 1932) on gill lamellae and filaments and termed 'chloride-secreting cells', because he studied Cl^- secretion by the isolated, marine eel (*Anguilla anguilla*), heart-gill preparation (Keys 1931). Since then, a variety of studies have determined that these cells are found in both marine and freshwater fish species, characterized by distinct basolateral infoldings, significant activities of the transport enzyme, Na^+, K^+-ATPase (NKA), and numerous mitochondria; hence, the current name: mitochondrion-rich cell (MRC). Generally, MRC are most common on the interlamellar surfaces of the filaments, but they may appear on lamellae in some freshwater fish species, and their specific position and morphology may change with salinity changes (Evans *et al.* 2005). Using immunohistochemical techniques, MRC are generally identified by intense staining with a monoclonal antibody raised against the alpha subunit of mammalian NKA (e.g. Hyndman & Evans 2009, Tang *et al.* 2010). MRC (and their associated 'accessory cells') usually account for <10% of gill epithelial cells; most adjacent cells (termed pavement cells; PVC) are much less complex and compose the majority (>90%) of the cellular architecture of the gill epithelium, especially on the lamellae. PVC generally express few mitochrondria and relatively low activities of NKA (Evans *et al.* 2005). Over 20 years ago, two morphological subtypes of MRC were identified in freshwater guppies (*Poecilia reticulata*) (Pisam *et al.* 1987), and, in the past few years, functional subdivisions of MRC have been identified and characterized (e.g. Galvez *et al.* 2002, Hiroi *et al.* 1999, reviewed in Hwang & Lee 2007).

Molecular evidence for an apical, vesiclular proton ATPase and Na^+ channel

Using heterologous antibodies, vesicular proton ATPase (V-ATPase) has been localized to the apical surface of MRC (and/or adjacent PVC) in the gill epithelium of the rainbow trout (Lin *et al.* 1994, Sullivan *et al.* 1995), tilapia (Wilson *et al.* 2000a), and mudskipper (*Periophthalmodon schlosseri*) (Wilson *et al.* 2000b), and antibodies raised against killifish (*Fundulus heteroclitus*) V-ATPase localize to acid-secreting cells in the skin of larval zebrafish (Lin *et al.* 2006), and homologous antibodies localized V-ATPase to the apical surface of NKA-poor cells in the gill of the zebrafish (Yan *et al.* 2007). A single study that localized a putative Na^+ channel (heterologous antibodies to ENaC) to both MRC and PVC in the tilapia gill (Wilson *et al.* 2000a) has not been corroborated. Messenger RNA transcripts for V-ATPase were also localized (by *in situ* hybridization) to what appear to be PVC in the rainbow trout (Sullivan *et al.* 1996), and to cells in the zebrafish gill that are NKA-poor (Yan *et al.* 2007). Two subtypes of MRC have now been isolated from rainbow trout gills, using a magnetic cell separation technique and binding to peanut lectin agglutinin (Galvez *et al.* 2002). The subpopulation that does not bind to peanut lectin agglutinin (PNA^-) appears to share some morphological characteristics with PVC, but has relatively high levels of mitochonrdria, as determined by fluorescence microscopy and staining with Mitotracker Green and the expression of the mitochondrial protein Sco1. The authors therefore termed these cells PNA^- MRC, and these cells express relatively high levels of both NKA and V-ATPase protein, determined by Western blots using heterologous antibodies (Galvez *et al.* 2002). Moreover, these PNA^- MR cells also displayed a bafilomycin-sensitive, and phenamil-sensitive Na^+ uptake (Reid *et al.* 2003), prompting the authors to name these cells α-MR cells, to note similarities with the acid-secreting (A or α), intercalated cells in the mammalian, inner medullary collecting duct, which also are PNA^- (LeHir *et al.* 1982) and express apical V-ATPase (Brown *et al.* 1988).

Some of the strongest evidence for a role of V-ATPase in Na^+ uptake comes from recent studies using the larval zebrafish (reviewed in Hwang & Lee 2007, Hwang 2009). Using *in situ* hybridization, immunohistochemical and ion-sensitive probe techniques, this group has localized acid-secreting, V-ATPase-expressing cells in the skin of larval zebrafish, that are sensitive to bafilomycin (Lin *et al.* 2006). Moreover, knockdown of V-ATPase mRNA by injecting morpholinos (modified antisense oligonucleotides; e.g. Corey & Abraqms 2001) impaired acid secretion and reduced the Na^+ content of the embryos (Horng *et al.* 2007).

Finally, it is important to note that, although partial cDNA sequences for fish V-ATPase have been published (Perry *et al.* 2000, Katoh *et al.* 2003, Lin *et al.* 2006), no sequences for the putative, phenamil-sensitive, Na^+ channel (ENaC) have been published, and none of the published fish genomes has nucleotide sequences homologous to ENaC, despite the presence of ENaC homologues in other invertebrate and vertebrate groups (Alvarez de la Rosa *et al.* 2000). So, it is unclear what molecular entity mediates the Na^+ uptake that is phenamil sensitive in fishes.

Molecular evidence for apical Na^+/H^+ exchange

Despite thermodynamic considerations that argue against the function of a Na^+/H^+ exchanger in Na^+ uptake (for a recent review, see Parks *et al.* 2008), there is considerable molecular evidence for the presence of such an exchanger (NHE family; Slepkov *et al.* 2007) on the apical surface of the fish gill epithelium. The first fish NHE (NHE3) was cloned from a freshwater (acid-tolerant) Osorezan dace (*Tribolodon hakonensis*) and (using homologous antibodies) was localized to the apical surface of a cell that expressed NKA on the basolateral surface. Both transport proteins were upregulated (analysed by Northern blots) when the dace was acclimated to a low pH (3.5) medium. The authors proposed that the activity of the basolateral NKA provided an electrochemical gradient sufficient to drive the apical NHE (Hirata *et al.* 2003), making these cells analogous to those providing Na^+ uptake in the proximal tubule in the mammalian kidney (e.g. Vallon *et al.* 2000). Previously, heterologous antibodies had localized NHE3 in what appeared to be MRC in the rainbow trout gill (Edwards *et al.* 1999) and in NKA-rich cells in tilapia (Wilson *et al.* 2000a). In addition, transfer of killifish from 10% seawater to freshwater was accompanied by a significant (1.7-fold) increase in the expression of mRNA for NHE2 in killifish gill tissue (Scott *et al.* 2005). Interestingly, in this study, the expression of V-ATPase mRNA in the gill tissue was unchanged by transfer from 10% seawater to freshwater. Moreover, hypercapnic acidosis in the killifish stimulated the expression of NHE3 protein in gill tissue (via Western blot; heterologous antibodies; Edwards *et al.* 2005). NHE3 has been cloned from the euryhaline, Atlantic stingray (*Dasyatis sabina*), and it was found that the mRNA expression increased upon acclimation to freshwater and the protein (using a homologous antibody) was localized to the apical surface of NKA-rich cells (Choe *et al.* 2005). The apical localization of V-ATPase in NKA-poor cells in the zebrafish gill has already been noted, but, using antibodies against another fish (dace) exchanger, NHE3 can be localized to the apical surface of the same cell (Yan *et al.* 2007). The fact that low pH (4.0) up-regulated V-ATPase mRNA transcripts (measured with qRT-PCR), while down-regulating NHE3 transcripts, and low Na^+ medium had the opposite effect on both transcripts, suggests that these two transporters can be differentially regulated under specific environmental stresses (Yan *et al.* 2007).

Molecular evidence for basolateral extrusion of Na^+ via coupled $Na^+ + HCO^-$ cotransport

There is emerging evidence that the basolateral transport of Na^+ (cytoplasm to plasma) is mediated by the cotransport of Na^+ plus HCO_3^- via proteins of the NBC (SLC4A) family, which are electrogenic (approx. 2 : 1; HCO_3^- : Na^+; Romero & Boron 1999). An NBC1 homolog has been cloned from the dace and homologous antibodies localized it to the basolateral membrane of NKA-rich cells (Hirata *et al.* 2003). Rainbow trout NBC1 also has been cloned, and transcripts were localized to gill tissue, and found to increase during respiratory acidosis (Perry *et al.* 2003a). Using heterologous antibodies, NBC has been localized to the basolateral membrane in rainbow trout, lamellar gill cells (NKA not localized; Parks *et al.* 2007). An NBCe1 (SLC4A4) has been cloned from the zebrafish, mRNA transcripts localized to the gill of embryos, and protein localized by heterologous antibodies to a subpopulation of gill cells, but not compared with NKA localization. The expression of the zebrafish NBCe1 was reduced by specific morpholino injection during development, and this was associated with oedema in the embryo, indicating 'altered fluid and electrolyte balance' (Sussman *et al.* 2009). The localization of mRNA transcripts of NBC1 to specific gill and skin cells has been confirmed, but these cells have not been further characterized (Hwang 2009).

Apical Cl^- uptake

Krogh had proposed that Cl^- uptake was coupled with HCO_3^- extrusion to facilitate gas exchange, but it is now accepted that the major pathway for CO_2 extrusion by the fish gill epithelium is via the gas transfer, not as an ion (Perry 1986, Gilmour & Perry 2009b). Nevertheless, it is clear that gill cell carbonic anhydrase produces intracellular HCO_3^- that is important in both fish acid-base regulation and Cl^- uptake (Gilmour & Perry 2009a). In some species (e.g. killifish, eel, and bluegill (*Lepomis macrochirus*), no Cl^- uptake is observed (e.g. Tomasso & Grosell 2005), but other species (goldfish, rainbow trout, zebrafish, etc.) do extract Cl^- from the environmental across the gill epithelium. In recent years, molecular approaches have

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provided evidence for two pathways for Cl^- uptake: Cl^-/HCO_3^- exchange and coupled $Na^+ + Cl^-$ uptake. The coupled transport is somewhat surprising since both Krogh (1937) and Maetz (Garcia-Romeu & Maetz 1964) provided convincing evidence for unlinked Na^+ vs. Cl^- uptake, and the external Na^+ and Cl^- concentrations in many freshwaters appear to be too low to drive either Cl^-/HCO_3^- exchange or a passive co-transporter. Either uptake mechanism could, however, function in freshwaters with higher salt concentrations or brackish waters.

Evidence for an apical Cl^-/HCO_3^- exchange

Infusion of HCO_3^- was found to stimulate Cl^- uptake by the rainbow trout gill (Kerstetter & Kirschner 1972), and Cl^- uptake was correlated well with base secretion in the goldfish (De Renzis & Maetz 1973). Moreover, kinetic analysis suggested a 1 : 1 Cl^-/HCO_3^- exchange mechanism in the rainbow trout gill (Wood & Goss 1990), and the anion exchange inhibitor SITS (4-acetoamido-4′-isothiocyanato-stilbene-2′, 2′-disulphonic acid) in the external bath inhibited Cl^- uptake 77% in the rainbow trout (Perry & Randall 1981), and converted Cl^- uptake to Cl^- loss in the goldfish and inhibited Cl^- uptake by 60% in the neon tetra (*Paracheirodon innesi*) (Preest *et al.* 2005).

Transcripts of the mRNA of a Cl^-/HCO_3^- exchanger (AE1, Band 3, SLC4A1) have been localized to both lamellar and filamental cells in the rainbow trout gill by *in situ* hybridization (Sullivan *et al.* 1996), and trout anti-AE antibodies have localized the protein to the apical surface of MRC in tilapia gill, but not trout gill (Wilson *et al.* 2000a). Using a polyclonal antibody raised against tilapia AE1, it has been found that expression of the protein (measured via immunoblot) in gill tissue increases 23-fold in freshwater vs. seawater-acclimated, euryhaline pufferfish (*Tetraodon nigroviridis*) (Tang & Lee 2007). Another class of Cl^-/HCO_3^- exchangers (pendrin, SLC26A4) has been localized (using heterologous antibodies) to the apical surface of cells that also express basolateral V-ATPase protein (but not NKA) in the euryhaline stingray in freshwater (Piermarini *et al.* 2002). Interestingly, the immunoreactivity of both proteins was greatest in the freshwater acclimated stingrays. More recently, mRNA transcripts for another Cl^-/HCO_3^- exchanger, SLC26A3, have been localized to filamental cells, but immunohistochemistry (using a homologous antibody) localized the protein to cells that often did not express high levels of NKA; nevertheless, the authors proposed that they were MRC (Perry *et al.* 2009). The expression of mRNA transcripts for SLC26A3 increased significantly after transfer to low Cl^- freshwater (0.02 mM), as it did after transfer to media containing elevated HCO_3^- concentrations (10–20 mM), which also elevated Cl^- uptake (Perry *et al.* 2009). The same group has now localized transcripts for SLC26A (A3, A4 and A6c) to gill primordia in larval zebrafish, and the protein for SLC26A3 was localized (homologous antibody) to a subpopulation of cells that often also expressed NKA (Bayaa *et al.* 2009). Raising embryos in low Cl^- medium (0.02 mM), resulted in an increased uptake of Cl^- and expression of SLC26A3 transcripts. Selective knockdown of transcripts for either A3 or A4 with morpholinos resulted in reduced Cl^- uptake rates, as well as HCO_3^- excretion. In addition, morpholino knockdown also inhibited the normal stimulation of Cl^- uptake by high HCO_3^- media (Bayaa *et al.* 2009). The authors also found that SLC26A6 may be involved, because low Cl^- or high HCO_3^- was associated with increased expression of transcripts for SLC26A6 and morpholino knockdown resulted in significant reduction in HCO_3^- excretion and nearly significant reduction in Cl^- uptake (Bayaa *et al.* 2009). In mammals, SLC26A6 is normally a proton-coupled amino acid transporter, but it may also function as a Cl^-/HCO_3^- exchanger that may be electrogenic (Ohana *et al.* 2009), and recent studies have found that at least intestinal Cl^-/HCO_3^- exchange in fishes is electrogenic (Kurita *et al.* 2008, Grosell *et al.* 2009). The electrochemical gradients driving either a neutral or electrogenic Cl^-/HCO_3^- exchange across the apical surface of gill cells remain to be determined, but V-ATPase has been localized to the basolateral membrane of MRC in the killifish (Katoh *et al.* 2003) and trout (Tresguerres *et al.* 2006), and to cells that expressed pendrin (but not NKA) in gill epithelium of the Atlantic stingray in freshwater (Piermarini & Evans 2001). If this is the case, this cell would be analogous to the base-secreting, B-type (β) intercalated cell in the mammalian cortical collecting duct, where basolateral proton extrusion provides intracellular HCO_3^- to drive apical Cl^-/HCO_3^- exchange. Moreover, apical extrusion of H^+ by V-ATPase could produce the electrochemical gradient to drive Cl^-/HCO_3^- exchange. Indeed, the V-ATPase inhibitor bafilomycin inhibited Cl^- uptake by tilapia (Fenwick *et al.* 1999) and zebrafish (Boisen *et al.* 2003).

Evidence for apical $Na^+ + Cl^-$ cotransport

Somewhat surprisingly, data have been accumulating recently that suggest Na^+ and Cl^- uptake may be mediated by a cotransporter that carries both ions. Both Na^+ and Cl^- uptake by the goldfish were inhibited by furosemide (75 and 90% respectively), which inhibits the $Na^+ + K^+ + 2\ Cl^-$ cotransporter (NKCC; e.g. Gimenez 2006). Interestingly, Cl^--free medium inhibited Na^+ uptake and Na^+-free medium inhibited Cl^- uptake in goldfish also (Preest *et al.* 2005). Using a heterologous antibody directed against the mammalian NKCC or

NCC ($Na^+ + Cl^-$) cotransporter, a subtype of MRC has been identified in the gill epithelium of tilapia that expresses apical NKCC/NCC and basolateral NKA (Hiroi *et al.* 2005). Four putative transporters (NKCC1a, NKCC1b, NKCC2 and NCC) have now been cloned from tilapia gill tissue and only the mRNA for NCC was expressed (qRT-PCR) in the gills of freshwater acclimated tilapia, as were transcripts for NHE3. And expression of both NCC and NHE3 increased when seawater acclimated tilapia were transferred to freshwater (Hiroi *et al.* 2008). Using homologous antibodies to NCC and NHE3, the authors confirmed that NCC was expressed apically in NKA-expressing MRC (termed type-II MRC), which were distinguishable from apical NHE3-expressing MRC, which also expressed basolateral NKCC and NKA (termed type-III MRC; Hiroi *et al.* 2008). Further morphological studies have determined that NCC is expressed in MRC with convex surfaces, while NHE3 is expressed in MRC with concave surfaces. Acclimation to low Cl^- freshwater was associated with increased expression of NCC and increased gill surface morphology of convex-surface MRC, while acclimation to low Na^+-freshwater was associated with increased NHE3 expression and surface area of concave-surface MRC (Inokuchi *et al.* 2009). Thus, it appears that the two subtypes of MRC that mediate Na^+ and Cl^- uptake may be differentially regulated, at least in tilapia. An NCC cotransporter (specifically SLC12A10.2) has been cloned from zebrafish, and shows high expression (via qRT-PCR) in gill tissue (Wang *et al.* 2009). The mRNA transcripts of this NCC are expressed in MRC separate from those that express protein for either V-ATPase or NKA, and this expression was up-regulated in embryos in low Cl^- medium, but down-regulated in low Na^+ medium. Incubation of embryos in medium containing a specific NCC inhibitor (metolazone; thiazide-like diuretic) reduced both Na^+ and Cl^- uptake. Specific knockdown of mRNA for SLC12A10.2 decreased both Cl^- influx and Cl^- content of injected embryos, but stimulated Na^+ influx and Na^+ content of embryos, as well as expression of mRNA for NHE3 (Wang *et al.* 2009), once again demonstrating at least partially independent regulation of Cl^- vs. Na^+ uptake mechanisms, and the importance of NCC in Cl^- vs. Na^+ uptake. This is confirmed by the recent finding that an inward Cl^- current could be measured over the convex MRC that express NCC immunoreactivity in the skin of larval tilapia, and the fact that current could be inhibited 90% by metolazone (Horng *et al.* 2009).

Evidence for basolateral Cl^- extrusion

Pathways for the basolateral extrusion of Cl^- across the gill epithelium are only recently being investigated. One putative Cl^- channel, CLC-3 (Chen 2005, Jentsch *et al.* 2005) has been cloned from tilapia and mRNA is expressed (via RNAase protection assay) in gill tissue (Miyazaki *et al.* 1999). A heterologous antibody localized (via immunoblots) CLC-3 to gill tissue in the euryhaline puffer and demonstrated increased expression in freshwater- vs. seawater-acclimated individuals (Tang & Lee 2007). More recently, CLC-3 has been cloned from the euryhaline pufferfish, localized to gill tissue (qRT-PCR), and immunolocalized (heterologous antibody) to the basolateral membrane of NKA-rich MRC (Tang *et al.* 2010). Although mRNA for CLC-3 (qRT-PCR) did not increase in freshwater vs. seawater, the protein level (immunoblot) did increase (2.7-fold), as it did in low Cl^- media (4.3-fold; 0.01 vs. 1.0 mM Cl^-; Tang *et al.* 2010).

Excretion of ammonia

Krogh proposed that Na^+ uptake was coupled to ammonia efflux, because he knew that fish ammonia excretion takes place across the gill epithelium (Smith 1929), and he found a rough correlation between Na^+ influx and ammonia efflux (Krogh 1939). A dominant role for apical Na^+/NH_4^+ exchange in ammonia excretion has been questioned by the extensive data that demonstrated that Na^+ influx was either via Na^+/H^+ exchange or apical Na^+ channels, driven by the electrogenic extrusion of H^+ via V-ATPase (see above). A substantial number of studies in the past 40 years have determined that ammonia excretion can be via a variety of pathways, including: diffusion of either NH_3 or even NH_4^+ across and between (leaky tight junctions) the gill epithelial cells, via NH_4^+ substituting for H^+on apical NHE or for K^+ on basolateral NKA or NKCC. These pathways are especially prevalent in seawater acclimated fishes (e.g. Evans & Cameron 1986, Wood 1993, Wilkie 2002). In the past 5 years, however, ammonia transport has been somewhat clarified (reviewed in Weihrauch *et al.* 2009, Wright & Wood 2009) by the discovery that Rh blood group glycoproteins (homologous to Amt proteins in plants and fungi) play a role in ammonia transport in cells from a variety of animal groups (Peng & Huang 2006).

Various Rh genes (Rh30, Rhag, Rhbg, Rhcg1, Rhcg2, RhP1 and RhP2) were identified in the gene database for pufferfish (*Takifugu rubripes*), trout, salmon (*Salmo salar),* zebrafish, and medaka (*Oryzias latipes*) (Huang & Peng 2005, Nakada *et al.* 2007b). Messenger RNA transcripts for Rhag, Rhbg, Rhcg1 and Rhcg2 were identified (via Northern blot) in gill tissue from the pufferfish, and all four could be localized (homologous antibody) to both MRC and PVC in the gill epithelium. While Rhcg1 was expressed on the apical membranes of MRC, Rhcg2 was

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expressed on the apical membranes of PVC, and Rhbg was expressed on the basolateral membranes of PVC (Nakada *et al.* 2007b). The same antibody has been used to localize Rhbg to PVC in the gill of zebrafish (Braun *et al.* 2009). Localization of Rhcg1 to the apical surface of V-ATPase-expressing MRC has been confirmed for the zebrafish (Nakada *et al.* 2007a). All four transcripts of the pufferfish genes stimulated methylammonium (ammonia analogue) transport when expressed in *Xenopus* oocytes (Nakada *et al.* 2007b), and this has been confirmed for the expressed rainbow trout proteins (Nawata *et al.* 2010). Full-length cDNAs of the genes for Rhbg, Rhcg1, and Rhcg2 have been cloned from the mangrove killifish (*Kryptolebias marmoratus*), and transcripts for all three were expressed (qRT-PCR) in gill tissue; transcripts for Rhbg were expressed in skin tissue. Exposure of individuals to high ammonia (1–2 mM) in the external medium increased body ammonia by 37% and upregulated gill Rhcg2 and skin Rhcg1 by 5.8- and 7.7-fold, respectively, suggesting that Rh proteins are important in both gill and skin excretion of ammonia (Hung *et al.* 2007). Seven, full-length cDNAs for Rh protein genes have been cloned in the rainbow trout and Rhbg, Rhcg1, Rhcg2 transcripts were expressed (qRT-PCR) in gill extracts, with Rhbg and Rhcg1 also expressed in skin extracts (Nawata *et al.* 2007). Exposure of the fish to high external ammonia (1.5 mM) was associated with significant upregulation (qRT-PCR) of Rhcg2 in the gill and Rhbg and Rhcg2 in the skin. Density-gradient fractionization of gill MRC vs. PVC after high-ammonia exposure showed that only PVC showed increased of expression of mRNA for Rhbg and Rhcg2; there was no change in expression of any Rh mRNA in the MRC. Also, PVC levels of V-ATPase mRNA, but not NHE2, increased during high-ammonia exposure, suggesting that gill boundary layer acidification plays a significant role in the excretion of ammonia by Rh glycoproteins (Nawata *et al.* 2007). The importance of boundary acidification was confirmed by the subsequent finding that both bafilomycin and phenamil, when added to the apical surface of cultured rainbow trout gill epithelial cells, inhibited ammonia efflux, as did HMA [5-(*N*,*N*-hexamethylene)amiloride], which is thought to inhibit NHE (Tsui *et al.* 2009). The mRNA expression of Rhbg and Rhcg2 was upregulated in these cells after exposure to medium containing 670–750 mmol L^{-1} ammonia, and expression of Rhcg2 and ammonia efflux was increased when the cells were exposed to low Na^+ freshwater (0–84 μmol L^{-1}; Tsui *et al.* 2009). The suggestion that apical Na^+ uptake is functionally linked to ammonia efflux was supported by the recent finding that the Na^+ uptake and ammonia loss that could be detected by ion selective microelectrodes over MRC in the skin of larval medakas were both reduced by the NHE blocker EIPA [5-(*N*-ethyl-*N*-isopropyl)-amiloride], but both stimulated by acclimation to low Na^+ (1 μmol L^{-1}) or high ammonia (5 mmol L^{-1}) media (Wu *et al.* 2010). Interestingly, bafilomycin had no effect on H^+ secretion in this system. *In situ* hybridization and qRT-PCR demonstrated that Rhcg1 and Rhbg expression were colocalized with mRNA expression of an NHE (SlC9a3) in MRC, and both increased upon low Na^+ acclimation (Wu *et al.* 2010).

Thus, these very recent studies, from a variety of laboratories, have demonstrated the critical importance of Rh glycoproteins in ammonia excretion by fish gills and skin, and suggest that apical proton transport by either V-ATPase or NHE probably acidifies the outside boundary layer, thereby facilitating the movement of NH_3 through an apical Rh glycoprotein (Wright & Wood 2009, Wu *et al.* 2010). The actual molecular species that traverses the Rh glycoproteins is still in dispute (Weiner & Hamm 2007; Wright & Wood 2009), but the apparent critical role of proton secretion suggests that, at least in the fish gill and skin, gaseous NH_3 is the dominant pathway (Fig. 2). Finally, it is important to note that this functional 'Na^+/NH_4^+ exchange complex' (Wright & Wood 2009) or 'NH_4-dependent Na^+ uptake' (Wu *et al.* 2010) confirms

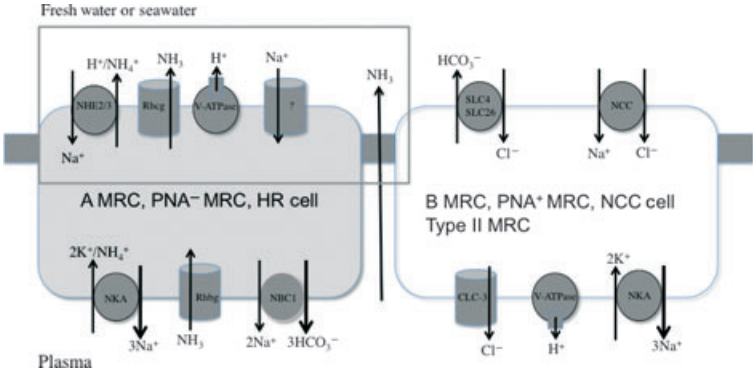

Figure 2 Current working model for ionic exchangers, channels and pumps mediating the uptake of Na^+ and Cl^- by the fish gill epithelium in freshwater or seawater (see Evans 2010 for evidence for these mechanisms in seawater fishes). Two mitochondrion-rich cells are diagrammed, but the actual distribution and nomenclature of the specific cells are debated and may be species specific. See text for details.

August Krogh's suggestion that Na^+ uptake in freshwater fishes is linked to ammonia efflux (Krogh 1939)!

Conflict of interest

There are no conflicts of interest in this study.

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