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⁺/NH₄⁺ and Cl⁻/HCO₃⁻ 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⁻/HCO₃⁻ 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 NH₃ diffusing through the paracellular junctions or NH₄⁺ 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, NaHCO₃ or NaNO₃) or cations (i.e. NaCl, KCl, NH₄Cl and CaCl₂) 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,
NH₄⁺ and HCO₃⁻ (Krogh 1937, 1938, reviewed in Krogh 1939).

Early support for independent Na⁺/NH₄⁺ and Cl⁻/HCO₃⁻ 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 (NH₄)₂SO₄ to the external medium inhibited both chemical and isotopic uptake of Cl⁻, and injection of this salt stimulated Cl⁻ uptake (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 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 was via exchange for intracellular NH₄⁺ and H⁺, and apical uptake of Cl⁻ is via exchange for intracellular HCO₃⁻. See text for supporting evidence. Redrawn from Evans (1975).

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 was via exchange for intracellular NH₄⁺ and H⁺, and apical uptake of Cl⁻ is via exchange for intracellular HCO₃⁻. See text for supporting evidence. Redrawn from Evans (1975).

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 (Oreochromis 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 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 (Murer 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 clamp- ing, 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.

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 NH₄⁺ or H⁺, and apical uptake of Cl⁻ is via exchange for intracellular HCO₃⁻. See text for supporting evidence. Redrawn from Evans (1975).
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, Groessl & Wood 2002), and two studies have demonstrated a phenamil-sensitive Na⁺ uptake into most 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 mitochondria 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, vesicular 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 (Periophthalmus 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 mitochondrial, 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 z-MR cells, to note similarities with the acid-secreting (A or z), 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) Osoresan dace (*Tribelodon bakonensis*) 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\(_3\)\(^-\) 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 (*Leopomis 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
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 STS (4-aminooctanoate-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, SLCA4A1) 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 & Evans 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 pufferfish 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+ /NH4+ 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 proton pump (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 NH3 or even NH4+ across and between (leaky tight junctions) the gill epithelial cells, via NH4+ 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...
The expression of Rh proteins in 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.

**Figure 2** Current working model for ion transport 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.
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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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