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Na^+, Cl^-, Ca^{2+} and Zn^{2+} Transport by Fish Gills: Retrospective Review and Prospective Synthesis

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ABSTRACT The secondary active Cl^- secretion in seawater (SW) teleost fish gills and elasmobranch rectal gland involves basolateral Na^+,K^+-ATPase and NKCC, apical membrane CFTR anion channels, and a paracellular Na^+-selective conductance. In freshwater (FW) teleost gill, the mechanism of NaCl uptake is more controversial and involves apical V-type H^+-ATPase linked to an apical Na^+ channel, apical Cl^-–HCO_3^- exchange and basolateral Na^+,K^+-ATPase. Ca^{2+} uptake (in FW and SW) is via Ca^{2+} channels in the apical membrane and Ca^{2+}-ATPase in the basolateral membrane. Mainly this transport occurs in mitochondria rich (MR) chloride cells, but there is a role for the pavement cells also. Future research will likely expand in two major directions, molded by methodology: first in physiological genomics of all the transporters, including their expression, trafficking, operation, and regulation at the molecular level, and second in biotelemetry to examine multivariable components in behavioral physiological ecology, thus widening the integration of physiology from the molecular to the environmental levels while deepening understanding at all levels. *J. Exp. Zool. 293:264–283, 2002.*

I. FRESH WATER (FW) ION TRANSPORT

To maintain high ion levels in the blood relative to the environment, FW teleosts take up ions (Na^+, Cl^-, Ca^{2+}) by active transport across osmoregulatory surfaces (primarily the gill epithelium) and produce large volumes of very dilute urine. In this way these animals compensate for osmotic gain of fluid and diffusive ion loss. The mechanism of ion uptake has been a matter of controversy in the past 25 years. Early models placed Na^+,K^+-ATPase in the apical membrane (Maetz, '74), but the enzyme was proven to be restricted to the basolateral membrane of the mitochondria-rich cells of the gill (Karnaky et al., '76). Parallel Na^+–H^+ and Cl^-–HCO_3^- exchangers in the apical membrane were major features of an accepted model for over 10 years yet seemed energetically questionable (Kirschner, '83). The most recent model involves an apical ATPase (like the original model), but a H^+-ATPase linked indirectly to Na^+ uptake. What follows is a description of the development of this current ion uptake model.

A. NaCl uptake mechanisms

1. V-type H^+-ATPase

There now is a compelling case for involvement of the V-type proton ATPase (EC 3.6.1.34) in providing the driving force for NaCl uptake in FW teleosts (Fig. 1: Perry, '97; Randall and Brauner, '98). Na^+ and Cl^- uptake by FW animals in their environment is thermodynamically active and each has an apparent K_m in the range of 0.2–0.5 mM (Kirschner, '83) but neither of the previous models for ion uptake (featuring either apical Na^+ channel or apical Na^+/H^+ exchange) adequately described particularly the in vivo FW flux data (Kirschner, '83). A new model arose in work on frog skin (Ehrenfeld et al., '85; Harvey, '92) that proposed involvement of a H^+-ATPase that would generate a large transmembrane potential and drive Na^+ from the medium into the tissue. Lin and Randall ('93) reported ATPase activity in FW rainbow trout (*Oncorhynchus mykiss*) gill that was inhibited by the sulfhydryl reagent *N*-ethylmaleimide and by bafilomycin, a specific inhibitor of V-type H^+-ATPase. The ATPase activity was activated by external Ca^{2+} and by chronic cortisol treatment. They inferred the linkage between the pump and Na^+ uptake because high external Na^+ caused a down-regulation of this H^+-ATPase

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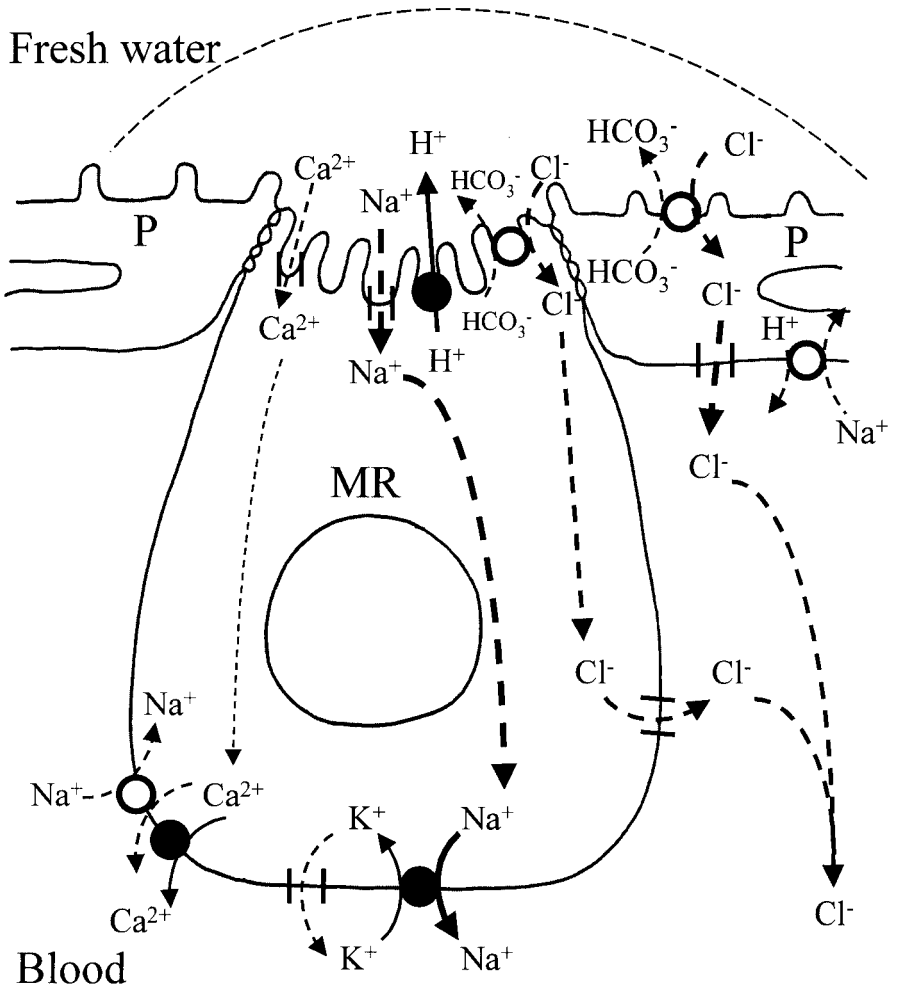

Fig. 1. Model of ion uptake in freshwater (FW) teleost fish gill involves mitochondria-rich (MR) and pavement cells (P), where the former is involved in Na^+ and Cl^- uptake while the latter is involved only in Cl^- uptake. Solid lines indicate active transport; dashed lines show diffusion or exchange through membrane channels or across the cell. ATPase pumps are represented by solid circles; co-transporter and exchangers by open circles; and ion channels by parallel lines. Carbonic anhydrase and its products are present in both cell types, but these features are excluded. In the MR cells, a vacuolar type H^+-ATPase in the apical membrane (typically with microvilli) generates a favorable electrochemical gradient for the passive diffusion (uptake) of Na^+ through an epithelial Na^+ channel and acidifies the boundary layer. In the apical membrane a Cl^-–HCO_3^- exchange (band III), possibly driven by low HCO_3^- in the boundary layer, drives Cl^- into the cell and Cl^- exits via an anion channel, likely the CFTR channel (Fig. 4). Na^+ uptake is completed at the basolateral membrane via Na^+, K^+-ATPase. K^+ pumped into the cell by Na^+,K^+-ATPase recycles via channels in the basolateral membrane. Ca^{2+} uptake is via apical channels and basolateral Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange. In the P cells of some species, Cl^- uptake is linked to bicarbonate secretion presumably driven by the low HCO_3^- generated locally by the H^+-ATPase of neighboring cells (the dotted "bubble" suggests this local microenvironment). Na^+–H^+ exchange in the basolateral membrane prevents acidification of the cytosol and Cl^- translocates across the basolateral membrane via anion channels. Between cells are well-developed tight junctions to minimize diffusive ion loss by the paracellular pathway.

activity. The V-type ATPase was immunolocalized to the apical portions of gill lamellae, confirmed by Western blotting (Lin et al., '94). Sullivan et al. ('95) reported immunoreactivity for the E subunit of V-type ATPase to be restricted to pavement cells, while workers using antibodies to the A subunit report immunoreactivity in pavement cells and in MR cells (Lin et al., '94; Wilson et al., 2000a), the difference perhaps being related to greater water hardness in the former study. In rainbow trout (Bury and Wood, '99) and in young (FW) carp (Fenwick et al., '99), addition of bafilomycin to the outside in vivo reversibly inhibited Na^+ uptake, thus demonstrating in vivo

a connection between the H^+-ATPase and Na^+ uptake. In larval FW tilapia, *Oreochromis mossambicus*, that lack gills, the yolk sac membrane expresses the V-type ATPase, as detected immunocytochemically (Hiroi et al., '98), hence the enzyme activity is not tissue- or life-stage-specific, rather it appears in the appropriate cells of ionregulatory organs of larval and adult fish. In rainbow trout, the V-type ATPase is present in the apical membranes of pavement cells but not MR cells, indicating the separation of H^+ pumping from HCO_3^--transporting cells (Perry, '98). The B subunit of the gene has been cloned and sequenced from rainbow trout gill (Perry et al., 2000) and from eel swim bladder (Niederstatter and Pelster, 2000). By Northern blot analysis there is increased expression in gill after hypercapnic acidosis, consistent with a role for the protein in acid–base balance (Perry et al., 2000; see Claiborne, 2002, this issue).

2. Na channel (ENaC)

Essential to the involvement of a H^+-ATPase in Na^+ uptake is the linkage indirectly with an epithelial sodium channel (ENaC) through which Na^+ can be driven down its electrochemical gradient into the cell. The linkage hinges on the development of a large (>100 mV, inside negative) transmembrane potential, sufficient to drive Na^+ from FW to typical intracellular Na^+ activities. The voltage and associated Na^+ uptake should be blocked by Na^+ channel blockers, particularly by amiloride and its analogues. There is also immunological evidence for the ENaC in the transporting tissue.

a. Electrical potential.

The ENaC would require a favorable electrical gradient to drive Na^+ into gill cells from FW. Transgill potentials measured in vivo for FW teleost fish and larval amphibians are usually inside negative (Maetz, '74; Potts, '84) and become more positive with added external calcium (Kerstetter and Kirschner, '72; Eddy, '75; Potts, '84) and potassium (Potts and Eddy, '73; Pic, '78). Microelectrode measurements of pavement cell apical membrane potentials in isolated flounder (*Platichthys flesus*) gills are significantly smaller than predicted by the model (-36.8 mV, Clarke and Potts, '98). While this electromotive force is the right direction to drive Na^+ through an apical channel, the voltage is too small, unless cytoplasmic Na^+ activity is extremely low. There may be technical problems in impaling the very small volume pavement cells that yield suboptimal potentials. Frog skin expresses the ENaC at high levels and responds to addition of isotonic NaCl to the apical side by a large increase in a serosa positive transepithelial potential connected to electrogenic Na^+ uptake that fits the ''Ussing'' model of sodium uptake (Kirschner, '83). FW killifish (*Fundulus heteroclitus*) opercular epithelium (a model of the FW gill epithelium; Marshall et al., '97) and isolated flounder gill (Clarke and Potts, '98) show a significant reduction in the serosa negative transmembrane potential with addition of external NaCl, but not the generation of a large serosa positive potential in symmetrical saline or the development of electrogenic Na^+ uptake. To explain this ''fish to frog'' discrepancy, it may be that the level of ENaC expression and/or operation is much less in FW fish than in amphibians or that a passive ion shunt in parallel masks the electrogenicity of the Na^+ uptake. Alternatively, the estuarine killifish (and flounder) may not conform in FW to the generally accepted model for typical FW resident fish (Patrick et al., '97). Refinement of technique will likely support the existence of an apical membrane potential of appropriate polarity and magnitude.

b. Amiloride sensitivity.

Amiloride is an inhibitor of epithelial Na^+ channels that has many analogues with differential affinities for Na^+ channel and the Na^+–H^+ antiporter. Amiloride inhibits Na^+ uptake with a K_i less than 0.1 mM in rainbow trout (Kirschner et al., '73), indicating Na^+ entry via an amiloride-sensitive pathway. Treatment of isolated flounder gill preparations with amiloride (0.1 mM) significantly reduced the measured apical membrane potential and 0.05 mM benzamil (an analogue with a high affinity for ENaC) inhibited Na^+ uptake (Clarke and Potts, '98), consistent with the presence of an apical Na^+ channel. In brown trout (*Salmo trutta*), apical amiloride in vivo inhibited Na^+ uptake (but not ammonia or proton transport) at 0.01 mM (Nelson, '97) and produced a transient hyperpolarization of the transgill potential, strongly suggestive of inhibition of a conductive channel rather than the Na^+–H^+ antiporter (that generally is inhibited by mM concentrations of amiloride). Higher amiloride doses (0.5 mM) produced an additional depolarization of the transgill potential (Nelson et al., '97). In FW killifish opercular epithelium in vitro (with FW outside), 0.1 mM amiloride produces a significant depolarization of the transmembrane

potential (Marshall et al., '97) but no significant decrease in Na^+ influx. Bury and Wood ('99) found that the amiloride analogue phenamil (0.1 mM) blocked Na^+ uptake in FW rainbow trout in vivo. Although phenamil has a high affinity for the adult amphibian ENaC (K_d = 0.65 μM, Li et al., '95) and has a lower affinity for Na^+–H^+ and Na^+/Ca^{2+} exchangers (Frelin et al., '88), the teleost pathway has an lower apparent affinity (approximately 10–100 μM; Nelson et al., '97; Bury and Wood, '99). Given that amiloride affinity and ion selectivity are different for larval versus adult amphibian versions of ENaC (Hillyard and Van Driesche, '91) and the notion that larval amphibians are closer physiologically to FW fish, the pharmacology of the teleostean ENaC ought to be different from the adult amphibian channel.

c. Immunolocalization.

Antibodies to the mammalian epithelial sodium channel (ENaC) α and β subunits cross-react with trout MR cells and lamellar cells of trout and tilapia gills (Wilson et al., 2000a) and, in almost all cases, appear to co-localize with immunoreactivity to the V-type ATPase. Immunoreactivity was confirmed by Western blot and localization confirmed by ImmunoGold reactions with electron microscopy (Wilson et al., 2000a). There remains a possibility that heterologous antibodies could label a different product, especially as this complex channel is known to have at least three subunits (Li et al., '95). Thus there is building evidence for the ENaC, but still needed are direct observations of ENaC operation in FW gill membranes and molecular identification of the teleostean ENaC.

3. Na^+–H^+ antiporter

Until recently, it was thought that the Na^+–H^+ exchanger (NHE), operating in parallel with the anion exchanger in the apical membrane of gill cells could drive NaCl uptake, but energetically this model is unlikely (Kirschner, '83). However, the gene for the Na^+–H^+ antiport is now cloned from two species of teleosts and has a major role instead in acid–base balance in some species (see Claiborne, 2002, this issue). Evidence for the presence of Na^+–H^+ exchange in FW teleost gill epithelia includes immunocytochemical identification of a subpopulation of gill cells in rainbow trout (and in a marine fish, the blue-throated wrasse) identified using rabbit polyclonal antibody directed against human NHE (Edwards et al., '99). These results were confirmed by Wilson et al. (2000a), using the same antibody (1380 and 1381), but the fluorescence was found to be nonspecific. In contrast, a polyclonal antibody to NHE_2 protein (antibody 597) specifically stained lamellar and interlamellar epithelial cells and accessory cells of FW adapted tilapia (but not rainbow trout) gills and in Western blots NHE_2-reactive bands did not co-migrate with fractions rich in Na^+,K^+-ATPase (Wilson et al., 2000a). The apparent absence of Na^+–H^+ immunoreactivity in the trout supports the current model involving V-type ATPase and a sodium channel (exclusive of the Na^+–H^+-antiport), but the presence of the antiporter immunoreactivity in tilapia may illuminate a fundamental difference in ionoregulatory strategies of euryhaline teleosts that adapt temporarily to FW (e.g., tilapia and killifish) as opposed to FW residents (e.g., trout, carp, and goldfish). Because NHE is present in some species and apparently not in MR cells, the exchanger is included in the model (Fig. 1) in the basolateral membrane of pavement cells.

4. Na^+,K^+-ATPase

The driving force for Na^+ uptake across the basolateral membrane is provided by Na^+,K^+-ATPase and the enzyme is present in virtually all animal cells. Na^+,K^+-ATPase is generally measured and reported to be in higher concentrations and more activated in SW resident teleosts, thus the main discussion appears in the ion secretion section (below). Clearly, however, basal levels of Na^+,K^+-ATPase are present in FW teleosts and are detected immunologically in FW tilapia and rainbow trout mitochondria-rich cells (Wilson et al., 2000a).

5. Cl^-–HCO_3 exchange and CFTR

There is considerable debate regarding Cl^-–HCO_3^- exchange location and function in teleosts but clearly it is present, as removal of Cl^- and addition of inhibitors of the exchange produce metabolic alkalosis (Perry, '97; Goss et al., '98; Evans et al., '99). In this limited discussion, the recent work on the role in Cl^- uptake is considered. In the FW NaCl uptake model (Fig. 1), Cl^-–HCO_3^- exchange occurs in the apical membrane of MR cells. Theoretically, local acidification in the apical crypt (or at the base of apical microvilli) of MR cells by H^+-ATPase will lower HCO_3^- activity sufficiently to drive the exchange and thus also Cl^- uptake (Fig. 1). In pavement cells, Cl^-–HCO_3^- exchange might operate instead in series with a basolateral anion conductance in a

low affinity Cl^- uptake system. Consistent with the latter idea, mRNA of the band III exchanger localizes to the filament and lamellar epithelium (i.e., MR cells and pavement cells) of rainbow trout gill (Sullivan et al., '96). Also, cultured gill epithelia from trout that resemble pavement cells take up Cl^- but not Na^+ (Wood et al., '98). CFTR immunofluorescence in FW killifish opercular epithelium is present in the basolateral membranes of pavement cells and in MR cells (Marshall et al., 2002; Fig. 4B) but not in the apical membranes of MR cells (where CFTR immunofluorescence is concentrated in SW MR cells). The band III anion exchanger has been cloned from rainbow trout erythrocytes and was shown to be involved in taurine transport and volume regulation in that tissue (Fievert et al., '95), but thus far the isoform from gill has not been cloned and sequenced. The band III anion exchanger has been immunolocalized (by heterologous antibodies) to the apical membranes of MR cells of tilapia gills but is apparently absent from apical membranes of pavement cells in this species; immunocytochemistry was confirmed by Western blot analysis that indicated a single 110-kDa band (Wilson et al., 2000a). There would appear to be substantial species variation in the placement and operation of the anion exchanger.

B. Divalent ion uptake

1. Ca^{2+} uptake

Calcium metabolism in teleosts includes active uptake of Ca^{2+} across the gill (Fig. 1) and intestinal epithelia, because teleost bone is extracellular and unable to provide sufficient calcium from bone. This is particularly true in FW, but even in SW fish, gill Ca^{2+} uptake is important. The Ca^{2+} uptake mechanism and its regulation was reviewed recently (Flik et al., '95, '97) and for SW chloride cells (Flik et al., '95, '97; Marshall and Bryson, '98). As Ca^{2+} transport in SW teleost gills appears to share the same mechanism with the FW condition, both salinities are considered here. Because intracellular calcium of vertebrate cells is extremely low, it is thought that Ca^{2+} entry across the apical membrane is passive and may involve a Ca^{2+} ion channel. There is sufficient driving force for Ca^{2+} uptake even in very soft water (Ca^{2+} activity of 10^{-5} M). Exit of Ca^{2+} across the basolateral membrane is certainly an active step and involves a Ca^{2+}-ATPase. There is Na^+ dependence of Ca^{2+} uptake from vesicle experiments (Flik et al., '97) and in isolated epithelia (Verbost et al., '97), demonstrating that Na^+–Ca^{2+} exchange contributes to Ca^{2+} uptake. Acclimation of rainbow trout to 70% SW evokes an 8-fold increase in Ca^{2+}-ATPase and a 5-fold increase in Na^+–Ca^{2+} exchange, far beyond requirements for transepithelial Ca^{2+} transport (Flik et al., '97), implying other Ca^{2+} transport functions. Thus far, the Ca^{2+}-ATPase and putative Na^+-Ca^{2+} exchanger have not been cloned from fish.

Ca^{2+} uptake occurs through MR cells, based on the variation of Ca^{2+} uptake rate with increasing density of MR cells (McCormick et al., '92; Marshall et al., '92, '95b) and the covariation of Na^+,K^+-ATPase with the activity of Ca^{2+}-ATPase in isolated vesicle experiments (Flik et al., '84, '97).

2. Zn^{2+} and Mg^{2+}

Zinc and magnesium are essential minerals and thus are expected to be transported and regulated physiologically. Some other heavy metals (Cu, Cd, Ag, Pb, and Hg) may be transported vicariously by gill and intestinal epithelium but are only important as toxins. The gill transport of other trace metals (Se, Co, Fe, and Mn) have not been studied, thus only Zn and Mg will be considered here.

Zinc uptake by FW rainbow trout is significantly inhibited by La^{3+}, as is Ca^{2+} uptake, and Zn^{2+} competitively inhibits Ca^{2+} uptake, raising the K_m for Ca^{2+} uptake significantly in the picomolar concentration range of Zn^{2+} (Hogstrand et al., '94, '96; Alsop and Wood, '99). There is non-specific competitive inhibition of Zn^{2+} by a number of cations including magnesium (Alsop and Wood, '99). In the converse experiments, Zn^{2+} uptake was competitively inhibited by Ca^{2+} (Spry and Wood, '89). Experimental hypercalcemia, invoked in juvenile rainbow trout by injection of $CaCl_2$, presumably operates via stanniocalcin and inhibits both Ca^{2+} and Zn^{2+} uptake across the gill (Hogstrand et al., '96) and low Ca^{2+} water up-regulates Ca^{2+} and Zn^{2+} uptake in parallel (Hogstrand et al., '98). Meanwhile, Zn^{2+} chronic exposure up-regulates Ca^{2+} uptake (Hogstrand et al., '98). These results collectively point to a common regulated active transport pathway for Zn^{2+} and Ca^{2+} uptake. There are low-affinity sites for binding of Zn^{2+} and other divalent ions that are evident at high (mM) concentrations, but at physiologically relevant Zn^{2+} activities (<100 μM), the K_m of the high affinity binding of Zn^{2+} is approximately 10 μM (Galvez et al., '98; Hogstrand et al., '98), in the correct range for

physiologically relevant Zn^{2+} uptake. At the basolateral membrane, Zn^{2+} does not seem to be transported by the Ca^{2+}-ATPase because the inhibition is mixed competitive and non-competitive; neither is Zn^{2+} transported by a Na+ dependent mechanism (Hogstrand et al., '96), so the translocation mechanism here is not yet known. Recent findings link Zn^{2+} and Cd^{2+} transport. Cadmium uptake in zebrafish (*Danio rerio*) is saturable but inhibited by Zn^{2+} and conversely Cd^{2+} inhibits Zn^{2+} uptake (Glynn, 2001). In summary, Zn^{2+} apparently enters gill cells by the same passive uptake pathway as does Ca^{2+}, but the presumably active basolateral exit mechanism for Zn^{2+} is unknown, but perhaps by its own ATPase.

Magnesium uptake by the gills of teleosts was suspected in experiments with Mg^{2+} depleted animals that could take up more than the Mg^{2+} content of the food (rainbow trout [Shearer, '89]; tilapia [Dabrowska et al., '91; Bijvelds et al., '96]). The extraintestinal Mg^{2+} uptake was presumably branchial. Depletion of Mg^{2+} in the water reduces total body Mg^{2+} content (Shearer and Asgard, '92; Bijvelds et al., '96), suggestive of the need for extraintestinal Mg^{2+} uptake. Passive diffusional loss of Mg^{2+} across the gills is negligible (Bijvelds et al., '96) or $<1\%$ of renal Mg^{2+} loss (Oikari and Rankin, '85), demonstrating effective impermeability of the integument to Mg^{2+}. The intestinal brush border membrane, a general model for Ca^{2+} and other divalent uptake mechanisms, has a Na^+-dependent Mg^{2+} uptake across the intestinal mucosa (Bijvelds et al., '98) that is driven by the apical membrane electrochemical potential (Bijvelds et al., 2001), but the mechanism for Mg^{2+} uptake in the gill specifically is unknown. Because Mg^{2+} (unlike La^{3+} and Zn^{2+}) fails to inhibit Ca^{2+} uptake in killifish (Patrick, '97), it would appear that Ca^{2+} and Mg^{2+} uptake pathways are separate from each other.

II. SW ION TRANSPORT

A. NaCl secretion

1. The model

NaCl secretion by teleost gill involves a basolateral Na^+,K^+-ATPase as the source of driving force, a basolateral Na^+,K^+,2Cl-co-transporter (NKCC) to effect Cl^- entry across the basolateral membrane and an anion channel homologous to cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane where Cl^- flows down its electrochemical gradient into SW (Fig. 2). Na^+ secretion follows that of Cl^- passively down its electrochemical gradient via a cation-selective paracellular pathway (see reviews: Marshall, '95; Karnaky, '98; Marshall and Bryson, '98; Evans et al., '99). This paracellular path is localized to thin tight junctions between mature chloride cells and accessory cells (Sardet et al., '79).

2. Na^+,K^+-ATPase

a. Cloned sequences

Na^+,K^+-ATPase (EC 3.1.4.3) is a ubiquitous enzyme with an α (catalytic) subunit that is the locus for ATP and ion binding as well as binding of ouabain to the K^+ (extracellular) site. Inhibition of this enzyme by ouabain demonstrated the relationship to NaCl secretion in marine teleosts (Silva et al., '77) and ouabain blocks Cl^- secretion by isolated opercular epithelium of SW killifish (Degnan et al., '77). There is also a smaller β subunit that is glycosylated and serves to facilitate trafficking of the complex to the plasma membrane. In most epithelia the enzyme appears only in the basolateral membrane. Na^+,K^+-ATPase has been localized to the basolateral membrane of MR chloride cells by tritiated ouabain autoradiography (Karnaky et al., '76) and fluorescent anthroyl ouabain (McCormick, '90b) and is localized to the tubule system of these cells (Hootman and Philpott, '80).

The catalytic α_1 subunit has been cloned from Pacific electric ray (*Torpedo californica*, Kawakami et al., '85), White sucker (*Catostomus commersoni*, Schonrock et al., '91), and European eel gill (*Anguilla anguilla,* Cutler et al., '95). The α_1 subunit shows high levels of expression in the gills (kidney, intestine, and brain) of eels with an increase in expression in gill tissue on adaptation to SW and hypersaline conditions (Cutler et al., '95). Antibody to the tilapia α_1 subunit cross-reacts with a 110-kDa band in gill, brain, and heart of tilapia and mudskipper (*Periophthalmus cantonensis*, Hwang et al., '98). There also is, submitted from the Hwang laboratory, a partial sequence of an α_3 subunit (NCBI accession number AAF75108, '98), indicative of more than one isoform of the gene. Further, Na^+,K^+-ATPase has been immunolocalized to the basolateral side of MR cells of the gills (tilapia [Lee et al., '98]; eel [Cutler et al., 2000]; rainbow trout [Wilson et al., 2000a]; and mudskipper *Periopththalmodon schollosseri* [Wilson et al., 2000b]) using antibodies directed against various α subunits.

The β subunit in two isoforms has been cloned from European eel (Cutler et al., 2000) and the β_1 and β_{233} isoforms share 70% amino acid serial identity. Generally, β subunits are believed to be involved in postprocessing and trafficking of the enzyme complex to the plasma membrane. The subunit is expressed strongly in gill (intestine, kidney, and brain) and is glycosylated to varying extents (Cutler et al., 2000). The β_1 subunit is up-regulated by acclimation of silver eels to SW but not in yellow (nonmigratory) eels, suggesting developmental differences in expression (Cutler et al., 2000).

b. Regulation.

Na^+,K^+-ATPase activity is up-regulated by transfer of fish to SW (*Fundulus heteroclitus* [Epstein et al., '67; Jacob and Taylor, '83; Mancera and McCormick, '99]; *Salmo trutta* [Madsen et al., '95]; *Dicentrarchus labrax* [Jensen et al., '98]) and by administration of cortisol and IGF-1 (*Salmo trutta* [Madsen et al., '95]; *Fundulus heteroclitus* [Mancera and McCormick, '99]). As early as 12 hr after transfer of brown trout to SW there is elevation of α_1 subunit mRNA, before standard activity measurements can detect changes in activity (Seidelin et al., 2000). Na^+,K^+-ATPase is involved in preadaptation of salmonid smolts to downstream migration and activity is increased during smolting of Atlantic salmon (*Salmo salar* [D'Cotta et al., 2000]), brown and seatrout (*Salmo trutta* [Pirhonen and Forsman, '98]), masou salmon (*Oncorhynchus masou* [Ura et al., '97]), and Arctic charr (*Salvelinus alpinus* [Lysfjord and Staurnes, '98]). However, proliferation of immunochemically detected chloride cells occurs in advance of detectable increases in enzyme activity (Ura et al., '97). Also, downstream migratory behavior and elevated enzyme activity are not tightly correlated (Pirhonen and Forsman, '98). Acclimation of sea bass (*Dicentrarchus labrax*) to isosmotic environment (15 ppt brackish water) down-regulates Na^+,K^+-ATPase such that subsequent transfer to FW or to SW increases Na^+,K^+-ATPase activity (Jensen et al., '98). Similarly, brackish water acclimation of killifish makes more pronounced the augmentation of Na^+,K^+-ATPase with cortisol and growth hormone treatment (Mancera and McCormick, '99). Up-regulation of rainbow trout gill Na^+/K^+-ATPase activity by cortisol is dependent on the density of cortisol receptors and is impeded by nonspecific stressors (handling) that diminish corticosteroid receptivity (Shrimpton and McCormick, '99). Gill basolateral membrane lipid composition in American eels (*Anguilla rostrata*) varies little with salinity, hence membrane composition does not appear to regulate enzyme activity (Crockett, '99), yet fatty acids from algal blooms may be highly active inhibitors of Na^+/K^+-ATPase in tilapia (Bury et al., '98). Further, in vitro activity measurements are affected by detergents (Mancera and McCormick, 2000; see rapid activation), suggestive of a regulatory role for membrane lipids. It would be interesting to follow lipid composition of developing salmon during the parr–smolt transformation.

c. Isoform expression.

Na^+,K^+-ATPase has several different isoforms even in homeotherms. The use of selected heterologous antibodies to mammalian isoforms has pointed to the existence of multiple isoforms in teleost fish as well. Tilapia in SW have a higher ratio of α_1 to α_3 isoform expression, based on quantitative Western immunoblots (Lee et al., '98), although the α_1 isoform is the predominate form in both FW and SW. Atlantic salmon parr and smolts appear to cross-react best with antibodies to the α_3 subunit and much less so to antibodies directed against the α_1 and α_2 isoforms (D'Cotta, 2000). It will be very interesting to see selective regulation of different isoforms in salinity and temperature acclimation and in development.

d. Rapid activation.

Na^+,K^+-ATPase expression and proliferation of chloride cells of premigratory smolts before the animals have experienced SW and while their Na^+,K^+-ATPase activity is low (Ura et al., '97) suggests that the enzyme, although present in presmolts, is not fully active until exposure to SW. A post-transcriptional activation process is therefore likely. Rapid activation of Na^+,K^+-ATPase has now been observed in killifish and in salmonids within a few hours of transfer to SW (Towle et al., '77; Mancera and McCormick, 2000; Tipsmark and Madsen, 2001). This increase in enzyme activity occurs in some cases within 1 hr (Towle et al., '77) or only after 3–6 h, the latter increase being dependent on protein synthesis (Mancera and McCormick, 2000). The early activation may be associated with phosphorylation via protein kinase A and cAMP (Tipsmark and Madsen, 2001). Curiously, the rapid activation is more detectable with the inclusion of detergent in the isolation medium (Mancera and McCormick, 2000), sug-

gesting a role of lipids or lipid metabolites in this effect. Because CFTR is activated by protein kinase A via cAMP-mediated pathways (Singer et al., '98) and as NKCC in shark rectal gland is activated by tyrosine phosphorylation (Lehrich and Forrest, '95) and cell shrinkage (Greger et al., '99), it would appear that all three transporters can be up-regulated in chorus. There is good reason to expect the simultaneous stimulation of the three. The turnover rate for cellular Cl^- in a SW chloride cell, assuming a whole-cell current of approximately 0.65–2.0 nA (cf. Marshall and Nishioka, '80; Karnaky et al., '84) and an intracellular Cl^- activity of approximately 50 mM, is only about two minutes. Thus the cell pumps its entire Cl^- content approximately every 2 min. This phenomenal Cl^- transport rate will require balanced activation of all transporters involved, not only to optimize Cl^- secretion but also to maintain cell volume.

e. Metabolic cost.

Na^+,K^+-ATPase is the primary driving force for active ion transport in FW and in marine teleosts, yet there have been very few attempts to measure the metabolic costs of osmoregulation. Estimates based on isolated perfused gill arches from SW and FW trout predict that in SW 2.4% and in FW 3.9% of total resting O_2 consumption derives from gill O_2 consumption, including the osmoregulatory component (Morgan and Iwama, '99). Clearly gill osmoregulatory based metabolism is a small percentage of the total, but these estimates exclude the cost of operating other ion regulatory structures (intestine, kidney, skin, etc.).

3. Na^+,K^+,2Cl co-transporter (NKCC)

The gill chloride cells of marine teleosts are sensitive to basolateral addition of furosemide and its analogues (e.g., Eriksson and Wistrand, '86). Basolateral membrane vesicles from FW and 70% SW rainbow trout have bumetanide and furosemide sensitive co-transport with 1:1:2 stoichiometry and a high sensitivity to plasma-side K^+ (Flik et al., '97). The Na^+ dependence of Cl^- secretion was shown in opercular epithelium of the killifish (Degnan et al., '77) and the jaw skin of a euryhaline goby, the long-jawed mudsucker (*Gillichthys mirabilis*; Marshall, '81), both preparations containing a high density of chloride cells (Karnaky and Kinter, '77; Marshall and Nishioka, '80; Foskett and Scheffey, '82). The K^+ dependence of NaCl secretion was shown in intact epithelium by comparison between K^+-free and ouabain inhibition of Cl^- secretion in killifish opercular epithelium (Marshall and Bryson, '98). Hence, the basolateral membranes of chloride cells in marine teleosts and the shark rectal gland contain NKCC. The development of antibodies to NKCC (Lytle et al., '95) included a widely applicable antibody (T_4) that is useful in immunofluorescence and Western blotting (Fig. 1). Pelis et al. (2001) demonstrated immunocytochemically the expression of NKCC in juvenile Atlantic salmon. Parr acclimated to SW showed large increases in NKCC expression and many immunofluorescent chloride cells in the gill epithelium, compared to presmolts. Increased expression of NKCC was coincident with the development of SW tolerance. Taken together, there is strong evidence for NKCC involvement in NaCl secretion in euryhaline and anadromous teleosts. The gene product in the rectal gland and teleost gill may be the NKCC1 isoform (Haas and Forbush, '98), as the sensitivity to bumetanide is greater than that to furosemide (Eriksson and Wistrand, '86) but Flik et al. ('97) observed thiazide-sensitive Na^+ uptake, so there also may also be a NaCl (1:1) co-transporter present. To date, the phosphorylation and dephosphorylation as well as up- and down-regulation of expression of NKCC in teleosts have not been demonstrated, but they are well known in other systems (Haas and Forbush, '98), and in the shark NKCC is phosphorylated at tyrosines (Greger et al., '99) and possibly also at serine–threonine locations (Waldegger et al., '98).

4. CFTR anion channel

A low conductance (8 pS) anion channel was identified in MR cells of opercular epithelium of SW killifish (a model of the SW gill epithelium) and the channel was activated by cAMP and inhibited by anion channel blockers diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Marshall et al., '95a). A full-length clone of the killifish homologue of CFTR (kfCFTR) was sequenced and shown by Northern blot analysis to be expressed in gill, opercular epithelium, and posterior intestine of SW killifish. Microinjection of kfCFTR mRNA into amphibian oocytes resulted in expression of a cAMP-activated anion conductance (Singer et al., '98). CFTR has been cloned from puffer fish (*Fugu rubripes*) and found to be closely related to killifish (Davidson et al., 2000). Killifish CFTR is the most divergent CFTR form, sharing exon amino acid sequences 57%, 59%, and 84%

with human, shark (*Squalus acanthias*), and pufferfish (*Fugu rubripes*), respectively. Two other isoforms have been cloned from Atlantic salmon (sCFTR I and sCFTR II) that share 95% identity at the amino acid level and group with the other teleostean forms (Chen et al., 2001).

Immunofluorescence of an antibody directed against the full-length human CFTR showed CFTR-like immunolocalization to the apical crypts of chloride cells in mudskipper gills adapted to 50% SW (Wilson et al., 2000b). Immunofluorescence of a monoclonal antibody directed to the C-terminus (a sequence shared by human and killifish CFTR; Singer et al., '98) confirm the apical crypt localization in SW killifish chloride cells (Marshall et al., 2002; Fig. 3). In sum, an apically located CFTR-like anion channel, activated by cAMP and up-regulated during SW adaptation, is responsible for Cl^- exit in Cl^- secretion by euryhaline teleost chloride cells.

5. Electrical potential

The majority of SW teleost fish have a blood positive electrical potential relative to the environment. In most cases the potential measured across the body wall is +10–+35 mV and appears to be close to the Nernst equilibrium potential for Na^+ (Potts, '84). The potential, however, is likely not evenly distributed across the mosaic of cell types that make up the surface of the body (particularly pavement cells and MR cells), so pockets of higher potentials must exist where the electrical gradient exceeds the diffusion potential and Na^+ thus can be secreted. In circumstances where the passive diffusional shunt across the large surface areas of gill are eliminated, such as in an isolated goby skin epithelium (Marshall, '81) or the killifish embryo that lacks gills (Guggino, '80), the measured electrical potential in SW is +40–+50 mV, higher than the whole animal potentials. Because the shunt through which Na^+ moves is localized to the junctions between accessory cells and chloride cells (Sardet et al., '79; Foskett and Scheffey, '82), the local electrical potential that drives Na^+ secretion may be +50 mV or greater. This large electrical driving force means that even in extreme hypersaline conditions (2.5–3.0 times SW) Na^+ would still be secreted. The energetic source for the potential is likely the high permeability of the apical membrane to Cl^- and Cl^- diffusion across the apical membrane, linked indirectly to the operation of Na^+,K^+-ATPase in the basolateral membrane. The inhibition of in vitro TEP across opercular membrane by anion channel blockers, DPC, and NPPB is evidence for Cl^- involvement in the potential, and the lack of effect of DIDS (that can block ClC-type anion channels but not CFTR-like channels) indicates that the conductance is the result of CFTR anion channels.

Those few teleosts with negative inside electrical potentials in SW are a great curiosity, as the driving force for Na^+ is reversed and hence their Na^+ secretion mechanism may be very different. The toadfish characteristically has a negative inside potential, but this animal is known to excrete urea intermittently (see Walsh, 2002, this issue), and thus it may intermittently operate ion secretion as well. The negative readings could represent a time when the ion secretion is voluntarily turned off.

6. Chloride cell dynamics

a. FW and SW cell types.

The emerging picture is that morphologically and functionally, MR cells change during adaptation to different salinities. Tilapia transferred to SW develop larger and more numerous MR cells in the opercular epithelium correlated with development of ion secretion in SW (Foskett et al., '81). Subtypes of MR cells have been recognized by ultrastructural differences (Pisam et al., '87; Pisam and Rambourg, '91), and there are α and β type cells in the gills of the guppy (*Lebistes reticulatus*) with the larger α cells, with smooth apical membrane and lighter staining, predominating in SW. The α cells have more intense immunocytochemical reaction to antibodies to the α subunit of Na^+-K^+-ATPase and increase in size and number during SW adaptation of guppies (*Poecilia reticulata*), while the β cells have no or weak immunoreactivity and dwindle during SW adaptation (Shikano and Fujio, '98b). Conversely, β cells proliferate during adaptation to FW while the mean cell size decreases (Shikano and Fujio, '98b). In killifish, FW MR cells of the opercular epithelium and gill are larger than their SW counterparts (Marshall et al., '97; Katoh et al., 2001) and have apical membranes that are thrown into microvillar folds. In the Japanese eel (*Anguilla japonica*) dispersed gill tissue yielded, by flow cytometry, three different classifications of MR cells based on size and granularity: two small cell types in FW, a transitional cell type during adaptation to SW, and two large cell types in fully adapted marine eels (Wong and Chan, '99).

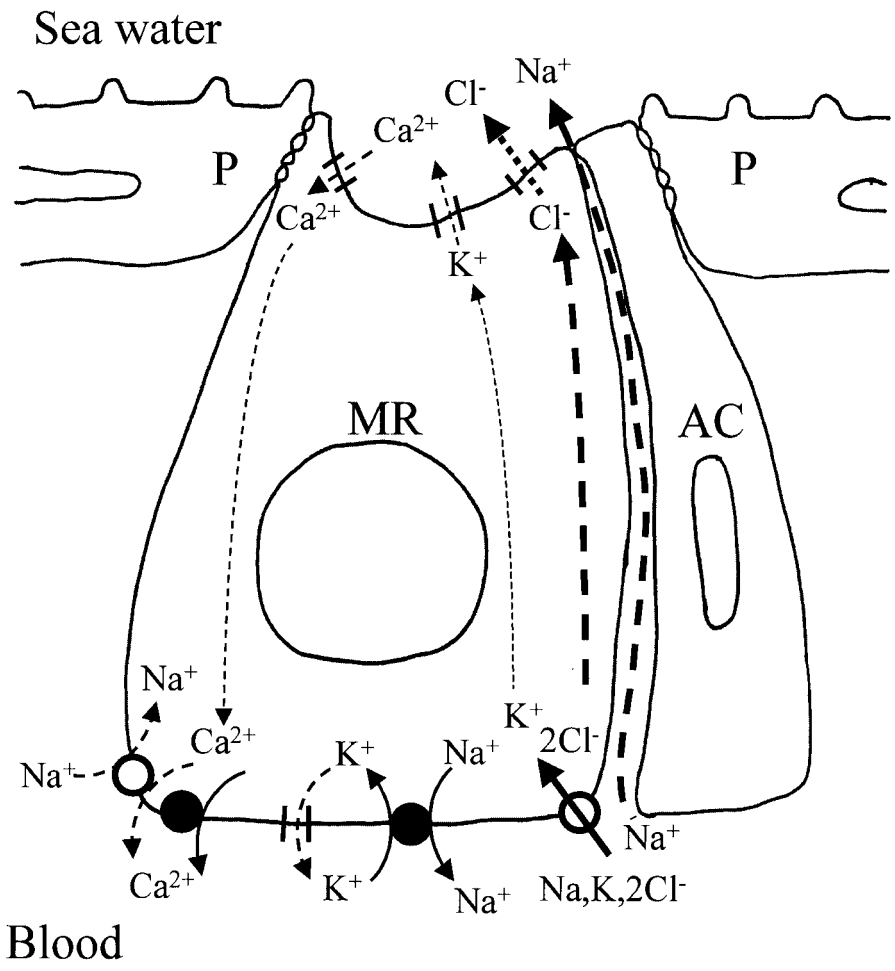

Fig. 2. Model of transporter placement in gill epithelium of seawater (SW) teleosts where intercellular junctions between accessory (AC) cells and mitochondria-rich (MR) chloride cells are single-stranded and cation selective. Symbol definitions are the same as for Fig. 1. Basolateral Na^+,K^+-ATPase provides driving force in the form of transmembrane Na^+ gradient to transport Cl^- into the cell via the Na^+,K^+,$2Cl^-$ co-transporter (NKCC type 1). K^+ mostly recycles across the basolateral membrane but a small amount is secreted passively. Cl^- exits across via CFTR-type anion channels in the apical membrane (typically a smooth, cup-shaped apical crypt) while Na^+ is secreted following its electrochemical gradient by the local paracellular pathway between chloride cells and accessory cells. Ca^{2+} uptake is by a mechanism similar to that described for FW. In SW, pavement cells (P) are much less active in ion transport.

Dispersed MR cells from FW rainbow trout have been separated by selective binding of a subpopulation of MR cells to peanut lectin (Goss et al., 2002) with the lectin-positive MR cells being unresponsive to cortisol treatment. In FW tilapia, two populations of chloride cells were distinguished by the relative immunostaining for Na^+,K^+-ATPase, and the weaker staining cells were interpreted to be apoptotic and/or necrotic (Dang et al., 2000), indicating a possible role for cell cycle in producing distinct subtypes. In chum salmon fry increasing salinity increased the proportion of mitochondria-rich cells that were immunopositive for $\alpha_{1,2,3}$ Na^+/K^+-ATPase (Shikano and Fujio, '98a) concomitant with increased hypo-osmoregulatory ability. The changing numbers of the two immunologically distinct MR cell types was interpreted as maturation of chloride cells in preparation by chum for seaward migration. The development and enlargement of MR cells in tilapia yolk sac larvae transferred to SW over 96 hr has been followed individually, and three distinct stages have been identified (Hiroi et al., '99). From this study it is clear that 75% of MR cells survive for 96 hr and engage in this process. Subpopulations of MR cells certainly exist, and there is considerable complexity and species differences.

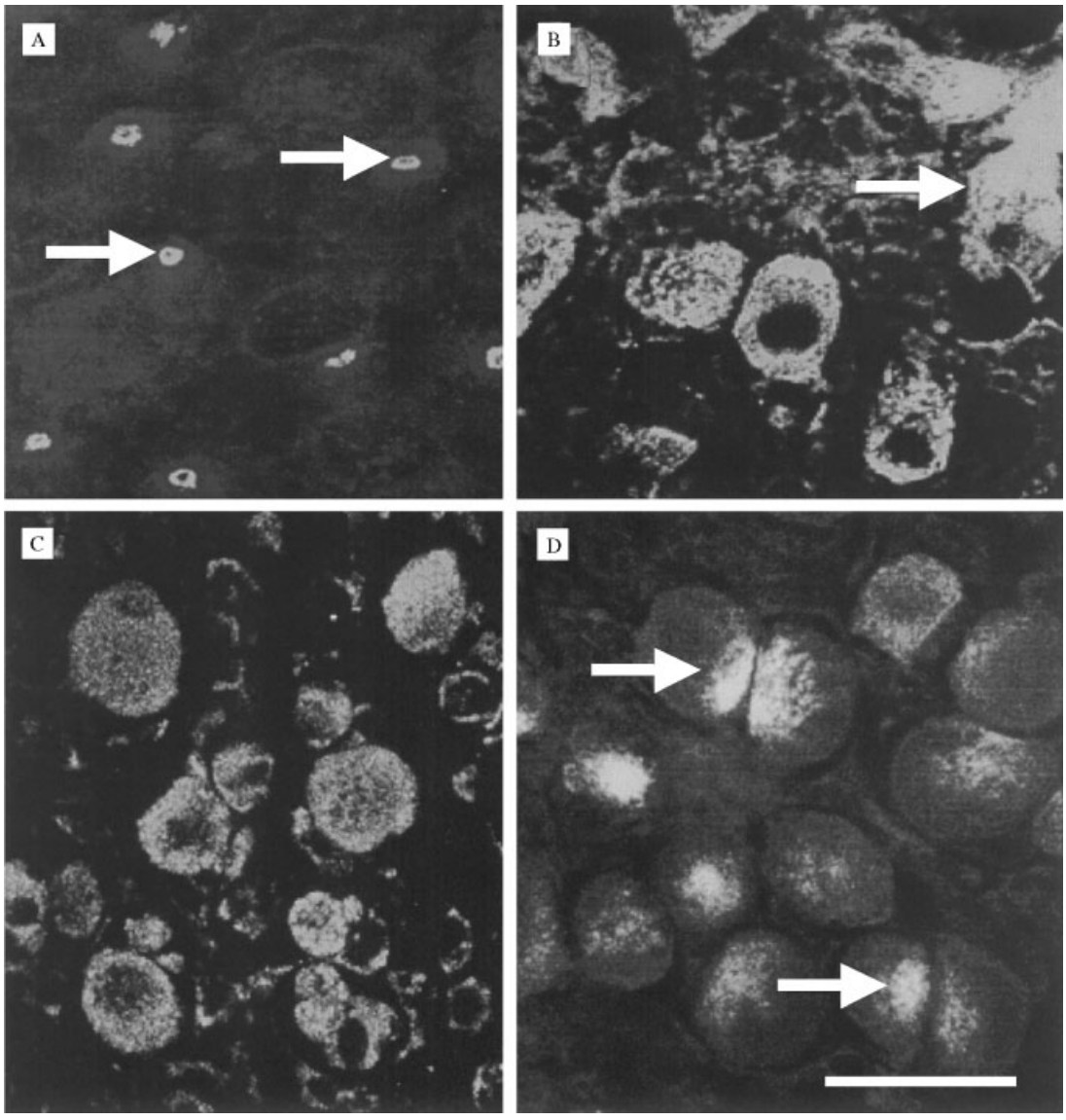

Fig. 3. Transport proteins CFTR and NKCC apparently move during salinity adaptation. (**A**) Immunofluorescence where the primary antibody is mouse monoclonal anti-hCFTR (the epitope for this antibody is −dtrl, a sequence shared by human and killifish CFTR) and the second antibody is goat anti-mouse IgG complexed with Oregon Green 488 and counterstained with Mitotracker Red. CFTR immunofluorescence is bright, and mitochondria stain gray. The confocal image is taken at the plane of the apical crypts of chloride cells in the opercular epithelium of SW-adapted killifish (*Fundulus heteroclitus*). Methods as per Marshall et al. (2002). Note the bright ring of CFTR fluorescence (arrow) in the apical crypts. The rings overlie mitochondria rich cells as detected by Mitotracker Red. Pavement cells and deeper parts of MR cells had no CFTR immunofluorescence. (**B**) CFTR immunofluorescence in FW-adapted killifish opercular epithelium is absent from the apical crypt and instead appears in the central cytoplasm of the MR cells (arrows) and in pavement cells, apparently in the basolateral membrane or tubular system (invaginations of the basolateral membrane). (**C**) In contrast, NKCC immunofluorescence (mouse monoclonal T_4 antibody against shark NKCC, same second antibody) is evenly distributed in the cytoplasm in MR cells (arrows) in opercular epithelium from SW acclimated animals but in FW-adapted animals (**D**) is condensed to areas eccentric from the nucleus (arrows) of MR cells. Bar is 20 μm.

Clearly, the functionally distinctive features between different subtypes of ion transporting MR cells are not primarily in the Na^+,K^+-ATPase pump that is present at one level or another in all, but rather in the differential distribution of channels, exchangers, co-transporters, and specialized ATPases (for Ca^{2+} and H^+) that distinguish one functional subtype from another. This chal-

lenging subfield will likely illuminate the dynamics of cellular differentiation and turnover not only for fish gills but also for vertebrate epithelia in general.

b. Morphological and functional variation.

Pavement cells with microridge patterns that are stabilized by actin (Fig. 4A) form the majority of the exposed surface. At periodic intervals, apical

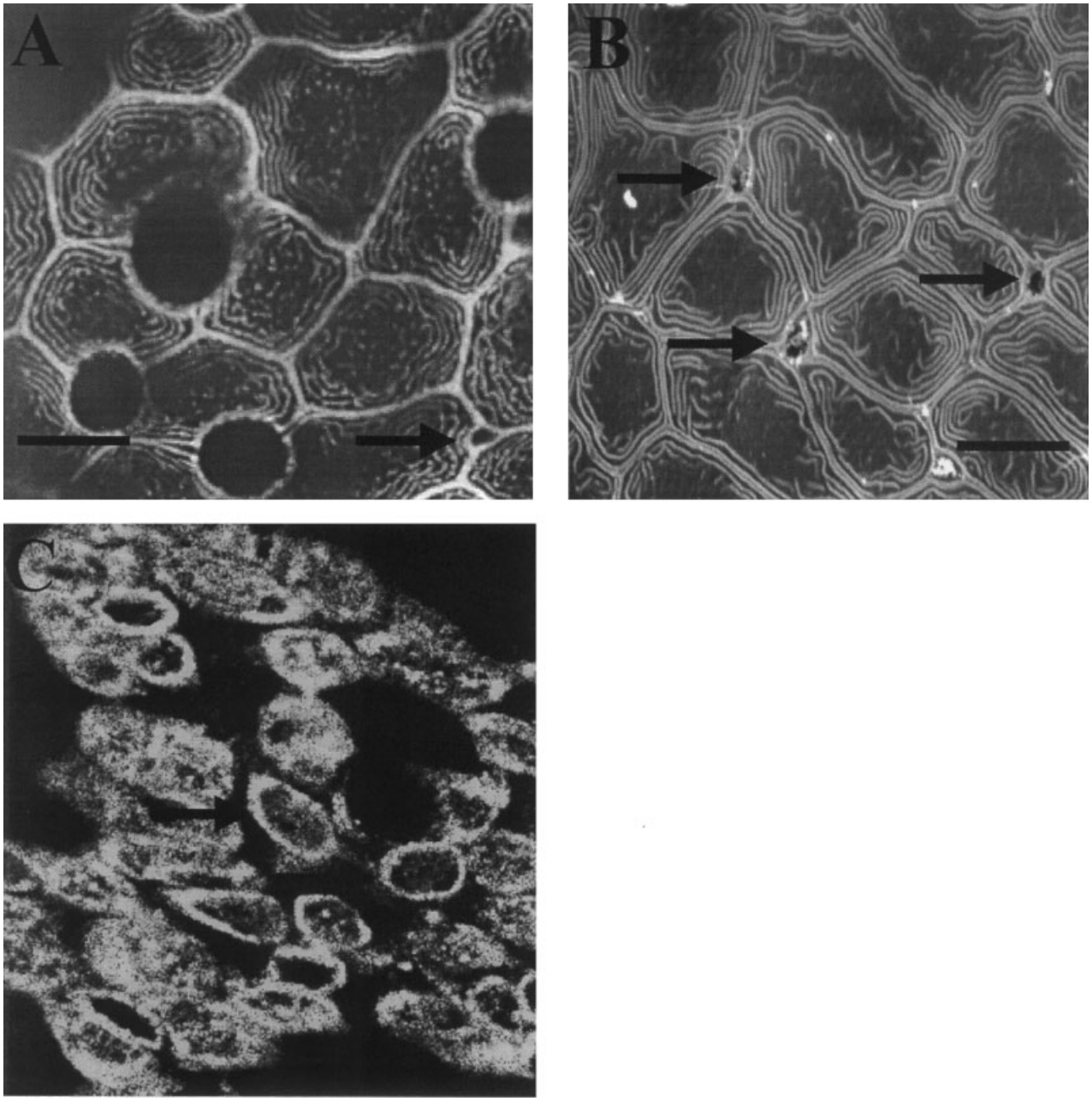

Fig. 4. Pavement cells overlie MR cells in killifish opercular epithelium. (**A**) Oregon Green phalloidin fluorescence at the plane of the pavement cells, showing the microridge pattern and polymerized actin within these microridges (bar = 10 μm; methods as per Daborn et al., 2001). Arrow indicates an apical crypt opening. The large round holes are not apical crypt openings, rather, they are artifacts of breakdown of intercellular junctions around some apical crypt openings after fixation. (**B**) Scanning electron microscope image of pavement cells, same magnification as (A), also showing the microridge pattern and apical crypt openings (arrows) (bar = 10 μm). (**C**) Immunofluorescence of CFTR (same antibodies as for Fig. 2A; methods as per Marshall et al., 2002) in the cytoplasm/basolateral membrane of pavement cells (arrows) 4.5 μm from the apical surface of opercular epithelium from freshwater-adapted killifish opercular epithelium. Pavement cell nuclei are negative for CFTR immunofluorescence (bar = 20 μm).

crypt openings locate the exposed apical membrane surface of MR cells. The microarchitecture of the apical crypts of chloride cells is highly variable and appears to be regulated. The depth of apical crypts increased in SW adapted killifish (Hossler et al., '85). The fractional surface area of MR cells varies with acid–base status (Goss et al., '98) in rainbow trout, to such an extent that the increased surface allotted to ion transport actually impeded gas exchange (Perry and Goss, '94; Goss et al., '98). The depth of the apical crypt increases with increasing salinity in tilapia (Lee et al., '96, 2000; Chang et al., 2001) such that ''wavy convex'' morphology typical of low NaCl FW contrasts the high-density of ''shallow basin'' morphology in low Ca^{2+} FW (Lee et al., '96; Chang et al., 2001) while SW adaptation produced ''deep hole'' apical crypts (Lee et al., 2000). Conversely, in armored catfish (*Hypostomus plecostomus*), distilled water produces deep crypts compared to normal tap water (Fernandes et al., '98), so extremes of salinity seem to induce reduced apical membrane exposure. Basolateral osmolality appears to be a major rapid regulator of ion transport rate by chloride cells, in that hyperosmolality stimulates (Zadunaisky et al., '95) and hypotonicity inhibits (Marshall et al., 2000) Cl^- secretion by opercular membranes from killifish. Transfer of euryhaline teleosts, mudskippers and killifish, to FW causes covering over of some chloride cells such that they are not exposed to the environment and are effectively removed from ion transport (Sakamoto et al., 2000; Daborn et al., 2001). This effect is reversible (Sakamoto et al., 2000), can be mimicked in vitro by basolateral hypotonic stress (Daborn et al., 2001), and is inhibited by cytochalasin D, an inhibitor of actin microfilament assembly (Daborn et al., 2001). A well-developed actin ring in the apical crypt suggests that it is the chloride cell that initiates the withdrawal from the surface (Daborn et al., 2001). From this recent evidence it is clear that chloride cells dynamically alter their exposure to the environment and the available membrane area for transport and are responding to osmotic and other cues. This is consistent with the early stage of FW acclimation when there is a rapid decrease in ion efflux from in vivo killifish transferred to FW (Wood and Marshall, '94). The possibly unique microenvironment created by the protected apical crypt is totally unexplored and could provide key information as to the local ion and pH gradients affecting transport.

Transfer to SW of euryhaline teleosts produces a changeover of transport mechanisms to secretion. SW transfer enhances de novo differentiation and turnover of MR cells (Uchida and Kaneko, '96) implying new cells are necessary for full acclimation, but clearly there is also ''retooling'' of existing cells. Within 12 hr of transfer killifish to SW, when the plasma sodium and osmolality are elevated, there is enhanced expression of CFTR (Singer et al., '98). CFTR up-regulation is followed by the generation of ion secretion by chloride cells in the opercular epithelium (and gill) and correction of plasma osmolality by 48 hr, while there was little change in Na^+,K^+-ATPase activity (Marshall et al., '99).

7. Cultured gill-like epithelia

Because of the architectural complexity of the gill, many types of transport experiments are not possible with this complicated system. Ion transport by simple flat membranes is more easily studied. The existence of flat membranes with chloride cells (opercular membrane and skin) provide good models of gill SW chloride cell function for ion transport, NaCl secretion and Ca^{2+} uptake (e.g., Karnaky et al., '76, '84; Degnan et al., '77; Marshall and Nishioka, '80; Karnaky, '91; McCormick et al., '92; Zadunaisky et al., '95; Marshall and Bryson, '98). Whole organ culture of tilapia opercular membranes has been used to show that cortisol in the medium was important in maintaining differentiated FW (and SW) chloride cells (McCormick, '90a). Efforts to develop a FW model of operation of MR cells using opercular epithelium have been more challenging. In killifish, FW opercular epithelia actively take up Na^+ and Cl^- in symmetrical saline and have non-agreement with the Ussing flux ratio in asymmetrical conditions (FW outside), but there is not a net flux in the uptake direction against gradients as steep as normally seen in vivo in FW (Marshall et al., '97). Trout skin and FW tilapia opercular membranes have few chloride cells and actively transport Ca^{2+} but not NaCl (McCormick et al., '92a; Marshall et al., '92).

The first teleost gill epithelial membranes that were cultured on permeable substrates from sea bass (*Dicentrarchus labrax*) produced confluent, vigorous primary epithelia that had high electrical resistance, low permeability and with obvious physiologically regulated NaCl secretion (Avella et al., '94, '99). The epithelia resemble confluent pavement cells rather than MR cells. These

cultures from marine teleosts produced patch clamp evidence for apical membrane CFTR-like anion channels (Duranton et al., '97) and for stretch-activated K^+ channels (Duranton et al., 2000). From these works, it is apparent that pavement cells may contribute to ion transport in SW gill and opercular epithelium. These successes with marine systems led also to similar approaches using FW teleosts.

Rainbow trout gill cells in primary culture on solid substrates have low permeability and little indication of active ion uptake (Wood and Pärt, '97; Wood et al., '98), but the cultures are vigorous and retain the ability to volume regulate (Leguen et al., 2001). Cortisol treatment of cultures is helpful in maintaining epithelial integrity (Kelly and Wood, 2001) but does not initiate active ion transport. A maxi-chloride channel has been identified (O'Donnell et al., 2001) that could be involved in Cl^- or HCO_3^- permeation, but the channel has a low open probability at normal membrane potentials. Essentially these initial cultures were similar to monolayers of pavement cells and, in asymmetrical conditions with FW on the mucosal side, generated transepithelial potentials similar to the transgill diffusion potentials seen in vivo. Cultures that are subsequently seeded with a concentrated fraction of MR cells over 2 days become heterologous mixtures of pavement cells and chloride cells similar to an opercular epithelium (Kelly et al., 2000) and with significant Na^+,K^+-ATPase activity. Thus far, culture conditions have not produced activation of ion uptake in these cultures (Fletcher et al., 2000). Cultured epithelial preparations do show particular promise in separating out the roles of specific cell types in ion and acid/base balance.

III. OSMOTIC PERMEABILITY

There are few recent studies that have measured osmotic permeability, but the old data seem adequate to describe the situation. The effectiveness of the SW gill to excrete salt with a minimum of accompanying water defines its success as an osmoregulatory organ, while in FW fish, low osmotic permeability would decrease osmotic water gain. Teleost gill (actually the total body surface in in vivo measurements) osmotic permeability is much lower, 0.16–1.29 μm/sec, than for representative amphibian skin (2.8–23.6 μm/sec) (Maetz, '74) and SW teleosts have lower osmotic permeability than do FW-acclimated specimens of the same species (e.g., 0.064 and 0.38 μm/sec for SW and FW yellow eels, respectively; Motais and Isaia, '72). Generally, amphibian abdominal skin absorbs water rapidly (it is the area used for absorbing water during terrestrial sorties), while SW teleosts osmotic permeability is extremely low, consistent with a function to secrete ions not water (Maetz, '74). As a result, water movement across the gills of fish can be largely ignored in hydromineral balance, and ion transport, especially in SW, occurs with a minimum of accompanying water.

IV. TWENTY-YEAR PROSPECTUS

A. Projected areas for growth

Comparative physiology has a strong philosophical base of evolutionary physiology that includes description of the variation in animals in adaptation of species to their habitats as well as observations on the process of evolution. The August Krogh principle, i.e., that for any given physiological question there exists the ideal model for that process, is core to comparative physiology (Somero, 2000) and it is unlikely to be abandoned. Thus a diversity of models will be applied to equally diverse questions. The discipline has also provided biomedical research community with many insights unattainable through the study of closely related (mammalian) model systems (for examples see Somero, 2000). With proven past value and forward momentum, successes are likely to be achieved so long as sufficient resources (human and monetary) are supplied to the endeavor. The recent tendency in health research to undervalue comparative animal research (as being too remote from health related mammalian models and concerns) needs to be opposed actively by evolutionary physiologists. Assuming sufficient resources then, scientific advances in gill ion transport will flourish.

Two extremes of dimension, extremely large and extremely small, will serve as foci for future research. At the molecular end, new techniques are arising almost daily, thus it is difficult to project where the new technology will take us. It is likely in a few years that we will be able to assay the relative or absolute expression of every gene of an animal in a single cell at any given time in an experiment. DNA microarrays that can assay transcription changes in upward of 6,000 open reading frames (ORF) at once are already in use (Xiang and Chen, 2000; Yale and Bohnert, 2001) and have been applied to the study of salt stress in

fungi, yeast, and plants (Bohnert et al., 2001). The euryhaline, eurythermic, and euryoxic teleost, the long-jawed mudsucker, has been studied by this method (5,376 ORF; Gracey et al., 2001) with the result that hypoxia invokes down-regulation of genes involved in cell growth and proliferation up-regulation of genes in oxygen transfer and glycolysis regulation. Equally large RNA microarrays are coming (Xiang and Chen, 2000; Cho and Campbell, 2000). The fine time resolution, on the order of minutes or seconds, will thus allow one to assess which transporter genes are transcribed and translated, post-processed, and trafficked to membrane rafts along with their diverse regulatory mechanisms, structural elements, and metabolic support. For now, however, microarray technology has provided us with hundreds of responsive genes for which no function is yet connected (Bohnert et al., 2001). Because of the emphasis on genetic basis of physiology, individual genetic variation in model organisms will be more noticeable and less desirable. Development of inbred fish strains with little (or no) genetic variability will be of large value. For instance, there are hermaphroditic tropical mangrove killifish (genus *Rivulus*) and clonal hybrids between *F. heteroclitus* and *F. diaphanus* (Dawley, '92).

At the other extreme, studies of animals in their environment will grow through application of sophisticated satellite and terrestrial based biotelemeters. Thus far, biotelemeters measure only a few variables (temperature, heart rate, and electromyogram), but these prototypes have provided important revelations, such as the fact that fish caught in a catch and release sports fishery require a full 16 hr to recover from the stress (Anderson et al., '98). Electromyogram and electrocardiogram data allow extrapolation to oxygen consumption and swimming speed in the wild (Thorstad et al., 2000) as well as cardiac output and indirect measurements of metabolic rate (Brodeur et al., 2001). Satellite-linked telemeters are large and their use is restricted to large animals (whales, bears, wolves, sharks, and seals) and one or two measured variables (e.g., time, position, depth; Goulet et al., 2001), but miniaturization will continue so that soon small animals will be monitored remotely. Soon also there will be multiple variable telemeters that will monitor environmental, tissue, cardiovascular, and blood characteristics in free-swimming wild fish will allow a new window on physiological ecology. In this way, the understanding of moment-to-moment decisions by wild animals in the environment can be revealed. Moving basic physiology to the field will have challenges, but the reward will be the immediate relevance to the biology of the animal in its environment.

B. Importance of integration.

The emergence of widely integrative studies from the molecule to the ecosystem will create a new type of freewheeling science. We may see an acceptance of this type of study, termed ''ecogenomics'' (Chapman, 2001), and the true representation of a complex animal in a multifaceted environment could produce a ''consilience'' (a jumping together of knowledge from widely different but related studies) for comparative physiology. Predicted also is a move beyond a few model species in new genome projects so that these studies could be applied to more species (Chapman and Almeida, 2001).

C. Conclusion

SW ion transport by MR cells has matured more rapidly than the more complicated FW situation in part because FW ion transport involves more cell types and more diverse transport mechanisms and also because SW is a constant acclimation medium worldwide, but FW composition varies widely, making interlaboratory scientific confirmation more difficult. In a geological sense, FW systems are all temporary, hence it is likely that FW osmoregulatory strategies evolved numerous times in diverse taxa, thus a single FW osmoregulatory strategy is unlikely. The future will be an exciting combination of expansion into molecular biology and functional genomics at one extreme and to physiological ecology at the other extreme. The challenge will be to maintain the ability to integrate across subdisciplines to obtain a clear image of the operation of fish gills as a whole.

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