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Ion Balance, Acid-Base Regulation, and Chloride Cell Function in the Common Killifish, *Fundulus heteroclitus*—A Euryhaline Estuarine Teleost

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ABSTRACT: The common killifish, *Fundulus heteroclitus*, is a euryhaline teleost common throughout estuaries of eastern North America. This symposium paper reviews the important contributions of the killifish to our present understanding of ionoregulation in seawater (SW) fish and their mechanisms of euryhalinity, and presents new data developing the killifish as a freshwater (FW) model system. Experiments on killifish have characterized (i) drinking in SW and its reduction in FW; (ii) the adaptive roles of the kidney to SW and FW conditions; (iii) the instantaneous (Phase I) and delayed (Phase II) reductions in Na^+ outflux that occur upon transfer from SW to FW; (iv) the importance of prolactin secretion in the Phase II effect; (v) the cortisol-stimulated induction of branchial Na^+,K^+-ATPase that occurs upon transfer from FW to SW; (vi) the accompanying changes in morphology of the mitochondria-rich (MR) or "chloride cells" on the gills; (vii) the localization of this Na^+,K^+-ATPase activity to the basolateral membrane of chloride cells; and (viii) the NaCl-secretory function of these cells in SW. The opercular epithelium, which is rich in chloride cells, has been used as an in vitro model to characterize the mechanisms and control of NaCl secretion in SW fish. Much less is known about gill function in fresh water (inward NaCl transport), primarily due to the absence of a comparable freshwater model. Here we show that killifish acclimated to dilute FW ([NaCl] = 1 mmol l^{-1}) possess large numbers of MR cells on the opercular epithelium. When mounted in vitro with FW on the outside, the preparation develops a large inside negative transepithelial potential (TEP) that is a Na^+ diffusion potential. By the Ussing flux ratio criterion, Na^+ fluxes are passive, but a small active influx of Cl^- occurs, an observation that supports the involvement of MR cells in active Cl uptake. This FW opercular epithelium if bathed with isotonic saline on both sides does not secrete Cl , indicating that the MR cells indeed are of the FW type. In vivo, the fish exhibits a high rate of Na^+ influx and outflux; Cl^- outflux is much lower, and there is no detectable Cl influx. Experimental variation of FW [NaCl] reveals a saturable, low affinity Na^+ uptake mechanism, a Cl influx mechanism that is activated only at much higher concentrations, and no evidence of exchange diffusion. Acid-base disturbance appears to be corrected by differential regulation of the outflux components only. Hence, the FW killifish ionoregulates somewhat differently from the few other FW teleosts that have been examined, and its opercular epithelium will serve as a very useful model system.

Introduction

NATURAL HISTORY AND EURYHALINITY

The common killifish or mummichog, *Fundulus heteroclitus*, lives in tidal marshes and estuaries along the eastern coast of North America from Texas to Newfoundland and the Gulf of St. Lawrence (Scott and Scott 1988). The species is extremely abundant and plays an important role as both predator and prey in the trophic dynamics of salt-marsh ecosystems (Kneib 1986; Dunson and Travis 1994). It is most commonly found in regions where the salinity fluctuates rapidly with the tidal cycle, but Griffith (1974) has shown that the killifish is capable of adapting to any constant salinity from 0‰ to 120‰ (almost 4-fold seawater!). Spawning generally occurs in the intertidal zone (i.e., in brackish water or seawater). The eggs are exposed to air for much of their incubation period but hatch only during periods of immersion by

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high tide (Taylor et al. 1977; DiMichele and Taylor 1980). However, neither saline water nor air emersion of the eggs are a prerequisite for successful reproduction (Atz 1986); landlocked, reproducing populations of *Fundulus heteroclitus* have been documented in freshwater lakes and streams (Denoncourt et al. 1978; Samaritan and Schmidt 1982). As with adults, newly hatched killifish are fully capable of ionoregulating in fresh water, seawater, or water of any salinity in between (Atz 1986). The species therefore demonstrates a remarkable degree of euryhalinity in all aspects of its natural history.

The Killifish as an Experimental Model

The killifish's euryhalinity, together with its abundance in the wild, ease of collection and maintenance in the laboratory, and general hardiness, has made the killifish a popular experimental animal for physiologists interested in ionoregulation. Indeed, *Fundulus heteroclitus* (together with a few congeners) has been the single most important species contributing to our current understanding of salt transport in the gills of seawater fish. However, for reasons to be outlined below, the killifish has contributed rather less to our understanding of ionoregulation in freshwater fish. We list below some of the landmark ionoregulatory discoveries that have been based on work with *Fundulus*. This list is not meant to be exhaustive; for example, we have omitted the immense importance of the killifish to our present understanding of Ca^{2+} homeostasis in teleost fish (e.g., Pang and Pang 1986). Our goal is to illustrate the remarkable contributions of this single species to one area of environmental and cellular physiology: NaCl homeostasis in fish.

(i) Potts and Evans (1967) demonstrated that *Fundulus heteroclitus* drinks a significant amount of the external medium when adapted to seawater, and that drinking rate is markedly reduced in fresh water. This was the first experimental confirmation of Smith's (1930) classic observation that a seawater teleost really does "drink like a fish." Today we recognize seawater drinking as the sole route of compensation for osmotic water loss across the gills and obligatory urinary water loss. As water and NaCl must be absorbed together through the gut, drinking is also the major route of *net* NaCl uptake by the seawater fish. This intestinal loading necessitates active NaCl excretion through the gills, described below.

(ii) Experiments by Stanley and Fleming (1964, 1966) and Fleming and Stanley (1965) on the plains killifish (*Fundulus kansae*) revealed dramatic changes in renal function upon transfer from fresh water to seawater. These included large reductions in glomerular filtration and increases in tubular reabsorption, which achieve >90% reductions in urine flow rate. The efficiency of this renal water conservation is emphasized by the fact that the killifish is the only teleost known to produce a urine *hypertonic* to the blood plasma during the period of seawater adaptation. Urinary Na^+ loss is small but significant in fresh water (about 20% of branchial outflux), but exhibits little quantitative change upon adaptation to seawater. Renal Na^+ excretion therefore makes little contribution to net salt excretion in seawater. Greater tubular Na^+ transport in fresh water that in seawater is supported by the finding of much higher Na^+,K^+-ATPase activities in the kidneys of *Fundulus heteroclitus* acclimated to fresh water (Epstein et al. 1969). The kidney changes from a Na^+-conserving, water-excreting role in hypotonic environments to a water-conserving role in hypertonic environments.

(iii) In a survey of four euryhaline teleosts (including *Fundulus heteroclitus*) and six stenohaline teleost species in seawater, Motais et al. (1966) found that all had high rates of Na^+ influx (J_{in}) and outflux (J_{out}) across the gills, amounting to 30–100% of the internal exchangeable Na^+ pool per hour. The excess of J_{out} over J_{in} accounted for the net excretion of Na^+ (negative J_{net}) across the gills referred to in (i). Upon transfer to fresh water, all species showed a dramatic and instantaneous reduction in J_{in}, but only the euryhaline forms were able to successfully reduce J_{out} by a comparable amount so as to restore Na^+ balance. Further work by Motais et al. (1966), Potts and Evans (1967), and Maetz et al. (1967a, b) demonstrated that this reduction in J_{out} was a two-stage process (Fig. 1). Phase I was an instantaneous reduction that appeared to be directly coupled to the fall in J_{in} ("Na^+-free" or "exchange diffusion" effect). Because in the early experiments the transgill electrical potential (TGP) was not measured, it was later recognized that the Phase I reduction in Na^+ outflux was at least in part due to an inversion of the TGP rather than exchange diffusion (e.g., Potts and Eddy 1973). The Cl^- outflux undergoes a similar reduction (Motais et al. 1966; Pic 1978), a point that cannot be explained on the basis of the change in electrical gradient. More recent analyses based on in vitro experiments with isolated epithelial preparations (see below) suggest that neither explanation is accurate, and that a third mechanism is responsible. Phase II was a slower progressive reduction seen after 30 min and reaching completion in 24–48 h (Fig. 1). Stenohaline forms lacked this delayed component. The time course of the latter, plus the fact that it was not readily reversed upon transfer back to seawater, suggested an endocrine mechanism. The strategy of *Fundulus heteroclitus* (Fig. 1A) was rather differ-

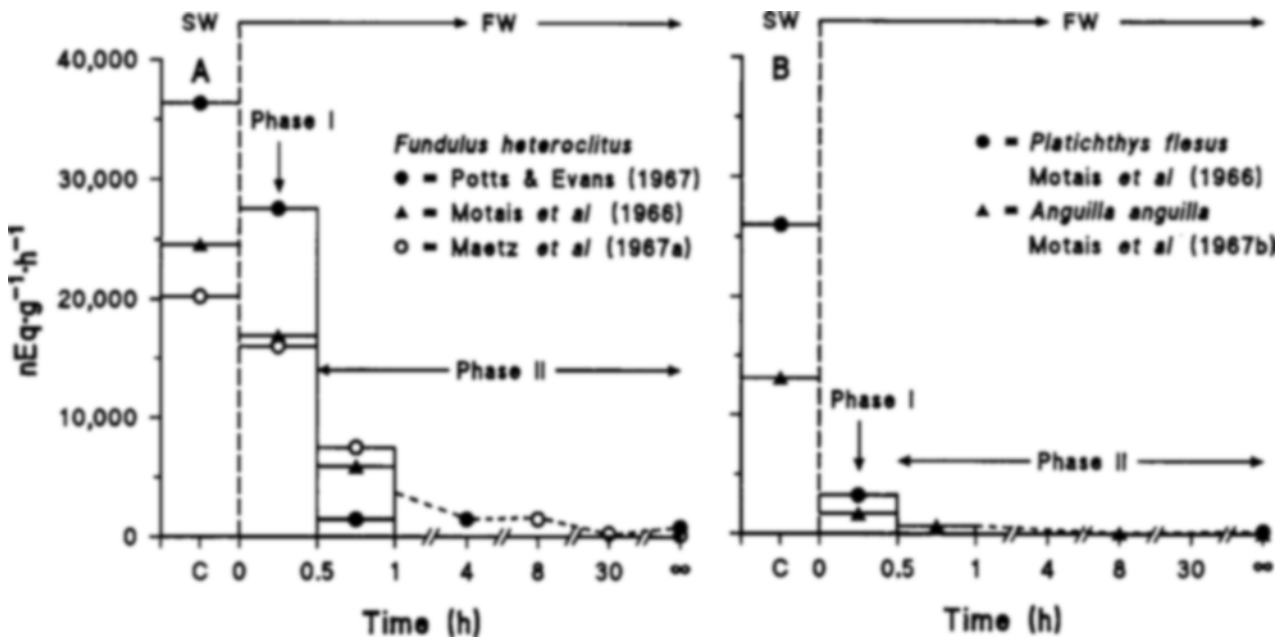

Fig. 1. Changes over time in Na^+ outflux (J_{out}) following transfer from seawater to fresh water in three freely euryhaline teleosts. (A) *Fundulus heteroclitus* (data recalculated from studies by Motais et al. 1966 [▲], Potts and Evans 1967 [●], and Maetz et al. 1967a [○]). (B) *Platichthys flesus* (from Motais et al. 1966 [●]) and *Anguilla anguilla* (from Maetz et al. 1967b [▲]). Phase I is an instantaneous reduction in J_{out} coupled to the reduction in external Na^+ concentration and J_{in}; Phase II is a slower and progressive reduction in J_{out} of apparent endocrine origin. Note the much smaller Phase I and larger Phase II components in *Fundulus* relative to *Platichthys* or *Anguilla*.

ent from that of the equally euryhaline flounder (*Platichthys flesus*) and eel (*Anguilla anguilla*) (Fig. 1B), with only a small instantaneous reduction (Phase I was 15–30% versus 85–90% in eel and flounder), and a much larger delayed Phase II component (70–85% versus 10–15%). This explains why the killifish suffers large reductions in internal Na^+ and Cl^- levels in the first few hours after transfer, even though steady state concentrations are very similar in fresh water and seawater once full adaptation has occurred. Later work by Pic (1978) suggested that the Phase I reduction is larger in killifish if particular care is taken to eliminate "stress" effects.

(iv) Burden (1956) demonstrated that hypophysectomy removed the ability of *Fundulus heteroclitus* to adapt to and ultimately survive in fresh water, while Pickford and Phillips (1959) showed that replacement therapy with ovine prolactin restored this ability. The protective effect of prolactin relates specifically to the control of internal Na^+, Cl^-, and osmotic pressure (Pickford et al. 1966); hypophysectomized fish die because they cannot maintain blood ion levels. Potts and Evans (1966), Maetz et al. (1967a), and Potts and Fleming (1971) presented evidence that increased secretion of prolactin was specifically responsible for the secondary, delayed reduction in branchial Na^+ outflux (i.e., Phase II; Fig. 1A) that occurs when *Fundulus* are transferred to fresh water. Effects of prolactin on Na^+ influx in fresh water are negligible. The mechanism of prolactin action in causing Phase II remains unknown, but reduction of permeability, increased mucous secretion (Burden 1956; Pickford et al. 1966), Ca^{2+}-binding (Potts and Fleming 1971), structural changes in the tight junctions (Ernst et al. 1980), and direct inhibitory effects on both cellular and paracellular conductances in the gills (Foskett et al. 1982, 1983) are all likely components of the response. Prolactin may also play a role in renal Na^+ conservation (Stanley and Fleming 1966, 1967) by stimulating Na^+,K^+-ATPase activity in the kidney (Pickford et al. 1970a). The "Na^+-retaining" test in hypophysectomized killifish transferred to fresh water has now been developed as a bioassay for prolactin-like principles in fish (Grau et al. 1984; Hasegawa et al. 1986).

(v) Epstein et al. (1967) showed that microsomal Na^+,K^+-ATPase activity is much higher in the gills of *Fundulus heteroclitus* in seawater than in fresh water. This was an important piece of evidence leading to the "Silva model" (Silva et al. 1977) for NaCl transport across the seawater gill (see Fig. 2). This observation was subsequently duplicated in a variety of other euryhaline species, and cortisol was shown to play an important role in the induction

of this activity (e.g., Pickford et al. 1970b; Forrest et al. 1973a, b). A decrease in the freshwater adaptive hormone prolactin (see iv above) during seawater adaptation may also be involved; Pickford et al. (1970a) found that prolactin therapy depressed gill Na^+,K^+-ATPase activity in hypophysectomized killifish. The change in enzyme activity occurs rapidly (within several days), though Jacob and Taylor (1983) were unable to confirm the finding of Towle et al. (1977) that Na^+,K^+-ATPase induction is complete within 0.5 h after transfer from fresh water to seawater!

(vi) Another key finding leading to the current model (Fig. 2) was the demonstration by Karnaky et al. (1976), using [^{3}H]ouabain-binding autoradiography, that the Na^+,K^+-ATPase activity induced in the gills of seawater *Fundulus heteroclitus* is located in the "chloride cells." The function(s) of these mitochondria-rich cells, originally identified as the putative sites ("chloride-secreting cells") of NaCl excretion by Keys and Willmer (1932), had long been the subject of controversy. Indeed, Bevelander (1935, 1936), Doyle and Gorecki (1961), and Fleming and Kamemoto (1963) had concluded (erroneously) that these cells were not involved in NaCl transport in either *Fundulus heteroclitus* or *Fundulus kansae*. On the other hand, Copeland (1948, 1950), Pettengill and Copeland (1948), Philpott and Copeland (1963), and Philpott (1965) identified the chloride cells as the sites of salt excretion in seawater killifish, and salt absorption in freshwater killifish. This conclusion was based on profound changes in cellular morphology and histochemistry in response to salinity variation and enteric NaCl loading in *Fundulus heteroclitus*, and differences between *Fundulus similis* (a marine species) and *Fundulus chrysotus* (a freshwater species). In particular, apical crypts which stained positively for Cl were present in the chloride cells of seawater killifish but absent in freshwater killifish. In addition, early ultrastructural work (reviewed by Philpott 1980) suggested that the chloride cells possessed not only the numerous mitochondria typical of transport cells, but also an extensive "smooth endoplasmic reticulum" throughout the cytoplasm. However, Philpott (1966) and Ritch and Philpott (1969) were able to show that this was not endoplasmic reticulum at all, but rather an extensive network of tubules that were contiguous with the basolateral plasma membrane.

(vii) Karnaky et al. (1976) also made the critical observation that Na^+,K^+-ATPase is located both on this network of internal tubules as well as on the basolateral membranes (i.e., on the interstitial fluid, blood side). The enzyme is not located on the apical membranes (i.e., water side) of killifish chloride cells. Based on both [^{3}H]ouabain autoradiography and measurements of enzyme inhibition, ouabain was effective only when applied from the blood side. As ouabain binds close to the K^+ site on the enzyme, this implied that Na^+,K^+-ATPase pumps Na^+ from the chloride cell into the interstitial fluid, an apparent paradox for a seawater fish where the net direction of NaCl transport is from interstitial fluid to water. Earlier work by Maetz's group had suggested an apical, Na^+-extruding location for the enzyme (Maetz 1971), an appealing idea but one that seemed unlikely because large amounts of K^+ uptake from seawater would be required to counter Na^+ secretion. It is now clear that Karnaky's observation was correct (e.g., Hootman and Philpott 1974; Ernst et al. 1980), and the basolateral location of the major ion pump became a key element in the model of secondary active Cl secretion by many epithelia (Frizzell et al. 1979; Fig. 2). The "Maetz model" has now been generally abandoned.

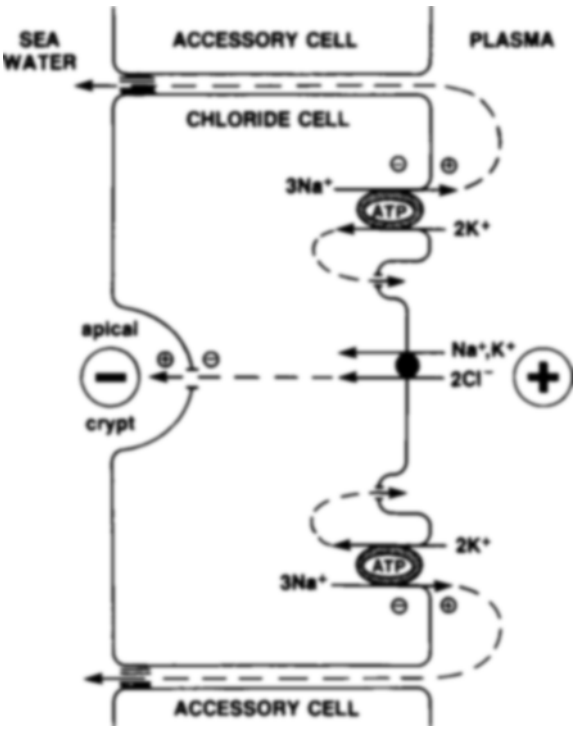

Fig. 2. The current model for NaCl transport through the chloride cells of the gills of a seawater fish, based on the original proposal of Silva et al. (1977) and confirming evidence from experiments with isolated opercular epithelia of *Fundulus heteroclitus* and analogous preparations. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines.

(viii) While the above observations on intact killifish and their excised gills were of immense value, there remained a pressing need for an in vitro model system. The complex geometry and blood

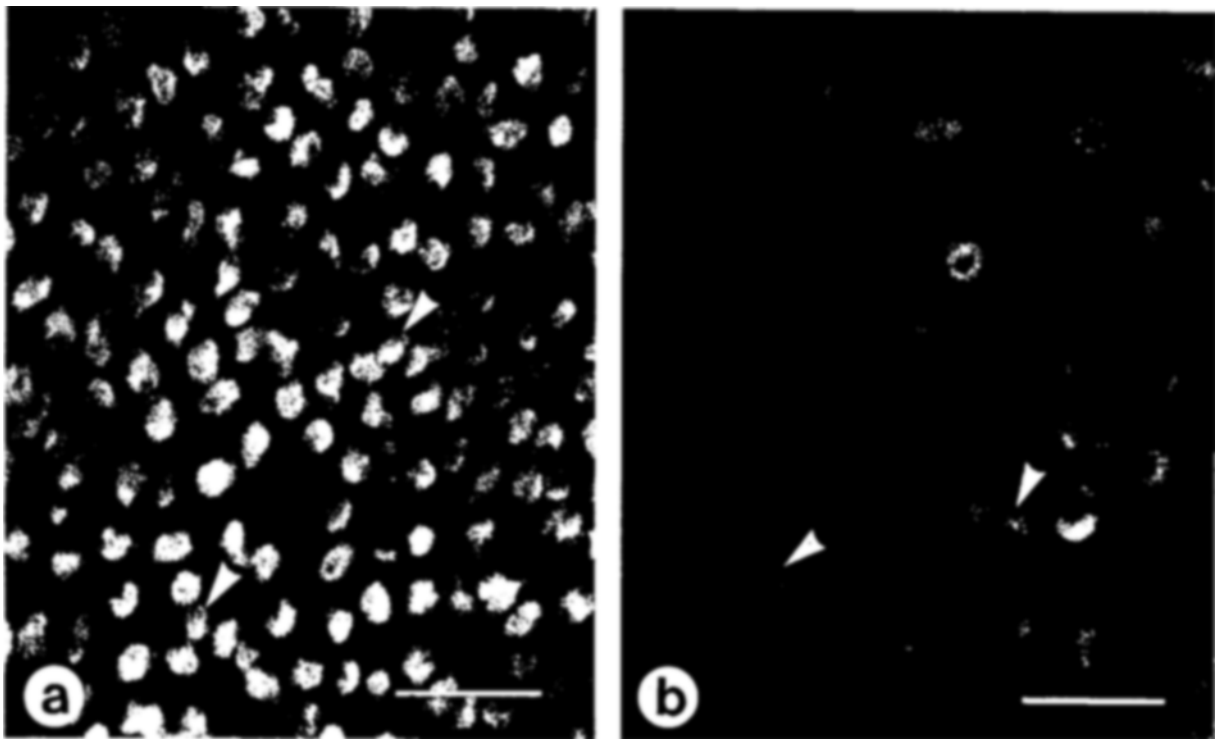

Fig. 3. Pictures of DASPEI-stained (30 min. in 10 μM DASPEI in oxygenated saline) opercular epithelia from (a) seawater-adapted and (b) freshwater-adapted *Fundulus heteroclitus*. In both salinities there are large numbers of mitochondria-rich (MR) cells and obvious cell groups (arrow). The MR cells in fresh water are larger than the corresponding cells in seawater, but there was no apparent difference in fluorescence intensity. Bars are 50 μm.

supply of the gills, the difficulties of rigorous electrical and thermodynamic analyses, and the complicating effects of stress in vivo were all impediments to further detailed understanding of the transport system. However, with the advent of epithelial preparations that could be mounted as flat sheets in vitro in Ussing chambers (Karnaky et al. 1977; Marshall 1977), many of these obstructions were overcome. In at least two marine species, the estuarine goby *Gillichthys mirabilis* (Marshall 1981a) and the intertidal shanny *Blennius pholis* (Nonnotte et al. 1979), the skin accounts for 20–50% of total body NaCl secretion and thus the skin is an important accessory ionoregulatory organ to the gill.

Burns and Copeland (1950) reported that the buccal epithelium and the skin lining the opercular bone (the "opercular epithelium") of seawater-adapted *Fundulus heteroclitus* was rich in chloride cells (Fig. 3). Karnaky and Kinter (1977) noted that these extrabranchial chloride cells were identical in ultrastructure to those of the gills, but far more numerous (30–70% versus <6% of total cell numbers); other cell types (mucous, pavement, and accessory) were also very similar to those in gills. The need for an in vitro model was therefore answered when, independently, Karnaky et al. (1977) using killifish opercular epithelium and Marshall (1977) using *Gillichthys* skin were able to dissect these epithelia, mount them in vitro in Ussing-style membrane chambers with isotonic saline (we use "saline" here to mean a modified Ringer's or Cortland's balanced saline solution that is a close mimic to plasma) on each side, and demonstrate NaCl transport from serosal (blood) to mucosal (water) surfaces. Over the past 15 years, the intensive use of these preparations and the opercular epithelium of the seawater-resident euryhaline tilapia *Oreochromis mossambicus* (e.g., Foskett et al. 1981) has allowed confirmation and elaboration of detail for the "Silva model" of NaCl transport across the seawater gill (Fig. 2). Without doubt, these preparations are the reason why our knowledge of seawater gill function has advanced so far ahead of understanding of freshwater gill function.

Epithelial Preparations as a Model for the Seawater Gill

Chloride cells can be readily identified in situ by the mitochondrial stain dimethylaminostyrylethylpyridinium iodide (DASPEI), a fluorophore that is purportedly specific for active mitochondria (Be-

reiter-Hahn 1976). The seawater opercular epithelium contains a high density of these cells (see Fig. 3), and the number of cells per mm^2 varies from preparation to preparation and from species to species. *Gillichthys* opercular epithelium and skin contains about 500–1,000 cells mm^{-2} (Marshall and Nishioka 1980), while *Fundulus* opercular epithelium has both a higher ion transport rate and a greater density of chloride cells (2,100–5,000 cells mm^{-2}, Karnaky et al. 1984). The killifish preparation mounted in vitro develops a transepithelial potential (TEP) of about +20 mV relative to mucosal saline, due almost entirely to the electrogenic transport of Cl^- (Péqueux et al. 1988). The TEP increases to +35 mV to +40 mV relative to mucosal seawater due to the Cl^- transport and a superimposed Na^+ diffusion potential. These potentials are about twice as large as in vivo measurements for intact killifish under comparable conditions (Pic 1978). Apparently the large surface area of the gill creates a low resistance shunt in vivo; *Fundulus* yolk sac embryos in seawater lack extensive gills and have a TEP of +35 mV to +40 mV (Guggino 1980) similar to that measured for the isolated opercular epithelium. If pH and P_{CO_2} are carefully controlled, net Cl secretion rates, unstimulated by hormones or neurotransmitters, can reach 1,550 nmol cm^{-2} h^{-1} under in vitro conditions that mimic in vivo seawater conditions. These conditions include saline on the inside, 500 mmol l^{-1} NaCl on the outside, and the preparation at open circuit (Péqueux et al. 1988), so Cl transport is occurring against strong electrochemical gradients. Hence, it is clear that the opercular epithelium contributes to the secretion of Cl^- by the whole animal in seawater, and possibly also in hypersaline conditions where these animals are often found.

Under short-circuit conditions—saline bathing both surfaces and the TEP clamped to zero—the epithelium actively secretes Cl^- at a high rate, about 4,000 nmol cm^{-2} h^{-1} (Karnaky et al. 1977; Péqueux et al. 1988). Net Cl extrusion is equivalent to the short-circuit current, which in turn is directly proportional to the number of chloride cells in the preparation (Marshall and Nishioka 1980; Karnaky et al. 1984). There is no net transport of Na^+. In contrast, under open-circuit conditions, equimolar net fluxes of Na^+ and Cl occur from mucosa to serosa; thus, Cl is transported actively, and Na^+ follows passively down its electrochemical gradient.

These experiments and others led to the model that is illustrated in Fig. 2. This model shares many characteristics with "secondary active Cl transport" in other salt-secreting epithelia such as mammalian airway and corneal epithelia and avian and elasmobranch salt glands (Frizzell et al. 1979). Only key points are summarized here based on a synthesis of many papers (e.g., Degnan et al. 1977; Karnaky et al. 1977; Marshall 1977, 1981a, b; Degnan and Zadunaisky 1979, 1980a, b; Marshall and Bern 1979, 1980; Ernst et al. 1980; Marshall and Nishioka 1980; Mayer-Gostan and Maetz 1980; Foskett et al. 1981, 1982, 1983; Foskett and Scheffey 1982; Foskett and Machen 1985; Degnan 1984, 1985; Eriksson et al. 1985). The reader is referred to reviews by Evans et al. (1982), Zadunaisky (1984), Karnaky (1980, 1986), and Péqueux et al. (1988) for additional details. The model arises from simultaneous work by Klyce and Wong (1977) on mammalian cornea and by Silva et al. (1977) on teleost gill. The model as applied to chloride cells has been reviewed extensively (cf. Zadunaisky 1984; Karnaky 1986; Péqueux et al. 1988) and is now widely accepted to be in some respects similar to that for other Cl^--secreting tissues, notably mammalian airway epithelium (e.g., Welsh and Liedke 1986) and corneal epithelium (Klyce and Crosson 1985).

Briefly, the transport enzyme Na^+,K^+-ATPase (inhibitable by ouabain on the basal side) is restricted to the basolateral membrane and the contiguous tubular system *only*, and this ion pump creates and maintains a Na^+ electrochemical gradient that strongly favours Na^+ entry into the cell on the basal side (Fig. 2). A cotransport mechanism that is inhibited by "loop" diuretics (furosemide and bumetanide on the basal side) carries Na^+ into the cell down its electrochemical gradient coupled with the uphill transmembrane transport of Cl^- and probably also K^+. In this way, Cl^- apparently accumulates above its electrochemical equilibrium in the cell to an extent sufficient that Cl^- conductive elements (channels) in the apical membrane allow Cl^- to leave the cell passively. The TEP of +35 mV to +40 mV is the combined result of the electrogenic Cl transport and a cation-selective paracellular shunt pathway. This TEP is large enough to carry Na^+ passively down its electrochemical gradient between the cells and into the seawater. Hence both Na^+ and Cl^- are secreted in equal amounts to satisfy the law of electroneutrality. Pivotal to this understanding of the operation of chloride cells were two discoveries—first the recognition of "leaky" junctions between chloride cells and accessory cells (Sardet et al. 1979; Hootman and Philpott 1980) that provided anatomic evidence for the paracellular secretion of Na^+. The second was the discovery that the Cl^- current was restricted locally in the epithelium to the apical crypts of the chloride cells as measured directly by the "vibrating probe" technique (Foskett and Scheffey 1982; Scheffey et al. 1983; Foskett and Ma-

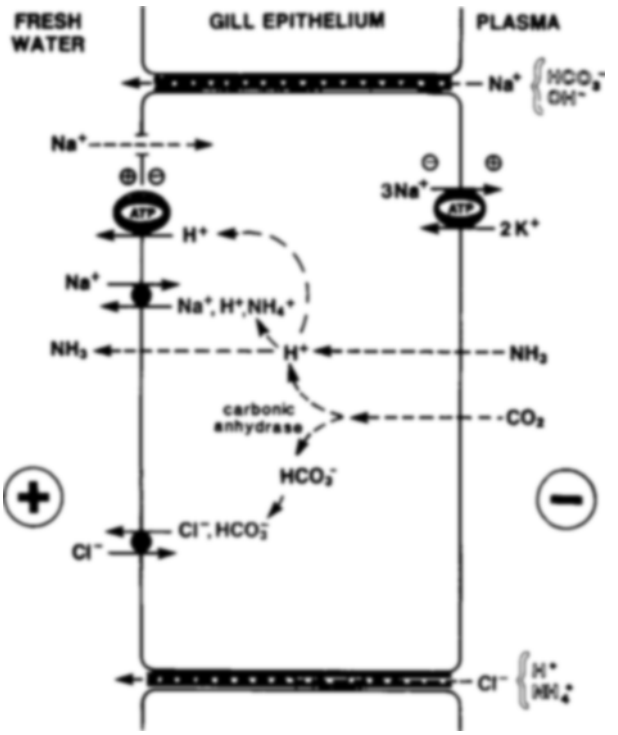

Fig. 4. Current ideas on the mechanism(s) for Na^+ and Cl uptake through the gills of a freshwater fish, based on experiments with intact fish and perfused gill preparations. The cell types involved have not been conclusively identified, hence they are not labelled. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines. See text for details. Paracellular movements of HCO_3^-, OH^-, H^+, and NH_4^+ (acid-base equivalents) are shown in white to indicate that they may be dependent variables constrained by differential strong cation (Na^+) and anion (Cl^-) outfluxes.

chen 1985). The original nomenclature of Keys and Willmer (1932)—"chloride-secreting cells"—was clearly correct. Major gaps in the model that still need to be filled are the identification and characterization of the anion channels involved at the apical membrane, actual measurements of the intracellular Cl^- activity and electrical potential, and the K^+ dependency of the basolateral cotransporter.

One drawback of the present model is that it does not include an acid-base component, in contrast to current freshwater models (Fig. 4) and the earlier "Maetz model" for the seawater gill (Maetz 1971). The gills are clearly the major sites of acid-base regulation in both freshwater and seawater fish (e.g., Heisler 1982, 1984). In vivo experiments by Evans and co-workers (summarized by Evans et al. 1982) have indicated the presence of Na^+/acid and Cl^-/base exchange mechanisms in the gills of several seawater species. It could be argued that these are not on the chloride cell and, therefore, would not be seen in isolated opercular epithelium experiments. However, Cl^- transport in the preparation is extremely sensitive to serosal acid-base status, and the anion channels involved in Cl^- transport in other epithelia are known to transport HCO_3^- as well as Cl^- (Tabcharani et al. 1990). To date there is no direct evidence of Na^+/acid or Cl^-/base exchange (Degnan et al. 1977; Karnaky et al. 1977; Zadunaisky 1984; Péqueux et al. 1988). The enzyme carbonic anhydrase, which plays a major role in ion and acid-base linkage in freshwater gills (cf. Fig. 4), is abundant in the chloride cells of seawater fish (Lacy 1983), but its role remains unknown. There is clearly a need for more work in this area.

Epithelial Preparations as a Model for Mechanisms of Euryhalinity

Usually overlooked is the fact that the epithelial preparations have been most commonly used with isotonic saline on both serosal and mucosal surfaces, a condition which mimics the gill in brackish water ([NaCl] $\simeq$ 140 mmol l^{-1} or 28% seawater) rather than the gill in seawater ([NaCl] $\simeq$ 500 mmol l^{-1}). On a qualitative basis, the preparation seems to behave in a similar manner to that when seawater is on the mucosal surface, although, of course, the contribution of the Na^+ diffusion potential to the TEP is eliminated. Unidirectional fluxes of both Na^+ and Cl decrease when mucosal salinity is lowered; there effects probably correspond to the instantaneous Phase I effects seen in vivo (Fig. 1). In the opercular epithelium, no evidence exists for the involvement of exchange diffusion, and the phenomena are commonly ascribed to a direct regulatory effect of external salinity on the conductances of the apical Cl^- channels and the paracellular pathway for Na^+ (Degnan and Zadunaisky 1980b; Marshall 1981a; Degnan 1984; Zadunaisky 1984). There are well-known voltage dependencies of apical Cl^- channels in Cl -secreting epithelia (e.g., in the corneal epithelium, Marshall and Hanrahan 1991) and instantaneous increases in paracellular ion conductance with increasing mucosal hypertonicity (e.g., in the toad urinary bladder, Finn and Bright 1978).

Injections of prolactin markedly reduce NaCl secretion in the opercular epithelia of seawater fish (Foskett et al. 1982), a finding that supports the idea that increased secretion of this hormone is responsible for the Phase II reduction seen in vivo (Fig. 1). As with reduced salinity itself, the effects of prolactin appear to be exerted on both the cellular and paracellular conductances. Both the active Cl pumping mechanism and the passive Na^+ shunt are shut down as the euryhaline fish moves from seawater to fresh water. Over several days,

prolactin may cause a dedifferentiation of the chloride cells (Foskett et al. 1983).

Foskett et al. (1981) studied changes in opercular epithelial properties of tilapia undergoing the opposite transition, from fresh water to seawater. Within 24 h of transfer, unidirectional and net secretory fluxes of Cl^- and total conductance across the epithelium (under short-circuit conditions) all increased, probably due to a reversal of the Phase II mechanisms discussed above. Chloride cell numbers increased over the first few days, followed by later increases in chloride cell size and internal complexity (i.e., differentiation). Full establishment of the secretory Cl^- current took 1–2 wk. Increased cortisol secretion may have played a role. Direct application of cortisol in vitro to epithelial membranes from freshwater tilapia maintained for several days in organ culture (i.e., isotonic salinity, not fresh water) increased their Na^+,K^+-ATPase activity (McCormick 1991). However, cortisol pretreatment of freshwater fish *maintained in fresh water* increased only the numbers of chloride cells on the opercular epithelium, not their size, internal structure, or Cl^- secretion rate in vitro (Foskett et al. 1981, 1983). Direct effects of salinity and reduction of prolactin secretion, in addition to increased cortisol, would appear necessary to cause full differentiation of the seawater function of epithelial chloride cells.

Epithelial Preparations as a Model for the Freshwater Gill

A number of authors (Karnaky and Kinter 1977; Evans et al. 1982; Zadunaisky 1984) have advocated using epithelial preparations from freshwater-adapted euryhaline teleosts as surrogate models for the gill in fresh water, but results to date have been disappointing. The common approach has been to mount these epithelia under short-circuit conditions—saline on both mucosal and serosal surfaces; the common finding has been an absence of net Cl^- absorption (Marshall 1977; Degnan et al. 1977; Foskett et al. 1981; Karnaky 1986). Either there is no net Cl^- transport, or else evidence of net transport from serosa to mucosa (as would occur in seawater!). We are aware of no published studies with freshwater opercular epithelia that have used fresh water in the mucosal bath to examine Na^+ and Cl^- transports. It is this absence of a model epithelium that has been the single biggest impediment to understanding the mechanisms(s) of Na^+ and Cl^- absorption in the freshwater gill.

The Current Model(s) for the Freshwater Gill

Our present understanding of freshwater gill function is both controversial and incomplete. Current model(s) (Fig. 4) are based entirely on flux measurements in intact fish and perfused gill preparations, most commonly the euryhaline salmonids (*Oncorhynchus, Salmo,* and *Salvelinus* sp.), and the stenohaline goldfish (*Carassius auratus*), channel catfish (*Ictalurus punctatus*), and European carp (*Cyprinus carpio*). To date, the killifish has contributed nothing to these models. The following account deals only with major points; the reader is referred to recent reviews by Péqueux et al. (1988), McDonald et al. (1989), Wood (1991), Goss and Wood (1991), and Goss et al. (1992) for additional detail and supporting references.

The uptake of Na^+ and Cl^- from the dilute external media has been traditionally viewed as active, independent processes mediated by electroneutral exchange carriers on the apical membranes of the transport cells. Na^+ is exchanged on a 1-for-1 basis for H^+ or NH_4^+ and Cl^- for HCO_3^-. These carriers are also thought to mediate a variable element of exchange diffusion for both Na^+ and Cl^-. The acidic (H^+) and basic (HCO_3^-) counter ions are provided by intracellular carbonic anhydrase, which catalyzes the hydration of respiratory CO_2. H^+ will tend to trap neutral NH_3 to form NH_4^+, which is also an acidic equivalent. A recently proposed alternate mechanism of Na^+ uptake (Avella and Bornancin 1989; Lin and Randall 1991), illustrated in Fig. 3, is based on analogy to the frog skin (Ehrenfeld et al. 1985). By this view, an active electrogenic extrusion of H^+ ions across the apical membrane by H^+-ATPase creates an electrochemical gradient for the entry of Na^+ through separate Na^+-selective channels. Whichever view eventually proves correct, both provide a 1:1 coupling of Na^+ uptake to acid excretion and Cl^- uptake to base excretion. Therefore differential alteration of the rates of Na^+/acid versus Cl^-/base exchange will alter the acid-base status of the fish. A large number of experimental studies support this concept. Selective inhibition of Na^+ or Cl^- uptake causes systemic acidosis or alkalosis respectively; conversely, the correction of induced acidosis is accompanied by increased Na^+ and decreased Cl^- uptake, while the correction of induced alkalosis is accompanied by exactly opposite changes.

Modulation of the rates of diffusive outflux of Na^+ versus Cl^- may provide another mechanism linking ion and acid-base balance. This is a new and somewhat controversial concept. However, recent studies of trout suggest that diffusive outflux adjustment accounts for 35–50% of the acid-base compensation during induced alkalosis (Goss et al. 1992a). Strong Ion Difference Theory (Stewart 1983), as well as the constraints of electroneutrality, dictate that any excess of strong cation (Na^+)

loss over strong anion (Cl^-) loss will constrain a net loss of base, while the reverse will constrain a net loss of acid. The major route of diffusive outflux for both Na^+ and Cl^- is thought to be the paracellular channels; both extracellular pH and environmental pH may modulate the cation versus anion selectivity of these channels. Possible mechanisms include a titration of net charge and/or a change in Ca^{2+} binding on the channels. Regardless of the acceptance of this idea, it is generally agreed that freshwater fish exhibit a TGP of 0 mV to −20 mV relative to the external medium, and that this is a diffusion potential caused by the greater permeability of paracellular pathways to Na^+ than to Cl^-. Ca^{2+} appears to play a critical role in stabilizing the tight junctions, and also perhaps in governing their selectivity. Diffusive outfluxes fall and TGP approaches 0 mV or may even become slightly positive as freshwater Ca^{2+} levels increase.

The mechanisms coupling transport processes to metabolic energy supply in the freshwater gill are also incompletely understood. Ouabain-inhibitable Na^+,K^+-ATPase, while much less abundant than in the seawater gill, is again located on the basolateral membranes (Philpott 1980). This is thought to be a major point of energy input into the transport system; the electrogenic extrusion of Na^+ would likely keep the intracellular Na^+ concentration low and the intracellular potential highly negative, thereby creating a strong electrochemical gradient for Na^+ entry through the apical membrane from the external fresh water. Whether this by itself would be sufficient to fully energize Na^+ transport is impossible to determine with currently available data. An electrogenic apical H^+-ATPase as proposed by Avella and Bornancin (1989) and Lin and Randall (1991) would clearly provide an additional energy supply, though its existence in the freshwater gill remains unproven. It is difficult to see how either of these pumps alone could explain "active" Cl^- transport. One possibility could be that a local pH gradient generated by apical Na^+-H^+ exchange would indirectly drive Cl^-/HCO_3^- exchange, though the exchanges would not be independent. Cl^-/HCO_3^--dependent ATPase has been detected in freshwater gills (e.g., Kerstetter and Kirschner 1974; Bornancin et al. 1977) and could provide an independent energy source for Cl^- uptake, although its precise localization, and linkage with the transport mechanism(s) remains uncertain. Other areas of uncertainty are the mechanisms by which acidic and basic equivalents and Cl^- move across the basolateral membrane, and whether the apical Na^+/acid and Cl^-/base exchanges are reversible under conditions of severe acid-base disturbance.

However, perhaps the most lively controversy surrounds the actual cell types in which these processes occur; until their function is better understood, we prefer to use the term mitochondria-rich (MR) to describe the freshwater equivalents of the seawater chloride cell. Early workers suspected that the MR cells on the gill filaments were the sites of both Na^+ and Cl^- uptake, but about 15 years ago attention shifted to the pavement cells on the respiratory lamellae as the major sites of transport (see Payan et al. 1984). However, we now know that MR cells occur on both filamental and respiratory surfaces. In the last few years, the pendulum has swung back the other way, with most investigations again pointing to the MR cells as the principal sites of both Na^+ and Cl^- uptake (e.g., Perry and Laurent 1989). On the other hand, the most recent studies (Goss et al. 1992a, b) suggest that while the MR cells transport Cl^-, the pavement cells may well be the sites of Na^+ uptake. These controversies illustrate the urgent need for a good in vitro model!

The Search for a New Model System for the Freshwater Gill

In light of the above discussion, we believe it essential that a freshwater epithelial model preparation should have asymmetrical solutions on mucosal and serosal surfaces, reflecting in vivo conditions (i.e., fresh water on the outside and saline of realistic acid-base status on the inside), and be directly validated against in vivo experiments on the same species.

Initially we focused on trout because of their common use in in vivo experiments. We found that both brook trout (Marshall 1985) and rainbow trout lack MR cells in the opercular epithelium, but that the rainbow, acclimated to very dilute fresh water ($Na^+ < 0.2$ mmol l^{-1}; $Ca^{2+} < 0.1$ mmol l^{-1}), has an MR cell population on the skin overlying the cleithrum bone (Marshall et al. 1992). However, we could not demonstrate active transport of either Na^+ or Cl^- when this preparation was mounted under realistic asymmetrical conditions in vitro. Nevertheless, we were able to demonstrate an active uptake of Ca^{2+} from mucosal to serosal surfaces in these preparations. A similar study by McCormick et al. (1992) using the opercular epithelium of freshwater tilapia also demonstrated active Ca^{2+} uptake. In both cases, Ca^{2+} influx was correlated with MR cell numbers on the epithelia, in agreement with in vivo studies (Perry and Wood 1985; Perry and Flik 1988) that have suggested that gill MR cells are sites of active Ca^{2+} uptake. Na^+ and Cl^- fluxes have not been examined in the tilapia preparation set up with fresh water on the mucosal surface, but it is known that the MR cell size is small in freshwater tilapia and

that there is no NaCl uptake across the epithelium with saline on the mucosal surface. (Foskett et al. 1981). Both of these euryhaline species are of freshwater origin, rather than the normally estuarine or marine origin.

A strongly euryhaline estuarine species such as *Fundulus heteroclitus* might provide a better epithelial model for freshwater NaCl transport. As outlined previously, the killifish has the ionoregulatory machinery to move rapidly between fresh water and seawater (e.g., Fig. 1), and maintains a MR cell population on its opercular epithelium in both media (Fig. 3). Our results to date, while preliminary, indicate that the opercular epithelium of the freshwater-adapted killifish may be a useful model system for the study of Na^+ and Cl transport in fresh water. However, there are very few previous flux data on the intact killifish in fresh water for comparison. We have therefore simultaneously studied Na^+ and Cl fluxes of *Fundulus heteroclitus* in vivo in the same freshwater environment.

In Vivo Studies on the Freshwater-adapted Killifish

Fundulus heteroclitus, collected in brackish water estuaries near Antigonish, Nova Scotia, were adapted to a very soft fresh water of defined composition (Table 1) at 20–25°C for at least 4 wk. The NaCl level (1 mmol $l^{-1} \simeq 0.2\%$ seawater) was well below the threshold (1% seawater) reported to cause the persistence of the seawater-type morphology of gill chloride cells (see above) but was sufficient to maintain the animals in good condition indefinitely in fresh water. Feeding was suspended 4 d prior to the experiment. Unidirectional and net flux rates of Na^+ and Cl^- of killifish in the acclimation medium were measured using standard radioisotopic techniques in which the isotope (^{22}Na, ^{36}Cl) is placed in the water and its disappearance into the fish is monitored. Methods were virtually identical to those outlined for rainbow trout by Goss and Wood (1991), apart from the fact that the fish were neither cannulated or blood-sampled, and water volumes during the flux measurements were scaled down in proportion to the much smaller size of the killifish.

Surprisingly, there was a vigorous turnover of Na^+ at the gills but no detectable uptake of Cl^- (Table 2). To our knowledge, the only other freshwater-adapted fish that have been reported to exhibit no Cl uptake are the eels *Anguilla anguilla* (Garcia-Romeu and Motais 1966) and *Anguilla rostrata* (Hyde and Perry 1987). Eels, like killifish, are euryhaline but exhibit a distinct catadramous lifestyle different from that of the killifish, which spends most or all of its life in estuaries. In the killifish, J_{in} and J_{out} for Na^+ were approximately equal, so J_{net} was not significantly different from zero (i.e., the fish were in Na^+ balance). For Cl^-, J_{out} was only about 20% of the corresponding Na^+ value, in spite of the presumably much larger electrochemical gradient for Cl^- outflux (cf. Fig. 4, TGP negative inside). Thus, despite the absence of detectable J_{in}, the fish were almost able to achieve Cl balance thanks to their very low permeability to this anion. Table 2 compares our data with the only other measurements available in the literature for freshwater-adapted *Fundulus heteroclitus.* For Cl^-, the single measurement of J_{out} by Potts and Evans (1967) was much larger than our value; however, as the methods were very different, and neither J_{in} nor J_{net} were determined by Potts and Evans (1967), it is difficult to assess whether this difference is real. For Na^+ fluxes, agreement with the measurements of Potts and Evans (1967) was reasonable, but Maetz et al. (1967a) reported much lower turnover rates. A likely explanation is that Maetz et al. (1967a) acclimated and tested their fish in a much lower concentration of NaCl (< 200 μmol l^{-1}) but a 20-fold higher Ca^{2+} concentration. The high [Ca^{2+}] would be expected to reduce paracellular J_{out}, whereas the low [Na^+] might limit uptake. Indeed the authors of both papers suggested anecdotally that J_{in} appeared to be dependent on external [NaCl].

We therefore examined the possible concentration-dependence of both Na^+ and Cl exchanges in freshwater-adapted *Fundulus heteroclitus* using acute short-term (<1 h) alterations in external [NaCl] within the traditional freshwater range (<10 mmol $l^{-1} \simeq 2\%$ seawater). All other water chemistry properties were held constant at the values in Table 1. Methods followed those outlined in Goss and Wood (1991). The results (Fig. 5) demonstrated that the concentration-dependence of J_{in} was very different for Na^+ than for Cl^-, and that the acclimation concentration (1,000 μmol l^{-1}) was above the threshold for Na^+ influx (close to 0 μmol l^{-1}; Fig. 5A) but below the threshold for Cl^- influx (about 2,000 μmol l^{-1}; Fig. 5B). Only at about 6,000 μmol l^{-1} did Cl influx approach Na^+ influx. For Na^+, influx rates varied with the external substrate concentration in a hyperbolic man-

TABLE 1. Composition (μmol l^{-1}) of the defined freshwater medium in which killifish were acclimated and tested in the present study.

Na^+	1,000	K^+	20
Cl	1,000	SO_4^{2-}	140
Ca^{2+}	100	Mg^{2+}	60
Titration alkalinity[a]	280	pH	6.8–7.2

[a] Titration to pH = 4.0.

TABLE 2. Unidirectional and net Na^+ and Cl fluxes of freshwater-adapted *Fundulus heteroclitus* in the present study and two previous studies.

	Present Study (n = 13–17)	Potts and Evans (1967)	Maetz et al. (1967a)
Freshwater composition			
[NaCl] (μmol l^{-1})	1,000	1,000	<200
[Ca^{2+}] (μmol l^{-1})	100	~25	~2,000
Ion fluxes			
$J_{in}^{Na^+}$ (nequiv g^{-1} h^{-1})	914.5 ±95.4	580	100
$J_{out}^{Na^+}$ (nequiv g^{-1} h^{-1})	−1,046.6 ±123.6	−825	−149
$J_{net}^{Na^+}$ (nequiv g^{-1} h^{-1})	−132.1 ±79.2	−245	−49
$J_{in}^{Cl^-}$ (nequiv g^{-1} h^{-1})	32.6 ±15.2	—	—
$J_{out}^{Cl^-}$ (nequiv g^{-1} h^{-1})	−204.9 ±41.0	−970	—
$J_{net}^{Cl^-}$ (nequiv g^{-1} h^{-1})	−172.3 ±36.0	—	—

ner. Such saturable first-order kinetics are well described by classical Michaelis-Menten analysis (reviewed by Wood 1991; Goss et al. 1992a):

$$J_{in} = \frac{J_{max} \times [X]_{ext}}{K_m + [X]_{ext}}$$

where $[X]_{ext}$ is the external substrate concentration (e.g., Na^+ or Cl), and J_{max} is the maximum transport rate, which is a function of the availability of both transport sites and internal counter ions (e.g., HCO_3^-, in the case of exchange carries such as the Cl^-/HCO_3^- exchanger). K_m is the external substrate concentration that will yield $\frac{1}{2}J_{max}$, and is therefore an inverse index of affinity (the lower the K_m, the higher the affinity). For Na^+ influx (Fig. 5A), the relationship was well described by the Michaelis-Menten equation with $K_m \simeq 1{,}700$ μmol l^{-1} and $J_{max} \simeq 2{,}300$ nequiv g^{-1} h^{-1}.

For Cl^- influx, saturation did not occur within the range tested. Thus we can only conclude that for Cl uptake, the K_m is above about 4,700 μmol l^{-1} and the J_{max} is equal to or higher than that for Na^+. Relative to most other freshwater fish, these data are unusual: first, in the high K_m values (to our knowledge, the highest ever reported in freshwater-adapted fish), second, in the high J_{max} values, and third, in the great differences in K_m between Na^+ and Cl^-. For example, rainbow trout acclimated to similar water chemistry exhibit K_m values of about 100 μmol l^{-1} and J_{max} values of about 400 nequiv g^{-1} h^{-1} for both Na^+ and Cl^- (Goss and Wood 1991).

In contrast to J_{in}, unidirectional outfluxes (J_{out}) of both Na^+ (Fig. 5A) and Cl^- (Fig. 5B) were virtually unaffected by external [NaCl], indicating a clear absence of any exchange diffusion compo-

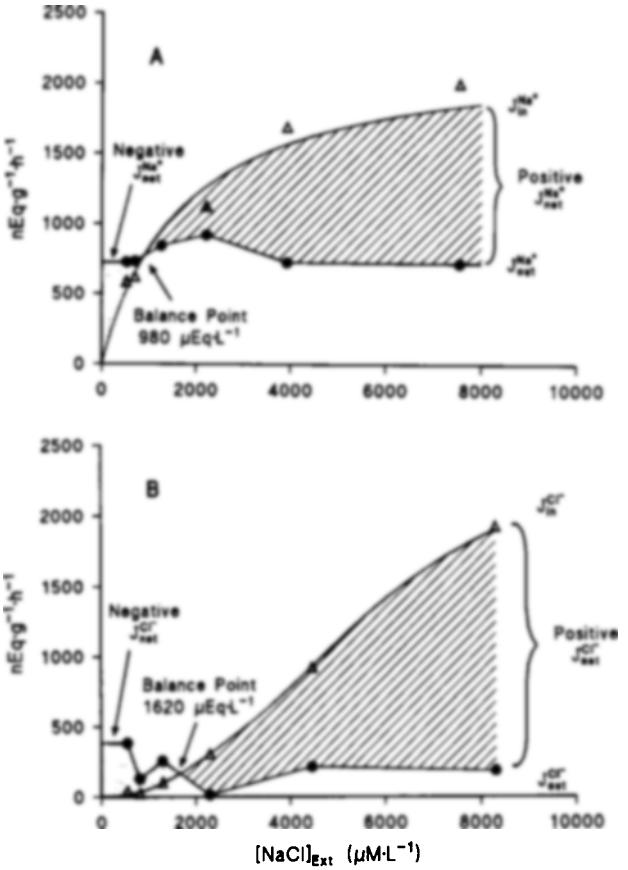

Fig. 5. Summary of relationships between unidirectional influx (J_{in}), outflux (J_{out}), and net flux rates (J_{net}, cross-hatched or stippled) of (A) Na^+ (n = 11) and (B) Cl (n = 6) versus external NaCl concentration over the freshwater range in freshwater-adapted *Fundulus heteroclitus*. Note that the acclimation concentration ([NaCl] = 1,000 μmol l^{-1}) was well above threshold for Na^+ influx, but below threshold for Cl influx. Thus the fish were able to achieve net balance for Na^+ but not Cl^- at this concentration. Note also that J_{out} for Cl was much lower than for Na^+, and that both were more or less independent of external [NaCl].

nents in the freshwater range. This again contrasts with most freshwater fish. The much lower Cl than Na^+ outflux was also unusual and persisted at all NaCl concentrations; this characteristically low permeability to Cl may be a remnant of the seawater origin of the killifish (i.e., preferential paracellular shunt permeability to cations; Fig. 2). The crossover or balance points for the J_{in} and J_{out} relationships occurred at 980 μmol l^{-1} [NaCl] for Na^+ (Fig. 5A), close to the acclimation concentration, but at 1,620 μmol l^{-1} for Cl^- (Fig. 5B). Above these points, the animals are in positive balance, and below these points in negative balance. Dietary Cl^- is probably an essential component of Cl^- balance when *Fundulus heteroclitus* are resident in dilute freshwater where branchial Cl^- influx is negligible.

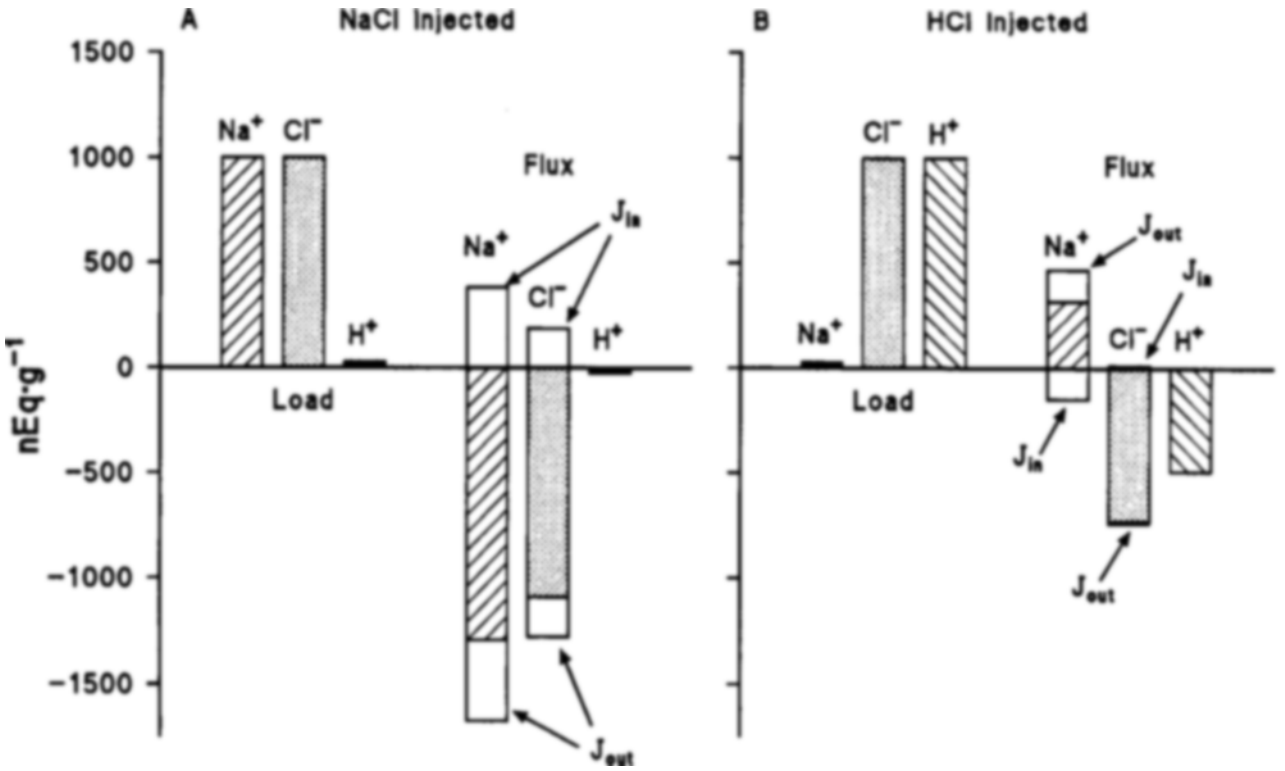

Fig. 6. Summary of cumulative changes in unidirectional and net fluxes of Na^+, Cl^-, and H^+ (net only) in freshwater-adapted *Fundulus heteroclitus*, over a 4-h period after intraperitoneal injection of either (A) 1,000 nmol g^{-1} NaCl (n = 14) or (B) 1,000 nmol g^{-1} HCl (n = 16). In each case the injected loads of Na^+, Cl^-, and H^+ are shown on the left, and the cumulative flux changes over 4 h are shown on the right. For Na^+ and Cl^-, the contributions of changes in influx (J_{in}) and outflux (J_{out}) to the cumulative net flux are shown separately. Note that after NaCl injection, approximately equal amounts of Na^+ and Cl^- were cleared, and net H^+ excretion was negligible. However, after HCl injection, the fish gained Na^+, lost Cl^-, and excreted H^+. Note also that in each case, the changes in Na^+ and Cl^- net fluxes were achieved completely by changes in J_{out}. Changes in J_{in} either opposed or made no contribution to the changes in net flux.

In a final in vivo test, we evaluated the linkage between ion and acid-base balance in *Fundulus heteroclitus* acclimated to the defined freshwater medium (Table 1). Killifish were injected intraperitoneally with an isotonic solution of either 1,000 nmol g^{-1} NaCl (control) or 1,000 nmol g^{-1} HCl (experimental), the latter inducing a systemic metabolic acidosis. Unidirectional and net fluxes of Na^+ and Cl^-, together with the net flux of acidic equivalents ("H^+"), were followed over the succeeding 4 h. The results, summarized in Fig. 6, were surprising. The standard response to acidosis seen in most freshwater teleosts would be a stimulation of Na^+ influx (i.e., Na^+ versus acid exchange) and an inhibition of Cl^- influx (i.e., Cl^- versus base exchange; McDonald et al. 1989; Wood 1991). These did not occur. Na^+ and Cl^- influx rates did not change significantly. Nevertheless, the killifish excreted approximately half of the H^+ load over the 4-h period, gained Na^+, and lost Cl^- (Fig. 6B). This was achieved by differential adjustment of Na^+ versus Cl^- outflux rates. Na^+ outflux decreased significantly, and Cl^- outflux increased significantly. As discussed earlier, the resulting excess of strong anion (Cl^-) loss over strong cation (Na^+) loss will constrain a net loss of H^+ by Strong Ion Difference Theory (cf. Fig. 4), assuming that these are the only major permeant ions. This pattern was very different from the NaCl-injected fish, which excreted almost equimolar amounts of Na^+ and Cl^- (approximately equal to the injected load) but no H^+ (Fig. 6A). However, again the compensation was achieved by adjustment of outflux rather than influx rates. The killifish in fresh water, lacking appreciable Cl^- influx and possessing only a low affinity Na^+ influx mechanism, seems to rely solely on outflux manipulation to adjust ion and acid-base balance. This adjustment presumably occurs via the paracellular pathway (Fig. 4).

These in vivo studies have indicated that the mechanisms of Na^+ and Cl^- transport in the freshwater killifish are very different from those of the classic "freshwater fish" shown in Fig. 4. However, it must be remembered that only a handful of the

TABLE 3. Unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratio of ^{36}Cl in isolated skin of 5% seawater-adapted *Gillichthys mirabilis* (n = 6) and freshwater-adapted *Fundulus heteroclitus* (n = 7) bathed on mucosal side with fresh water and saline water.

Bathing Solution Serosa/Mucosa	TEP (mV)[a]	G_t (mS cm^{-2})[b]	J_{sm} (nmol cm^{-2} h^{-1})	J_{ms} (nmol cm^{-2} h^{-1})	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	p[d]
5% SW adapted *Gillichthys*							
Saline/FW	+12.2 ± 6.9	0.27 ± 0.05	547 ± 69	14 ± 2.2	45.8 ± 11.3	45.8 ± 4.8	NS
Saline/Saline	+14.7 ± 2.3	4.58 ± 0.54	625 + 119	288 ± 45.6	2.3 ± 0.4	0.58 ± 0.05	<0.002
P[c]	NS	<0.001	NS	<0.001			
FW adapted *Fundulus*							
Saline/FW	−53.9 ± 3.8	1.80 ± 0.2	1,170 ± 100	90 ± 20.0	55.1 ± 27.8	884 ± 141	<0.001
Saline/Saline	+1.5 ± 0.4	3.40 ± 0.3	1,440 ± 150	1,190 ± 150	1.4 + 0.2	0.9 ± 0.03	NS
P[c]	<0.001	<0.05	NS	<0.001			

[a] Transepithelial potential with reference to the mucosal (outside) bathing solution and corrected for junction potentials; n = 12 for *Gillichthys*, n = 14 for *Fundulus*.
[b] Total tissue conductance corrected for solution resistance.
[c] paired *t*-test, two-tailed.
[d] Mann Whitney *U*-test, two-tailed.

1,000s of freshwater species in the world have been examined to date, and amongst these, many details remain controversial. The killifish pattern may prove to be just as "representative" eventually.

In Vitro Studies on the Killifish Opercular Epithelium

Development of a Freshwater MR Cell System

Epithelia from freshwater-adapted *Fundulus* (1.0 mmol l^{-1} NaCl, 0.1 mmol l^{-1} Ca^{2+}; at least 10 d acclimation) and 5% seawater-adapted *Gillichthys* were mounted in vitro with fresh water on the mucosal surface while unidirectional and net ion fluxes were determined. The preparations were at open circuit and the TEP (measured with the mucosal side grounded) and tissue conductance (G_t, a parameter that varies directly with total ion permeability) were monitored. The flux methods and electrophysiology were the same as in Marshall (1981a) for *Gillichthys* skin and Marshall el al. (1992) for *Fundulus* preparations. These open-circuit fluxes provide information on the character of ion uptake mechanisms that might be present. After these initial measurements, we changed the mucosal bath to saline and continued the flux, TEP, and G_t measurements. Seawater-type chloride cells under these conditions develop a net secretion of Cl^- that is equal to the short-circuit current (I_{sc} Degnan et al. 1977; Marshall 1980). We reasoned that the freshwater-adaptive MR cell would not be capable of Cl secretion under these conditions and the procedure then may be used as a means to distinguish *functionally* between freshwater-type and seawater-type MR cells.

Table 3 presents some previously unpublished data on the isolated jaw skin of *Gillichthys mirabilis* acclimated to 5% seawater and mounted with artificial fresh water (1% saline ≈ 0.3% seawater) on the mucosal side. There was very little Cl influx, and the observed flux ratio was not significantly different from that predicted for purely passive fluxes by the Ussing flux ratio criterion (Ussing 1949). When the external solution was then changed to isotonic saline (i.e., symmetrical conditions), there was net Cl *secretion* from serosal to mucosal surfaces (Table 1), indicating the presence of operational seawater-type chloride cells. However, it must be emphasized that in common with most previous investigations, the medium used for hypotonic acclimation was not real fresh water but rather a dilution of seawater (in this case 5% seawater). Several studies have shown that the chloride cells maintain a typical seawater-type morphology down to an acclimation salinity equivalent to about 1% seawater (Copeland 1950; Lacy 1983; Philpott and Copeland 1963; Karnaky 1986). It was clear from this and earlier studies that acclimation to very dilute fresh water was a necessary prerequisite to development of freshwater MR cells.

Opercular epithelia from *Fundulus* that had been adapted for at least 10 d to real fresh water have numerous MR cells (Fig. 3). By DASPEI fluorescence, the MR cells are comparable in size, shape, and density in fresh water and seawater (unlike tilapia freshwater MR cells that are much smaller than the seawater chloride cells). There have been several reports of ultrastructural differences between the MR cells of seawater and freshwater teleost gills. Two general distinctions are that freshwater MR cells lack obvious invaginated apical surfaces termed apical pits and have fewer adjacent cells associated with the large MR cells (Sardet et al. 1979). The normal freshwater *Fundulus* opercular epithelium frequently has pairs, triplets, and larger groupings of chloride cells (Fig. 3), hence these types of cell-cell interactions are clearly not unique to the seawater condition. The question is then whether these cells have different abilities to

TABLE 4. Unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratio of ^{36}Cl in isolated skin of freshwater-adapted *Fundulus heteroclitus* bathed on mucosal side with fresh water.

Bathing Solution Serosa/Mucosa	TEP (mV)[a]	G_t (mS cm^{-2})[b]	J_{sm} (nmol cm^{-2} h^{-1})	J_{ms} (nmol cm^{-2} h^{-1})	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	p[c]
Saline/FW Hour 1	−49.9 ± 5.6	1.46 ± 0.22	602 ± 70	44 ± 15	37 ± 24	903 ± 140	<0.002
Saline/FW Hour 2	−48.7 ± 5.7	1.97 ± 0.53	655 ± 70	30 ± 10	88 ± 55	844 ± 173	<0.002
Saline/FW Hour 3	−51.8 ± 4.9	2.51 ± 0.63	771 ± 106	22 ± 8	145 ± 76	923 ± 160	<0.002

[a] Transepithelial potential measured with respect to the mucosal bath and corrected for junction potentials; n = 12.
[b] Total tissue conductance corrected for solution resistance; n = 12.
[c] Mann-Whitney *U*-test, two-tailed.

transport ions or whether the freshwater MR cells are merely seawater-type cells that are somehow inhibited hormonally from secreting Cl^-.

The unidirectional Cl^- fluxes for freshwater *Fundulus* opercular epithelium mounted with fresh water on the mucosal side (Table 3) showed that the ratio of efflux to influx of Cl^- was much smaller ($p < 0.001$) than that predicted by the Ussing flux ratio equation. Such disagreement with the predicted ratio indicates that transport mechanism(s) other than passive diffusion are important and suggests that an active transport process is involved. When the mucosal bath was changed to saline, *Fundulus* opercular epithelium did *not* develop a positive TEP nor was there net secretion of Cl^- (Table 3), which we take as evidence that seawater-type MR cells were not present. Further, we conclude that MR cells are involved in Cl^- uptake but not Na^+ uptake, something that was suspected from in vivo studies with *Fundulus* (above) and brown bullhead, *Ictalurus nebulosus* (Goss et al. 1992a, b). Of interest is the observation that the Cl^- influx drops over time while the Cl^- efflux (and both Na^+ unidirectional fluxes) do not change (Table 4). Indirectly this decline of Cl^- influx over time is indicative of active ion transport involvement. The Cl^- influx could be supported hormonally or metabolically in a manner that is not sustained under our present in vitro conditions. It would be interesting to supplement the bathing solutions with freshwater-adaptive hormones or with metabolites that would change the redox state of the cells (reduced glutathione or ascorbate) to see if this transport could be maintained over longer periods.

The unidirectional and net fluxes of Na^+ (Table 5) indicate that this ion is passively distributed across the epithelium, inasmuch as the flux ratio observed under the concentration gradient that favors Na^+ efflux and an electrical gradient that favors Na^+ uptake is not significantly different from the flux ratio predicted on the basis of purely passive diffusion with the Ussing flux ratio equation. The Cl^- unidirectional and net fluxes (Tables 3 and 4) were smaller than the respective Na^+ fluxes (Table 5), which is consistent with the in vivo data (above) indicating the lower permeability to anions. The Na^+ efflux was in all cases larger than the influx, hence the epithelium was consistently losing Na^+. One would expect this of an in vitro preparation, inasmuch as even minor leak pathways opened during dissection and mounting will contribute to ion leakage. In previous work with trout opercular epithelium, however, the leaks created by this procedure were small, as gauged by

TABLE 5. Unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratio of $^{22}Na^+$ in isolated skin of freshwater-adapted *Fundulus heteroclitus* bathed on mucosal side with fresh water.

Bathing Solution Serosa/Mucosa	TEP (mV)[a]	G_t (mS cm^{-2})[b]	J_{sm} (nmol cm^{-2} h^{-1})	J_{ms} (nmol cm^{-2} h^{-1})	J_{sm}/J_{ms} Observed	J_{sm}/J_{ms} Predicted	p[c]
Saline/FW Hour 1	−52.4 ± 2.6	1.14 ± 0.18	789 ± 240	104 ± 20	9.2 ± 3.5	14.2 ± 2.0	NS
Saline/FW Hour 2	−50.7 ± 3.2	1.53 ± 0.38	1,052 ± 210	95 ± 20	13.4 ± 3.7	15.7 ± 2.3	NS
Saline/FW Hour 3	−43.1 ± 4.2	1.29 ± 0.23	958 ± 260	94 ± 20	15.7 ± 2.3	22.2 ± 4.6	NS

[a] Transepithelial potential measured with respect to the mucosal bath and corrected for junction potentials; n = 12.
[b] Total tissue conductance corrected for solution resistance; n = 12.
[c] Mann-Whitney *U*-test, two-tailed.

the low permeability of the nominally extracellular marker ^{14}C-mannitol (Marshall 1985).

The freshwater opercular epithelium in vitro has a large negative TEP, between −30 mV and −70 mV (Tables 3–5), that is steady over the 3-h incubation period (Tables 4 and 5). The TEP is larger than for in vitro brook trout opercular epithelium under similar conditions (−8.6 ± 0.8 mV; Marshall 1985) and could reflect the presence of MR cells in the *Fundulus* epithelium that are absent from the brook trout epithelium. The voltage is also greater than that seen in vivo for *Fundulus* larvae in 5% seawater (−11 ± 5.0 mV, Guggino 1980), for *Gillichthys* in 2% seawater (−24.8 ± 2.0 mV; Thompson 1972), and for many other freshwater species in whole animal TGP measurements (see review by Potts 1984). The TEP reflects a high degree of selectivity of the epithelium for cations over anions. The Nernst equilibrium potential for Na^+ across the epithelium is approximately −120 mV and the maximum potential we have seen is −72 mV, hence it would appear that the TEP is mostly a Na^+ diffusion potential. The TEP was not changed significantly by increasing mucosal Cl^- from 1 mM to 140 mM, indicating a low conductance for Cl^-, as expected (data not shown).

The transepithelial conductance (G_t) is a direct function of the sum of the single ion permeabilities of the epithelium. The G_t of *Fundulus* opercular epithelium with fresh water outside averaged 1.16–2.50 mS cm^{-2} (Tables 3–5) and was much larger compared to the brook trout opercular epithelium (0.2 ± 0.04 mS cm^{-2}; Marshall 1985) and the rainbow trout cleithrum skin (0.09 ± 0.1 mS cm^{-2}; Marshall et al. 1992) under similar conditions, indicating the higher permeability of the *Fundulus* epithelium. With saline on both sides (Table 3), both the *Fundulus* and *Gillichthys* preparations show a large increase in G_t (not due to solution resistance changes), consistent with the notion that high mucosal osmolarity increases the conductance of the paracellular pathway in epithelia (Finn and Bright 1978). It has been shown previously that the high TEP in freshwater in vivo measurements is related to low environmental calcium activity (Potts 1984). In these three studies of teleost skin in vitro we used low calcium bathing solutions (100 μM) routinely and we find that only those epithelia that contain large numbers of MR cells (i.e., *Fundulus*) have the large negative inside TEP and associated large ionic conductance. It seems that *Fundulus* in adapting to the freshwater medium does not (or cannot) eliminate entirely the cation-selective shunt pathway that is well recognized in the seawater-adapted animals. To this extent, *Fundulus* appears to be a marine fish that copes well with fresh water and the failure of the paracellular pathway to close may account for the weak Phase I response after transfer of *Fundulus* to fresh water (see Fig. 1).

Conclusions and Future Directions

The establishment of a model epithelium to study the operation of freshwater-type, teleostean MR cells has been attained. There appears to be active uptake of Cl^- but only passive movement of Na^+ across this epithelium when it is bathed with fresh water on the mucosal side. The cellular mechanism of the Cl^- active uptake remains to be revealed, as well as the connections with the acid/base fluxes seen in vivo. For example, no studies to date have examined the possible relationship between Ca^{2+} and Cl^- uptake or their connection to acid/base fluxes. This work extends the study of ion transport by the freshwater MR cell from the previous studies of calcium transport (McCormick et al. 1992; Marshall et al. 1992), and will allow examination of the effects of hormones and neurotransmitters that are purported to be involved in the adaptation of euryhaline fish to different salinities. Of particular interest would be studies of the development of seawater type chloride cells under the influence of growth hormone/IGF-I and cortisol (see McCormick 1994) and the development of freshwater-type MR cells under the influence of prolactin during freshwater adaptation (reviewed by Hirano 1986 and Bern and Madsen 1992). Also, the interesting possibility that rapid-acting hormones such as epinephrine may be involved in the temporary shutdown of Cl^- secretion can be examined using this system. This sort of ion regulation may be necessary in estuarine species such as *Fundulus* where rapid but temporary salinity fluctuations are experienced often. The judicious use of in vitro systems in physiology to corroborate and extend in vivo experiments is a powerful combination that provides physiological relevance and mechanistic details. Now the full extent of freshwater-seawater adaptation by a euryhaline teleost can be followed using both experimental approaches.

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