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Seawater acclimation causes independent alterations in Na^+/K^+- and H^+-ATPase activity in isolated mitochondria-rich cell subtypes of the rainbow trout gill

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Summary

Mitochondria-rich cells (MR cells) of the gills of rainbow trout undergo changes in relative distribution and biochemical function during acclimation to partial-strength (10‰) and full-strength (30‰) seawater. In isolated total gill cells, Na^+/K^+-ATPase activity increased fivefold and H^+-ATPase activity decreased fourfold when trout were acclimated to either 10‰ or 30‰ seawater. When total MR gill cells were separated based on differential binding to peanut lectin agglutinin (PNA), the PNA subtypes underwent a change in relative distribution in seawater-acclimated fish. In freshwater, the ratio of PNA^-:PNA^+ was 65:35 while in seawater the distribution changed to 20:80 PNA^-:PNA^+. Additionally, differential changes in Na^+/K^+-ATPase and H^+-ATPase activity in each of the independent cell types occurred during seawater acclimation; Na^+/K^+-ATPase activity in the PNA^- cells increased by 197% while in PNA^+ cells Na^+/K^+-ATPase decreased by 57%. However, H^+-ATPase activity was decreased in both PNA^- (84%) and PNA^+ (72%) subtypes during acclimation to seawater.

Key words: mitochondria-rich cells, MR cells, sodium, transport, Na^+/K^+-ATPase, H^+-ATPase, rainbow trout, *Oncorhynchus mykiss*, gill, seawater adaptation.

Introduction

Maintenance of an acid–base status within optimal limits is essential for proper cell function. In fishes, the principal regulatory organ for maintenance of blood pH is the gill. Various models of ion transport in the freshwater teleost fish gill have linked proton (H^+) secretion to sodium (Na^+) uptake and bicarbonate (HCO_3^-) secretion to chloride (Cl^-) uptake (Krogh, 1938; McDonald and Wood, 1981; Goss et al., 1998). Electron microscopy has allowed ultrastructural description of chloride cells (Karnaky, 1980; Laurent and Dunel, 1980). In these studies, chloride cells were characterized by their high numbers of mitochondria, dense vesicular–tubular network in the cytoplasm, ovoid nuclei and high levels of the transport protein Na^+/K^+-ATPase. However, the role of the chloride cell has been expanded to include transport of other ions including Na^+, calcium and acidic/basic equivalents (Maetz, 1976; Hobe et al., 1984). Therefore, we now refer to these 'chloride cells' as simply mitochondria-rich (MR) cells, although these terms still tend to be used interchangeably.

In freshwater, the current model for ion exchange links Cl^- uptake to HCO_3^- secretion *via* an anion exchanger. Meanwhile, Na^+ uptake is thought to occur *via* an Na^+ channel linked electrochemically to a coupled V-type H^+-ATPase on the apical membrane (Lin and Randall, 1991; Sullivan et al., 1995; Wilson et al., 2002). This model is based on the requirement to move both Na^+ and H^+ against their electrochemical gradients in low ionic strength media (Avella and Bornancin, 1989). However, in a higher ionic strength environment such as seawater, the Na^+ concentration gradient is favourable for linked electroneutral exchange. The mechanism by which the removal of protons is believed to occur is *via* an electroneutral sodium/proton exchange (NHE) system (Claiborne et al., 1999; Wilson et al., 2000), reducing the ATP requirement for acid–base regulation. NHE isoforms have been identified by molecular and immunological methods in the gills of a marine species (*Myoxocephalus octodecimspinosus*) and the euryhaline killifish (*Fundulus heteroclitus*) (Claiborne et al., 1999). Seawater acclimation results in many changes in gill function including increases in the activity of Na^+/K^+-ATPase in MR gill cells (Mancera and McCormick, 2000), concomitant reductions in H^+-ATPase (Lin and Randall, 1993) and the appearance of accessory cells on the gill epithelium (Laurent and Dunel, 1980).

Recently, it has been proposed that there are separate subtypes of the MR cells that perform different ionoregulatory functions (Galvez et al., 2002). Pisam and co-workers (Pisam et al., 1987, 1990), using morphological characteristics such as staining, shape, apical surface and location in the gill, have identified two different MR cells on the gill epithelium of freshwater teleost fish, which they termed α and β MR cells. Pisam et al. (1987) described two MR types (α- and β-cells) in the gills of freshwater teleost fish, but only one MR cell subtype in the gills of seawater teleost fish (consisting of the α MR cells). More recently, Wong and Chan (1999) used flow

cytometry to track the change in MR gill cell populations during seawater transfer using relative cell size, granularity and autofluorescence as the defining characteristics. They demonstrated that in freshwater Japanese eels (*Anguilla japonica*) there are two distinct populations of cells that undergo a transition into morphologically separate seawater cell subtypes. However, this study did not further assess the function of these presumed MR gill cell populations. Nonetheless, these studies suggest the existence of different MR cell subtypes during seawater acclimation.

There has been limited progress made in characterizing the role of MR gill cell populations in ion and acid–base regulation, partly due to the lack of any suitable techniques for differentiating between MR cell populations in live cells. In other epithelia, such as the mammalian cortical collecting duct (Turnheim, 1991), the frog skin (Smith, 1971) and the turtle urinary bladder (Rich et al., 2002), peanut lectin agglutinin (PNA) is known to differentially bind to MR cell populations based on differences in glycoproteins on the apical (mucosal) surfaces of these cells. All the aforementioned systems are tight epithelia that have low rates of passive ion loss across the epithelium against large electrochemical gradients, and the major cell type (called 'principal cells' in the mammalian collecting duct and 'granular cells' in the frog skin and turtle bladder) has similar features to the fish gill pavement cells (PVCs). It has been reported that the MR intercalated collecting duct cells could be differentiated based on the binding of PNA (Satlin et al., 1992). Only one type of intercalated collecting duct cell (β-type) was found to bind the PNA. Recently, our lab has demonstrated that a sub-population of MR gill cells from freshwater rainbow trout bind PNA (Goss et al., 2001). Furthermore, we have developed a method for separation and isolation of functionally different MR cell subtypes in freshwater rainbow trout based on differential binding to PNA (Galvez et al., 2002). We have also shown that phenamil-sensitive Na^+ transport is linked only to the PNA^- cells (Reid et al., 2003). Phenamil sensitivity suggests the presence of an epithelial sodium channel (ENaC family of Na^+ channels) on the PNA^- cells only and establishes a role for this MR cell subtype in Na^+ transport.

The purpose of the present study was twofold. The first objective was to examine changes in MR cell subtype populations (PNA^+ and PNA^-) during seawater acclimation to compare with the morphological data obtained by Pisam et al. (1987) and Wong and Chan (1999). The second objective was to use our ability to isolate sub-populations of MR cells to independently examine changes in Na^+/K^+- and H^+-ATPase activity in each of the MR cell subtypes during seawater acclimation.

Materials and methods

Experimental animals

Freshwater rainbow trout (*Oncorhynchus mykiss* Walbaum) of either sex were obtained from Alberta Trout Growers Ltd (Alberta, Canada) and held indoors in 450-litre fibreglass tanks with flowing dechlorinated Edmonton tap water (hardness 1.6 mmol l^{-1} as $CaCO_3$; total alkalinity 120 mg l^{-1}; pH 8.2). Water temperature was maintained at 15°C, and the photoperiod matched the natural regime of Edmonton. The fish were fed daily with dry commercial trout pellets.

Fish used in the seawater transfer experiments were removed from the holding facility and transferred to a recirculating system containing 10‰ (~30% full-strength) seawater. The water temperature and photoperiod were unchanged. Seawater was made by diluting Instant Ocean brand salts (Aquarium Systems Inc., Mentor, OH, USA) in dechlorinated Edmonton tap water. Fish were acclimated to 10‰ for at least one week before transfer to a second system containing full-strength seawater (30‰) for at least an additional week before sampling. Salinity of seawater was checked weekly and adjusted as necessary. Experimental animals were randomly selected from holding tanks and killed by overdose (1 g l^{-1} MS-222; Syndel, Vancouver, BC, Canada) followed by cephalic blow.

Materials

Streptavidin-conjugated Alexa fluor 594 and Mitotracker Green-FM were obtained from Molecular Probes (Eugene, OR, USA). The magnetic cell separation (MACS) system and streptavidin-conjugated microbeads used for MACS were purchased from Miltynei Biotech (Auburn, CA, USA). All other reagents used for experimentation were obtained from Sigma Chemicals, St Louis, MO, USA.

Gill digestion

Gill arches were removed from fish, rinsed in dechlorinated tap water and lightly blotted to remove excess water. Gill filaments were removed and placed into ice-cold Cortland's saline (143 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1.5 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $NaHCO_3$, 3 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} glucose, pH 7.8). Filaments were digested in 0.2 mg ml^{-1} collagenase (type 1A) in Cortland's saline for 20 min at 18°C with continuous agitation at 300 revs min^{-1}. Digested filaments were then scraped with glass slides and filtered through 254 μm (gravity) and 96 μm (vacuum) nylon mesh to remove large debris. The filtrate was then resuspended in Ca^{2+}-free phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 4.3 mmol l^{-1} Na_2HPO_4, 1.4 mmol l^{-1} NaH_2PO_4 and adjusted to pH 7.8) for centrifugation at 1500 ***g*** for 5 min at 4°C. Dispersed cells were washed twice with at least 10× volume PBS, pelleted by centrifugation and resuspended in 1 ml red cell lysis buffer (154 mmol l^{-1} ammonium chloride) for exactly 60 s to lyse the erythrocytes. After 1 min, the cells were rapidly diluted by the addition of 45 ml PBS and then centrifuged for 5 min (1500 ***g***). The resultant cell pellet was washed twice with 40 ml PBS and was finally resuspended in 2 ml PBS. The cells were then layered on a discontinuous Percoll density gradient (1.03, 1.05 and 1.09 g ml^{-1} Percoll) and centrifuged at 2000 ***g*** for 45 min. The MR cells from the 1.05–1.09 g ml^{-1} Percoll interface were collected, spun down and either used immediately in experiments or further separated into the PNA^- and PNA^+ cell fractions as outlined previously by Galvez et al. (2002).

Fluorescence microscopy

The percentage of MR cells binding PNA was determined by labelling cells with a biotin-conjugated PNA (40 μg ml^{-1}, 20 min), washing the cells in PBS, followed by a double labelling with streptavidin-conjugated Alexa fluor 594 (20 μg ml^{-1}, 15 min) and Mitotracker Green-FM (40 μg ml^{-1}, 15 min). Cells were washed in PBS to remove unbound dyes and placed on glass slides for differential interference contrast (DIC) microscopy (Nikon Eclipse TE300) and fluorescence imaging (TE-FM epi-fluorescence attachment). Fluorescence microscopy was performed using epi-illumination *via* a Xenon arc lamp (Lambda LS; Sutter Instruments, Novato, CA, USA). Plan-Fluor objectives at either 40× or 100× (oil immersion) were used and images were digitally captured on a 12-bit CCD camera (Cooke SensiCam, Kelheim, Germany). Mitotracker Green-FM and Alexa fluor 594 were excited at 495±5 nm and 560±2 nm, respectively, and emission was measured using 540±25 nm and 630±30 nm filters, respectively. Images were binned at 2×2 to increase the sensitivity of fluorescence capture, and final images were adjusted using Adobe Photoshop 6 for contrast and brightness only.

To determine the percentage of total MR cells made up of PNA^{+} or PNA^{-} cells in the gills of freshwater and seawater-acclimated trout, random view fields were selected on the slide and a DIC image captured for total cell counts. Serial images of each field were captured with fluorescence microscopy. Mitotracker Green-FM fluorescence was used to indicate the MR cells in the field, and Alexa fluor 594 fluorescence was used to determine the percentage of MR cells that were PNA^{+}. At least five random fields were captured and used for each fish. Cell sizes were measured using the calibrated measuring tool located in Slidebook v.3.1.2 (Intelligent Imaging Innovations; Denver, CO, USA) with the DIC images captured from the seawater adaptation experiments.

ATPase assay

An ATPase assay based on a method developed by McCormick (1993) was adapted to determine both the ouabain (Na^{+}/K^{+}-ATPase inhibitor)- and bafilomycin (V-type H^{+}-ATPase inhibitor)-sensitive ATPase activities. Gill cells were counted using a haemocytometer and used as either total MR cells or separated into PNA^{-} and PNA^{+} fractions and stored in SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2EDTA, 50 mmol l^{-1} imidazole and adjusted to pH 7.3) at –80°C until assays were performed. Cells were thawed and homogenized with the addition of 0.5% sodium deoxycholic acid on ice and immediately centrifuged at 5000 ***g*** for 30 s to remove insoluble material. Homogenate (10 μl) from each sample was added to nine wells in a 96-well plate. This provided three treatments for each sample [control, ouabain (500 μmol l^{-1}) and ouabain (500 μmol l^{-1}) + bafilomycin (50 nmol l^{-1})] with triplicate measurements of each treatment. Preliminary experiments demonstrated that H^{+}-ATPase activity in total MR cells was maximally inhibited between 10 nmol l^{-1} and 100 nmol l^{-1}. Therefore, 50 nmol l^{-1} was chosen as the appropriate dose for our assay. To each well was added 150 μl of assay mixture [50 mmol l^{-1} imidazole buffer, 2 mmol l^{-1} phosphoenol pyruvate (PEP), 0.16 mmol l^{-1} NADH, 0.5 mmol l^{-1} ATP, 3.3 U ml^{-1} lactate dehydrogenase (LDH), 3.6 U ml^{-1} phosphokinase (PK)], with appropriate drug treatment added, and 50 μl of salt solution (50 mmol l^{-1} imidazole, 189 mmol l^{-1} NaCl, 10.5 mmol l^{-1} $MgCl_2$, 42 mmol l^{-1} KCl). The microplate was read at a wavelength of 340 nm in a kinetic microplate reader (ThermoMAX; Molecular Devices, Sunnyvale, CA, USA) at 15 s intervals for 20 min. The average rate for each treatment was taken from the stable slope and calculated from a standard curve generated just prior to the assay. Na^{+}/K^{+}-ATPase activity was obtained by subtracting the ouabain-treated ATPase activity from control ATPase activity (see McCormick, 1993). We also modified the assay to assess H^{+}-ATPase activity by calculating the difference in ATPase activity between the ouabain- and the oaubain + bafilomycin-treated samples.

Statistical analysis

All statistics were performed with SPSS version 10, using analysis of variance (ANOVA) followed by multiple comparison tests using Tukey's HSD. In all cases, the level of significance was set at $P<0.05$.

Results

Seawater acclimation is commonly associated with elevated levels of gill Na^{+}/K^{+}-ATPase activity (Mancera and McCormick, 2000; Tipsmark et al., 2002). Using the total isolated gill cell population (i.e. cells from a 1.03–1.09 interface representing PVCs and MR cells combined but after removal of mucous cells), we found that acclimation to 10‰ or 30‰ seawater resulted in a 4–5-fold increase in Na^{+}/K^{+}-ATPase activity on a per cell basis when compared with that in freshwater fish (Fig. 1).

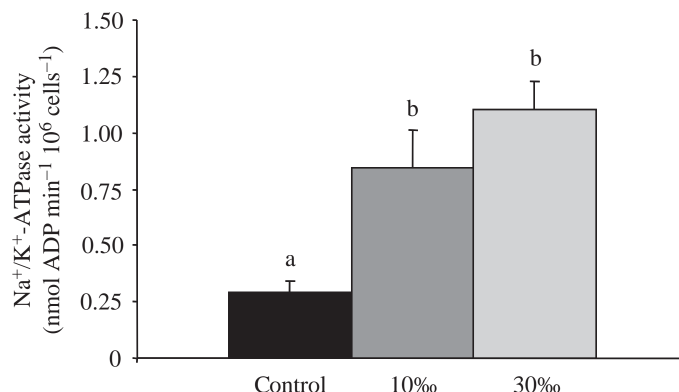

Fig. 1. Ouabain-sensitive Na^{+}/K^{+}-ATPase activity of control and seawater-adapted fish in a total cell fraction [i.e. cells collected from combined PVC/MR (pavement cell/mitochondria-rich) fraction, the 1.03–1.09 g ml^{-1} Percoll interface]. Both partial-strength seawater (10‰) and full-strength seawater (30‰)-adapted fish were used for testing. Significant differences are indicated by different letters. Values represent means ± S.E.M. (N=5 fish).

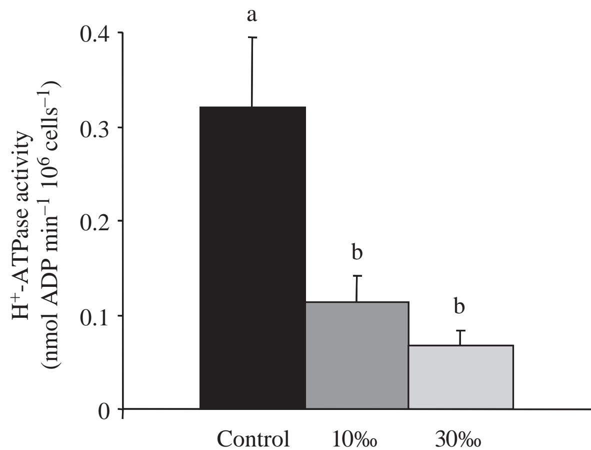

Fig. 2. Bafilomycin (50 nmol l^{-1})-sensitive H^+-ATPase activity in the total cell fraction [i.e. cells collected from combined PVC/MR (pavement cell/mitochondria-rich) fraction, the 1.03–1.09 g ml^{-1} Percoll interface]. Freshwater control fish compared with both 10‰ and 30‰ seawater-adapted fish. Significant differences are indicated by different letters. Values represent means ± S.E.M. (N=4–6 fish).

Similarly, reductions in bafilomycin-sensitive H^+-ATPase activity occur in the same total isolated 1.03–1.09 cell fraction. When the total gill cell population was examined, bafilomycin-sensitive H^+-ATPase activity declined 79% from 0.32±0.07 nmol ADP min^{-1} 10^{-6} $cells^{-1}$ in freshwater fish to 0.067±0.02 nmol ADP min^{-1} 10^{-6} $cells^{-1}$ in full-strength seawater-acclimated fish (Fig. 2).

Fig. 3A–C shows differential interference contrast (DIC; Fig. 3A) and fluorescence microscopic images (Fig. 3B,C) from the same field of view for total MR cells (Percoll density isolated from 1.05–1.09 interface) from freshwater rainbow trout. The fluorescence images permit the identification of cells as MR cells using Mitotracker (Fig. 3B) and further distinguish those MR cells as either binding PNA or not (Fig. 3C). Greater than 95% of the cells in the fields of view (Fig. 3A) were found to be MR cells, as demonstrated in Fig. 3B. The percentage of PNA^+ cells was calculated from the number showing PNA fluorescence (Fig. 3C). Fig. 3D–F shows DIC and fluorescence images of the same field of view for a 30‰ acclimated (seawater) trout. Clearly, the relative number of PNA^+ cells in the field of view has greatly increased from the freshwater situation (Fig. 3F). Also noted during the image capture of gill cells from seawater-acclimated rainbow trout is the ~30% increase in size of the PNA^+ cells relative to the size of PNA^+ cells from freshwater rainbow trout. Quantification of this size change was performed, and this increase in size concurs with the increase in size of MR cells during seawater acclimation (Fig. 4).

These fluorescence images were used to quantify the relative numbers of the different MR cell subtypes during transition from freshwater to seawater. In freshwater, the total MR cell fraction is primarily made up of PNA^- cells. However, in either the 10‰ (not shown) or the 30‰ acclimated fish, almost all MR cells in the observed fields are PNA^+ (Fig. 3). Quantification of these changes was performed by counting the total number of MR cells and the number of PNA^+ MR cells in each corresponding field, to give the percentage of the MR cells that were PNA^+ (minimum five fields per fish). In freshwater, approximately 35% of the MR cells are PNA^+. After seawater acclimation, 78–94% of the MR cells are PNA^+ (Fig. 5). Of note, these percentages represent only the relative distribution of the PNA^+ and PNA^- cells and cannot distinguish between an increase in one cell type and a decrease in another. Unfortunately, absolute cell counts are not possible due to variable loss of total cells throughout the protocol, with each step involving suction and/or handling of the cells. However, the relative distribution of cells should remain the

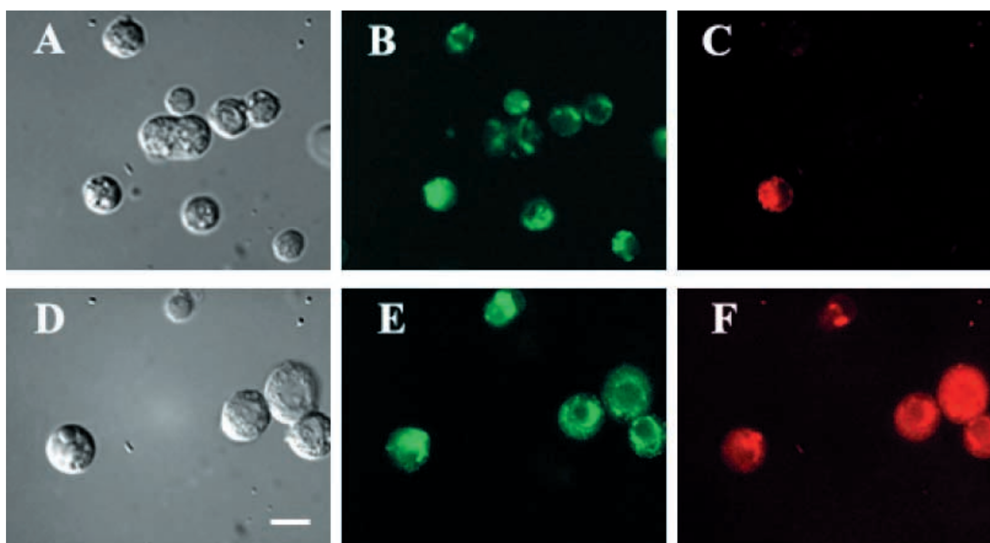

Fig. 3. Differential interference contrast (DIC) and fluorescence images of dispersed gill cells from freshwater rainbow trout (A–C) and seawater-adapted (30‰) trout (D–F). Cells were obtained from the mitochondria-rich (MR) 1.05–1.09 g ml^{-1} Percoll layer and stained with Mitotracker Green-FM (100 nmol l^{-1}, 20 min; B,E) or labelled with PNA Alexa fluor 594 (40 μg ml^{-1}; C,F) for identification of MR and PNA^+ cell types, respectively. All pictures in A–C and D–F are of the same field of view. Scale bar, 10 μm.

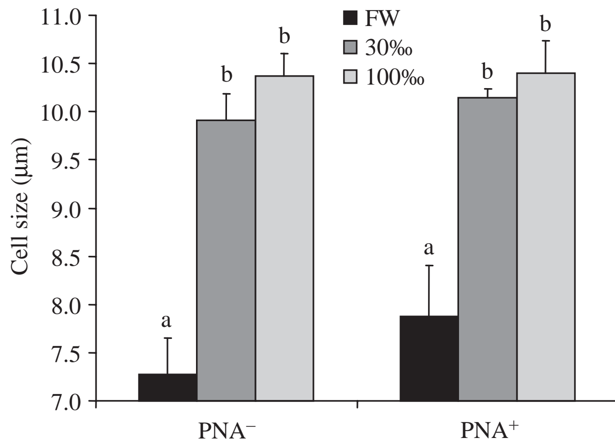

Fig. 4. Measured sizes of cells in freshwater and seawater-adapted trout. Cell sizes measured by calibrated measuring tool in viewing software from differential interference contrast (DIC) images taken for seawater adaptation experiments. Values represent means ± S.E.M. (*N*=30–40 cells for each population).

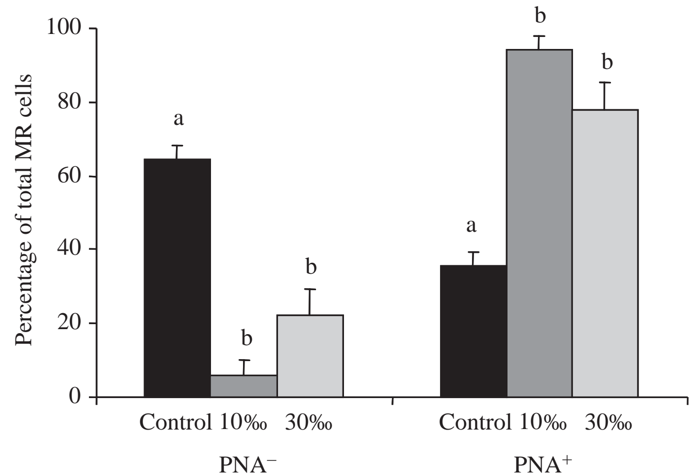

Fig. 5. Relative percentage of PNA⁻ and PNA⁺ cell types in populations of gill cells isolated from the MR 1.05–1.09 g ml^{-1} Percoll interface. Control fish (freshwater) are compared with seawater-adapted fish at partial-strength (10‰) and full-strength seawater (30‰). PNA⁺ and PNA⁻ cells counted under 100× objective of fluorescence microscope with a minimum of five fields of view counted for each freshwater and seawater fish. Values represent means ± S.E.M. (*N*=6 fish for each treatment group).

same within a single fraction in a single isolation. A potential complication was that the mobility of PNA⁻ cells in the Percoll gradients might have changed during acclimation of fish to seawater. However, MR cells were not detected in any of the other Percoll fractions (data not shown), indicating that seawater-acclimated MR cells are in fact all migrating to the same Percoll density fraction as found for freshwater MR cells and that monitoring the change in relative distribution of PNA⁺:PNA⁻ cells is valid.

The total MR cell fraction was separated into PNA⁻ and PNA⁺ using the MACS system (Galvez et al., 2002), and ouabain-sensitive (Na^+/K^+-ATPase) activity was measured. The PNA⁺ fraction showed a decrease in Na^+/K^+-ATPase activity, on a per cell basis, upon transfer of rainbow trout from freshwater to 100% seawater, while the PNA⁻ fraction demonstrated the opposite response. PNA⁻ Na^+/K^+-ATPase activity (on a per cell basis) was found to significantly increase during acclimation to full-strength seawater (Fig. 6).

We separated the total MR gill cells into PNA⁺ and PNA⁻ fractions and assessed bafilomycin-sensitive H^+-ATPase activity. Under freshwater conditions, H^+-ATPase activity in PNA⁻ cells was the same as in PNA⁺ cells but was substantially (~90%) decreased after acclimation to either 10‰ or 30‰ seawater (Fig. 7). Interestingly, we found that H^+-ATPase in the PNA⁺ cells of freshwater trout was also high and a similar trend, with a 73% reduction in activity, occurred during seawater acclimation.

Discussion

Previous reports from our lab have established that there are at least two functionally distinct sub-populations of MR cells in freshwater rainbow trout based on *in situ* electron microscope work (Goss et al., 2001) and fluorescence microscopy in live dispersed gill cells (Galvez et al., 2002). This previous work correlates with the earlier morphological studies of Pisam et al. (1987, 1990) and Wong and Chan (1999) suggesting different subtypes of gill MR cells. The present data are the first to show evidence of changes in function and relative distribution of specific MR cell subtypes during seawater acclimation. Pisam and colleagues, using transmission electron microscopy, demonstrated that there are lighter and darker staining MR cells in gills of freshwater fish, which they termed α- and β-cells, respectively (Pisam et al.,

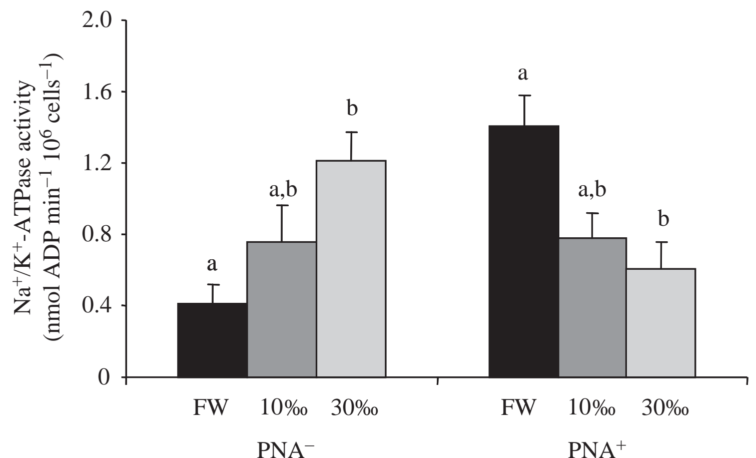

Fig. 6. Ouabain-sensitive Na^+/K^+-ATPase activity of control and seawater-adapted fish in the PNA⁻ and PNA⁺ cell fractions. Both partial-strength seawater (10‰) and full-strength seawater (30‰)-adapted fish used for testing. Values represent means ± S.E.M. (*N*=5–6 fish).

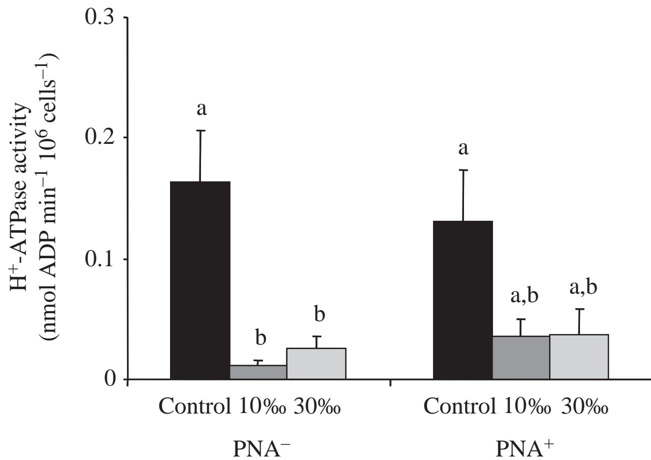

Fig. 7. Bafilomycin (50 nmol l^{-1})-sensitive H^+-ATPase activity in the PNA^+ and PNA^- gill cell fractions. Freshwater control fish compared with both 10‰ and 30‰ seawater-adapted fish. Values represent means ± S.E.M. (N=3–5 fish).

1987). Furthermore, during seawater acclimation, they found that β-cells were significantly reduced, leaving α-cells as the principal MR cell in seawater fish (Pisam et al., 1990). Similarly, using flow cytometry, Wong and Chan (1999) found in Japanese eels that there were two distinctly gated MR cell populations in freshwater, while during transition to seawater the size and internal complexity of the two MR cell populations increased. Furthermore, in freshwater, Wong and Chan demonstrated that two populations of MR cells existed: one type comprising ~67% of the population and the other ~33%. These values are very similar to the distribution of PNA subtypes seen in our freshwater trout (35:65 PNA^+:PNA^-). Furthermore, the major freshwater cell identified (F1 population) began disappearing by day 1 and was gone by day 3, a process that might appear in our study as a PNA^- cell-type reduction. Additionally, the increased size of the remaining MR cells after seawater transition correlates with the increased size noted for the PNA^+ cells isolated from seawater trout. These previous studies lend support to our finding that there is a transition of cell types during seawater acclimation in fish. The current study is the first to link these changes with alterations in biochemical function in independently separated cell types.

Levels of Na^+/K^+-ATPase have been used extensively as an index of transport capacity in fish exposed to a variety of conditions including seawater transfer (Yoshikawa et al., 1993; Mancera and McCormick, 2000). We have already demonstrated that the MR cell fraction possesses high levels of Na^+/K^+-ATPase activity compared with the other fractions in the Percoll density separation (Galvez et al., 2002). Activity and abundance of Na^+/K^+-ATPase in total gill homogenates have been demonstrated to increase with seawater acclimation and have been used numerous times as 'indicators' of readiness for migration or transition into seawater (see review by McCormick, 1995). Our data are consistent with these previous findings that Na^+/K^+-ATPase activity is elevated in the total gill cell fraction during transition to seawater (Fig. 1). We demonstrate that Na^+/K^+-ATPase activity is approximately fivefold higher in the gills of seawater-acclimated trout compared with that observed in the freshwater fish gill. This increase is in agreement with previously published figures (Epstein et al., 1980; Kultz et al., 1992; Wilson et al., 2002). However, the present paper is the first to separate and independently analyze this response into distinct MR cell fractions based on binding of PNA to the cell membrane. We found that the two cell fractions respond differently to seawater acclimation. While the Na^+/K^+-ATPase activity of PNA^+ cell types is significantly greater than that of the PNA^- cells under freshwater conditions, Na^+/K^+-ATPase activity in the PNA^- cells of seawater-acclimated fish is increased while that of PNA^+ cells is decreased.

The decrease in Na^+/K^+-ATPase activity during seawater adaptation in both PNA^+ and PNA^- cells seems contradictory given that total gill Na^+/K^+-ATPase activity is increased. We believe that there are at least two possible explanations for these changes. The first is that the noted decrease in Na^+/K^+-ATPase activity is a result of an overall decrease to half of the value on an individual cell basis. However, to have this occur and still be congruent with increases in total Na^+/K^+-ATPase activity on a whole gill basis, there must be a large increase in the total number of PNA^+ MR cells in seawater fish, thereby increasing total Na^+/K^+-ATPase activity. When examining sections of gills by light microscopy, although there has been a substantial (up to fivefold in some studies) increase in the total number of chloride cells in seawater-adapted fish (Borgatti et al., 1992; Shikano and Fujio, 1998) this cannot entirely account for the noted drop in the average cell Na^+/K^+-ATPase activities found in the present study. A second possible explanation for the noted decrease in Na^+/K^+-ATPase activity in PNA^+ MR cells during seawater adaptation is methodological. It is possible that accessory cells that are expressed only in seawater may also be PNA^+ and also migrate to the same fraction as the MR PNA^+ chloride cells in the Percoll density gradient. If accessory cells are found in the PNA^+ fraction then our technique for isolating PNA^+ cells in seawater fish would result in a mixed population of MR chloride cells and accessory cells, making interpretation of the results difficult. Accessory cells are known to possess lower Na^+/K^+-ATPase activity compared with MR cells, as demonstrated by ouabain-binding studies (McCormick, 1990), but can be considered relatively mitochondria-rich, at least when compared with surrounding PVCs (Laurent and Dunel, 1980). In our assay, the accessory cells would appear as MR, as the assay is only a relative comparison to the PVC fraction. By combining the two cell types into one population, we would predict a decrease in Na^+/K^+-ATPase activity depending on the relative number of accessory cells in each preparation. We cannot differentiate between PNA^+ MR cells and accessory cells once cells are dispersed. Accessory cells are usually identified under the electron microscope by their close association with 'MR chloride cells' in the base of the lamellae. The lack of specific gill localization of the accessory cells does not allow for distinct identification. This is a potential problem

with isolation and characterization of PNA^+ cells in seawater-adapted fish and makes final interpretation of these data difficult. We must find a specific marker to identify one of the cell types in seawater fish to resolve these differences.

The current model for ion exchange in the cells of freshwater fish links Na^+ uptake to a rheogenic H^+-ATPase. This H^+-ATPase drives the uptake of Na^+ through an apical epithelial Na^+ channel (probably a phenamil-sensitive ENaC type Na^+ channel, although direct molecular evidence is lacking) by creating a negative potential inside the cell (see review by Perry, 1997). We have previously shown that there is an acid-inducible, phenamil-sensitive Na^+ uptake attributed to the PNA^- cells in freshwater trout gills (Reid et al., 2003). Furthermore, western blot analysis shows that the expression of H^+-ATPase is elevated in the PNA^- cells, relative to that of PNA^+ cells, and increased during hypercapnic acidosis but not during infusion of bicarbonate. Of note, however, was the relatively high expression of H^+-ATPase in the PNA^+ cells under freshwater control conditions similar to that found earlier in our lab (Galvez et al., 2002).

In seawater, the requirement for H^+-ATPase-driven Na^+ uptake is reduced or eliminated due to a favourable Na^+ gradient into the fish. It has been shown that Na^+ movement in a seawater situation is more likely through an energetically favourable sodium/proton exchange mechanism (NHE; Claiborne et al., 1999). Reductions in H^+-ATPase immunoreactivity (Lin et al., 1994; Piermarini and Evans, 2001) and biochemical activity (Lin and Randall, 1993; Wilson et al., 2002) have been previously demonstrated during seawater acclimation in fish. These results are comparable to those seen in the present study (Fig. 2), with total MR cell H^+-ATPase activity decreased during seawater acclimation to about one-third of the activity of that of the freshwater fish.

An important contribution of the present paper is the analysis of independent changes in H^+-ATPase activity in the PNA^- MR cell population. When the total MR gill cell fraction was separated into PNA subtypes, we found that seawater adaptation resulted in a notable decrease in H^+-ATPase activity in both the PNA^- and the PNA^+ cells. The decreases in H^+-ATPase activity found in PNA^- cells during seawater adaptation were predicted based on previous attribution of Na^+ uptake to this particular cell type. In freshwater, the PNA^- cell type is responsible for Na^+ uptake, as demonstrated by Reid et al. (2003), where acid-stimulated, phenamil-sensitive Na^+ uptake could only be found in the PNA^- cell fraction and not in the PNA^+ fraction. Theoretically, Na^+ uptake from low ionic strength media requires active H^+ extrusion to the water *via* an apical H^+-ATPase to provide the driving gradient for Na^+ uptake. This mechanism for Na^+ uptake was first proposed by Avella and Bornancin (1989) based on the frog skin model of Na^+ uptake (Ehrenfeld et al., 1985; Harvey and Ehrenfeld, 1986, 1988). A similar mechanism has also been demonstrated for acid–base regulation in the mammalian kidney (Brown and Breton, 2000). In the kidney, two types of MR cells exist, termed α- and β- (acid- and base-secreting, respectively) intercalated cells. These cells have similar morphological and physiological characteristics to the MR cells in the fish gill. The results of this and previous experiments are consistent with this model and allow us to suggest that the PNA^- cell is the α-MR cell in the fish.

Of note, our terminology is inconsistent (opposite) with that of the α- and β-MR cells coined by Pisam et al. (1987). Our terminology follows functionally from the α- (acid-secreting) and β- (base-secreting) intercalated cells in the mammalian kidney and is based primarily on the fact that the PNA^- cell types in both tissues are responsible for Na^+ uptake and acid excretion while the PNA^+ cells in the kidney have been demonstrated to be involved in base secretion (apical Cl^-/HCO_3^- exchange). Pisam et al. (1987) based their observations on morphological (staining) characteristics that, unfortunately, do not appear to match the functional equivalents in the mammalian kidney.

During seawater acclimation, external Na^+ rises to a level that favours inward-directed Na^+ movement without the need for an energetically expensive apical H^+-ATPase. Our results, showing a reduction in PNA^- (α-MR cell) H^+-ATPase during seawater acclimation, are consistent with this model. Other authors (Lin et al., 1994; Piermarini and Evans, 2001) have also documented a reduction in H^+-ATPase immunoreactivity during seawater acclimation and also the appearance of immunoreactivity of the NHE-2 isoform of the Na^+/H^+ exchanger family in seawater-adapted fish (Wilson et al., 2002).

We were surprised at the relatively high levels of H^+-ATPase activity found in the freshwater PNA^+ cells and the concomitant reductions that occurred during seawater acclimation. The fact that PNA^- cells appear to perform Na^+ uptake/H^+ excretion in freshwater suggests that PNA^+ cells are responsible for Cl^- uptake/base excretion. While the reduction in H^+-ATPase activity during seawater adaptation in PNA^- cells may be due to methodological problems, with the possibility of accessory cell contamination in the seawater PNA^+, the high levels of H^+-ATPase activity in freshwater suggest that the H^+-ATPase may play a significant role in Cl^- uptake and acid–base regulation in freshwater fish. Chloride uptake from very dilute freshwater does not have a favourable driving electrochemical gradient from the water to the fish. Similarly, a strict gradient for HCO_3^- from the blood to the water is not sufficient to provide a driving gradient for HCO_3^- excretion under normal conditions. The relatively high levels of expression of H^+-ATPase in PNA^+ cells under freshwater conditions suggest that Cl^- uptake may be linked to the activity of H^+-ATPase, although the mechanism(s) is as yet undetermined. Immunocytochemical localization of the anion exchanger Pendrin through Pendrin-like immunoreactivity has been demonstrated for euryhaline stingrays (Piermarini et al., 2002) but has not yet been demonstrated for salmonids. Our future research focus will take advantage of our ability to isolate MR cells into separate populations and to use this technique to functionally characterize these individual cell populations and their adaptations during environmental changes. The power of this approach is demonstrated in the above results, whereby independent changes in biochemical

activity in separate cell types are unmasked by the ability to separate the MR cells.

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