Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout

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The gill epithelium of freshwater fish is a dynamic ion transporting epithelium that can regulate blood pH by manipulating the relative rates of Cl− and Na+ uptake. Current models suggest that Cl− uptake is linked to base extrusion, whereas transepithelial Na+ transport is likely coupled to H+ extrusion through an apical Na+ channel electrogenically coupled to a V-type H+-ATPase (7, 26). The cellular location of these transport processes, the molecular identification of the transporters involved, and how these transporters are regulated remain largely unknown. The cell types in the gill epithelium most often implicated in ion and acid-base transport include the chloride cell (CC) and, to a lesser extent, the pavement cell (PVC). Recently, Pisam and co-workers (30–32) suggested that a third cell type, the accessory cell, which is normally only found in seawater fishes, is also present in freshwater fishes. They suggested that the accessory cells may play a role in ion and acid-base transfer.

The role of each of these cell types in ion transport is still largely unknown. The heterogeneous nature of the gill makes studies of individual physiological function of various cell types and direct linkage of these transport processes to a specific cell type difficult. Evidence for only implicating the CC were the defined ultrastructural characteristics such as high mitochondria density, well-developed vesiculotubular network, and high levels of Na+–K+–ATPase expression, characteristics associated with transporting cells (13). However, when examining unidirectional Cl− uptake during acidosis and alkalosis, Goss and colleagues (4, 5, 10, 23) demonstrated a good correlation between Cl− transport and the CC fractional surface area of the gill epithelium. However, in similar treatments where acid-base status was disturbed, they were unable to demonstrate the same correlation for unidirectional Na+ uptake and H+ excretion, suggesting that the site for Na+ transport/H+ excretion is not the CC. Other studies have also suggested that PVCs are involved in ion and acid-base regulation and are potentially the site of the Na+–K+–ATPase or to the CC fractional surface area of the gill epithelium.

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rich (MR) cells in the gill epithelia of freshwater rainbow trout (3). This characterization is based on differential binding of MR cells to peanut lectin agglutinin (PNA). Similar subtyping of MR cells based on PNA staining has been demonstrated for the intercalated cells from the collecting duct of mammalian kidney (15, 34). Further research demonstrated that PNA binds specifically to the apical surface of base-secreting β-type intercalated cells (IC) of the inner medullary collecting ducts of mammalian kidney (21, 33). Recently, Tsuganezawa et al. (37) took advantage of these PNA binding properties to isolate pure populations of PNA+ β-intercalated cells. This isolation proved to be a requirement for successful cloning and characterization of the apical anion exchanger (AE4) expressed in these cells.

In freshwater fish, it has long been proposed that different subtypes of MR CCs exist based on ultrastructural differences and differences in autofluorescence (27–31, 40, 41). However, these classes are based mostly on staining characteristics and localization within the tissue. No conclusive physiological or biochemical evidence has been given to support that the subtypes of MR cells are, in fact, functionally distinct cell types. The objectives of the present study were to develop a technique for isolating populations of each MR gill cell type, to identify each cell type using transmission electron microscopy (TEM) analysis, and to characterize the resultant populations using Western blotting for differences in functional proteins and expression of these proteins during acid-base disturbances. We report that there exist at least two types of functionally distinct MR cells in the freshwater fish gill and suggest that these are involved in manipulation of acid-base and ionic status.

MATERIALS AND METHODS

Experimental animals. Adult rainbow trout (~200 g) (Oncorhynchus mykiss) from Alberta Trout Growers were maintained in flow-through 450-liter fiberglass tanks supplied with aerated dechlorinated Edmonton tap water (hardness 160 ppm as CaCO3, total alkalinity 120 mg/l; pH 8.2). Water temperature was maintained at 15°C, and the photoperiod matched the natural regime of Edmonton, Alberta, Canada. Fish were fed once daily with dry trout pellets.

For experiments to develop the gill cell isolation and magnetic separation protocols, fish were randomly selected from holding tanks and killed immediately by overdose (1 g/l MS-222, Syndel) followed by a cephalic blow. Fish used for the hypercapnia or base infusion experiments were removed from holding facilities and placed individually in darkened, aerated flow-through Plexiglas aquariums (~6-liter capacity). Fish were allowed to acclimate to the aquariums for at least 24 h before experimentation. To induce an acidosis in the fish, in-flowing water was switched to water rendered hypercapnic by bubbling with 1% CO2 in air. In addition, boxes were individually aerated with 1% CO2 in air. Control parallel experiments were run with fish maintained under normocapnic conditions. Mixing of gas for aeration of boxes and in-flowing water was accomplished using 100% CO2 (Praxair) and compressed air (1:99) using calibrated flow meters and a gas equilibration column. To induce an alkalosis in the fish, a base-infusion protocol similar to that of Goss et al. (7) was followed. Briefly, fish were lightly anesthetized with 0.1 g/l MS-222 and implanted with polyethylene cannulas (PE-50; Clay-Adams) into the dorsal aorta (35). Fish were allowed to recover from surgery for 48 h and then were infused for 24 h with either NaHCO3 (140 mM, pH 7.8) or, as a parallel control, NaCl (140 mM, pH 7.8) at a nominal rate of ~1,000 μmol·kg body wt·h–1.

Materials. Streptavidin-conjugated Alexa fluor 594, Mitotracker Green-FM and calcine-AM were obtained from Molecular Probes, Eugene, OR. The magnetic cell separation system and anti-FITC microbeads used for MACS separation were from Miltenyi Biotech (Auburn, CA). Antibodies to Scol1, a yeast mitochondrial integral membrane protein, were obtained from Dr. M. Glerum, University of Alberta. Antibodies to the 31-kDa subunit of the kidney V-type H+-ATPase were obtained from Dr J. N. Fryer, University of Ottawa. Antibodies against the α-subunit of the Na+-K+-ATPase (Drosophila) were obtained from Dr. Doug Fambrong, Johns Hopkins University. Horseradish-peroxidase-coupled goat anti-rabbit secondary antibody and Luminol enhanced development reagents were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Commercial BCA protein kit was obtained from Pierce (Rockford, IL). All other reagents used in the protocol were obtained from Sigma Chemicals, St. Louis, MO.

Gill digestion protocol. Gill arches were excised from the fish, rinsed in dechlorinated tap water to remove coagulated blood and mucus, and lightly blotted to remove excess water. Gill filaments were cut from the gill rakers and placed in ice-cold Cortland’s saline (in mM: 143 NaCl, 5.0 KCl, 1.5 CaCl2, 1.0 MgSO4, 5.0 NaHCO3, 3.0 NaH2PO4, 5.0 glucose; pH 7.8). Gill arches were digested in 0.2 mg/ml collagenase (type IA) in Cortland’s saline for 20 min at 18°C with continuous circular agitation (300 rpm). Gill filaments were scraped with a glass microscope slide, and the gill suspension was filtered through 254- and 96-μm nylon meshes to remove large debris. The final filtrate of dispersed cells was diluted in PBS (in mM: 137 NaCl, 2.7 KCl, 4.3 NaH2PO4, 1.4 NaHCO3; pH 7.8) and centrifuged at 500 g for 10 min at 4°C. The absence of Ca2+ from the media significantly reduced clumping and aggregation of dispersed cells during subsequent steps of the isolation protocol. Dispersed cells were washed twice with at least 10 vol of PBS and resuspended in 2 ml PBS, placed on a 1.09 g/ml Percol solution, and spun for 10 min at 500 g (4°C) to separate contaminating red blood cells (RBCs) from the gill cell suspension. The gill cells remained on top of the 1.09 g/ml Percol solution, whereas RBCs pelleted to the bottom of the tube. Gill cells were again collected, diluted in ~10 vol of PBS, centrifuged for 10 min at 500 g, resuspended in 2 ml PBS, layered over either one a two-step gradient consisting of 1.03 and 1.09 g/ml Percoll or a three-step gradient consisting of 1.03, 1.06, and 1.09 g/ml Percoll (4 ml each) and centrifuged at 2,000 g (45 min). Consistent with the findings of Goss et al. (3), distinct bands of cells were collected from the Percoll interfaces of the discontinuous gradients after centrifugation. Cells from each of the Percoll interfaces were collected independently, washed once in ~10 vol PBS, centrifuged (2,000 g; 10 min), resuspended in 0.5–2.0 ml PBS, and used for analysis as appropriate. Gill cells from the 1.06–1.09 g/ml Percoll interface of the three-step gradient were further separated into PNA– and PNA+ fractions using magnetic cell separation (MACS) as outlined below.

Fluorescence microscopy and fluorescence spectrophotometry. Dispersed gill cells (1.03–1.06 g/ml or 1.06–1.09 g/ml) were incubated in PNA-FITC in PBS (20 min; 40 μg/ml) to identify and quantify the PNA+ cells using fluorescence microscopy. Mitotracker green-FM (20 min; 100 nM) was used.
to characterize mitochondria staining in the different fractions of isolated dispersed cells using both an inverted microscope, to identify staining in single cells, and a fluorescence plate reader, to quantify mitochondria staining in gill cell populations. The percentage of MR cells binding PNA was determined by labeling cells first in biotin-conjugated PNA (PNA-biotin; 20 min, 40 μg/ml), washing the cells, and then double labeling the cells with streptavidin-Alexa fluor 594 (20 min; 20 μl/ml) and Mitotracker green-FM (20 min; 100 nM). Gill cells were washed twice in PBS to remove unbound dye(s). For fluorescence microscopy, cells were placed on glass slides for differential interference contrast (DIC) microscopy (Nikon Eclipse 300) and fluorescence imaging (TE-FM Epi-Fluorescence attachment) using an inverted microscope. The fluorescence microscope was equipped with epi-illumination via a Xenon arc lamp (Lambda LS, Sutter Instruments, Novato, CA). PNA-FITC, Mitotracker green-FM or Calcein-AM (excitation 495 ± 5 nm; dichroic 515 nm cut off; emission 540 ± 25 nm) and PNA-Alexa fluor 594 were imaged (excitation 560 ± 20 nm; dichroic 595 nm long pass; emission 670 ± 30 nm) using Plan-Fluor objectives at ×40 [0.6 numerical aperture (NA) air] and ×100 (1.3 NA oil immersion). Images were digitally captured on a 12-bit charge-coupled device camera ( Cooke SensiCam, Kelheim, Germany). Fluorescence images were often binned at 2 × 2 to increase the sensitivity of the fluorescence capture. Final images were adjusted for contrast and brightness only using Adobe Photoshop 6 software. To examine relative mitochondrial staining in the fractions, 20 × 10³ Mitotracker-labeled cells in 200 μl PBS (as above) from each fraction were loaded into a 96-well plate and relative fluorescence intensity was read (excitation 485 ± 25 nm; emission 538 ± 25 nm) using a fluorescence microplate spectrophotometer (Molecular Dynamics fmax).

**Magnetic cell separation protocol.** Magnetic cell separation was used to isolate PNA⁺ cells. Recent studies in our laboratory (3) showed that PNA⁺ cells fractionate exclusively to the 1.06–1.09 g/ml Percoll interface. Magnetic separation was only performed on cells from this fraction. Gill cells collected from the 1.06–1.09 g/ml Percoll interface were incubated in PNA-FITC and rotated continuously. Cells were incubated in a solution of anti-FITC antibodies covalently linked to the top of a positive selection iron column (LS/H11001) and anti-FITC microbeads bound would be retained in the column. The supernatant was given 3 and 2 ml of degassed PBS buffer. The cells were washed twice to remove unbound anti-FITC microbeads and resuspended in 1–2 ml of degassed PBS buffer. The gill suspension was passed through a 30-μm filter attached to the top of a positive selection iron column (LS⁺, Militynei held within a magnetic field). Cells with PNA-FITC and anti-FITC microbeads bound would be retained in the column as long as the magnetic field was present. The column was given 3 × 3 ml rinses with PBS. The cells passing through the column in the presence of the magnetic field were collected and termed the PNA⁻ fraction. The column was then removed from the magnet and 5 ml PBS was added to the column and the cells were eluted from the column using a plunger. The eluted cells were termed the PNA⁺ fraction. Both fractions were centrifuged at 500 g for 10 min at 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 0.5–1.0 ml PBS buffer.

**Flow cytometry analysis.** PNA⁺ cells, PNA⁻ cells, and the total 1.06–1.09 g/ml fraction (pre-MACS separation) from untreated cells were collected, diluted to 1 × 10⁶ cells/ml in PBS, passed through a 96-μm syringe filter, and subjected to flow cytometric (FC) analysis (Becton-Dickenson FACScan Calibur). Gill cells were analyzed for relative cell size [forward light scatter (FLS)], relative cell complexity [side light scatter (SLS)], and fluorescence emission (FL1). The flow cytometer was equipped with an argon laser (excitation 488 ± 5 nm, emission 530 ± 15 nm). Preliminary FC analysis of gill cells from the 1.06–1.09 g/ml fraction showed that distinct populations of cells could not be identified in rainbow trout solely on relative cell size or cell complexity. To further identify cell populations using FC analysis, the distribution of fluorescent-labeled cells incubated with PNA-FITC was analyzed using FL1. PNA-FITC-labeled cells were primarily gated to regions of increased relative cell size (high FLS) or cell complexity (high SLS). Percentage of total cells binding PNA-FITC and gating to the high-FLS and high-SLS region was analyzed in cells harvested from the total 1.06–1.09 g/ml, PNA⁺ and PNA⁻ fractions.

**TEM.** Isolated PNA⁺ and PNA⁻ gill cells were prepared for TEM to definitively identify the ultrastructural characteristics of these cells. Cells were pelleted in a microcentrifuge tube and fixed in a 2.5% glutaraldehyde-3% paraformaldehyde solution (Marivac, Halifax, NS) buffered with 0.15 M sodium cacodylate (pH 7.8; 290 mosM) for 2 h at 4°C. All subsequent procedures including washes, were performed with minimal disruption of the pellet. Cell pellets were post-fixed in 2% osmium tetroxide in water for 2 h, followed by dehydration steps in 20, 40, 70, 90, and 100% (3 × 10 ml) ethanol at 60°C for 2 days. Ultrathin sections (~90 nm) were prepared with an automatic ultramicrotome (Reichert Ultracut E) using a diamond knife. Sections were collected on nickel grids (300 mesh) and stained with 1% uranyl acetate (1 h, room temperature) and 0.02% lead citrate (1 min). Grids were examined in the TEM (Philips Morgani, model 268).

**Western blot analysis.** Samples collected from each of the fractions were placed in denaturing buffer (500 mM Tris-HCl, 5% glycerol, 10% SDS, 1% bromophenol blue, 5% mercaptoethanol), gelated, and analyzed by SDS-PAGE (10% acrylamide) followed by semi-dry horizontal transfer (BioRad) onto nitrocellulose membranes for immunoblotting. To ensure equivalent loading in each lane, equal cell numbers (Sco1 experiments) or equivalent total protein (for analysis of Na⁺/K⁺-ATPase and H⁺-ATPase expression) was loaded into each lane. After transfer, nitrocellulose sheets were placed in blocking buffer (5% skim milk powder in antibody buffer-140 mM NaCl, 10 mM Tris base, 0.03% Tween 20, pH 7.4) and incubated for 1 h at room temperature. After washing with antibody buffer (3 × 20 min), blots were incubated overnight (4°C) on a rocking platform in antibody buffer plus 1% skim milk powder containing antibodies raised against Sco1 (1:1,000), anti Na⁺/K⁺-ATPase (1:2,500), or anti V-type H⁺-ATPase (31-kDa subunit 1:2,500). The following morning, blots were quickly rinsed in double-distilled H₂O and washed in large volumes of antibody buffer (3 × 20 min). The secondary antibody, HRP-conjugated anti-rabbit IgG (1:5,000 in antibody buffer with 1% skim milk powder), was incubated with the blot on a rocking platform for 1 h at room temperature. The secondary antibody was removed by washing in antibody buffer (3 × 20 min). Detection of proteins was accomplished using a Luminol Western blotting detection kit (Santa Cruz) and detection on X-ray film. Resultant bands were quantified by densitometric analysis using Scion Image software.

**Statistical analysis.** One-way ANOVA and the post hoc Student-Newman-Keuls test were performed using SPSS version 8.0 software to test for significant differences between control and experimental groups. P < 0.05 was used as the fiducial limit of significance.
RESULTS

PNA binds to MR chloride cells in the gill epithelium. We previously demonstrated (3) that PNA binds in situ to the exposed surface of cells located in the interlamellar region of the gill filament. On the basis of migration patterns in a Percoll density gradient and EM studies, it was suggested that PNA binds to MR chloride cells. To determine if the cells that bind PNA are indeed mitochondria rich, we separated dispersed gill cells using a two-step Percoll gradient to separate the mucous cells from the mixed PVC and CC fraction. This fraction was stained with Mitotracker Green-FM, a vital mitochondria stain, to examine staining patterns in live dispersed gill cells. Figure 1, A and B, demonstrates paired differential interference contrast (DIC) and fluorescence microscopic images of total dispersed gill cells (from a 1.03–1.09 g/ml interface) from untreated rainbow trout. This fraction yielded a mix of MR cells and pavement cells without any mucous cells or cellular debris. In this fraction, cells varied considerably in size and internal complexity based on DIC microscopy (Fig. 1A). Additionally, there was a large range in mitochondrial density as illustrated by fluorescence microscopy using Mitotracker Green. Some cells had high amounts of fluorescence, while others displayed much lower levels (Fig. 1B). The PNA staining (red) is found over the entire surface of the cells, although it appears peripheral due to the nature of the focused optics of the microscope. Mitotracker staining is clearly intracellular and punctate (green). Classical features of pavement cells, such as surface microridges (Fig. 1A, arrow and inset) and large irregularly shaped nuclei (Fig. 1A, asterisk), can be seen in many of the gill cells when passing through sequential focal planes of each cell. It was noted that cells displaying these features did not bind PNA. However, identification of distinct cell populations was not possible using only DIC microscopy and combined fluorescence imaging of Mitotracker. To determine if PNA binding cells are indeed MR cells, we double stained the total gill cell (1.03–1.09 g/ml) fraction with both PNA-Alexa fluor 594 and Mitotracker Green-FM. Double staining of gill cells allowed us to characterize at least three populations of cells in the 1.03–1.09 g/ml gill cell dispersion (Fig. 1, C and D). Cells that weakly stained with Mitotracker Green never bound PNA specifically (PNA−, low mitochondria density). Other cells stained intensely for mitochondria using Mitotracker but did not also stain with PNA (PNA−, MR cell). However, ~6% of the MR cells also stained PNA strongly on their cell surface, as noted by the intense peripheral staining pattern of PNA-Alexa fluor 594 (Fig. 1D), representing a PNA+ MR cell subtype. With the use of DIC microscopy, the PNA binding MR cells were always characterized by high internal complexity and small ovoid nuclei.

Isolation of MR chloride and MR pavement cells by MACS. A three-step rather than a two-step discontinuous Percoll density gradient was recently shown to separate MR gill cells (combined PNA+/H11001 and PNA+/H11002) from untreated rainbow trout. Cells were harvested from a 1.03–1.09 g/ml Percoll interface and stained with either Mitotracker only (100 nM, 20 min; A and B) or double stained with PNA-biotin (40 μg/ml) and streptavidin-conjugated Alexa fluor 594 (20 μl/ml) (C and D) and the mitochondrial stain Mitotracker Green-FM (100 nM, 20 min). A: arrow indicates the surface microridges and the * represents an irregularly shaped nucleus. A, inset: a different focal plane of the cell identified by the arrow that more clearly shows the surface microridges. Scale bar, 2 μm.

Fig. 1. Differential interference contrast optics (DIC; A and C) and fluorescent images (B and D) of Mitotracker-stained dispersed gill cells from 2 representative untreated rainbow trout. Cells were harvested from a 1.03–1.09 g/ml Percoll interface and stained with either Mitotracker only (100 nM, 20 min; A and B) or double stained with PNA-biotin (40 μg/ml) and streptavidin-conjugated Alexa fluor 594 (20 μl/ml) (C and D) and the mitochondrial stain Mitotracker Green-FM (100 nM, 20 min). A: arrow indicates the surface microridges and the * represents an irregularly shaped nucleus. A, inset: a different focal plane of the cell identified by the arrow that more clearly shows the surface microridges. Scale bar, 2 μm.
from the less dense pavement and mucus cells (3). In the present study, a novel magnetic bead separation system (MACS) was developed to further fractionate MR cells (1.06–1.09 g/ml fraction) based on their differential staining to PNA. After MACS separation, we analyzed the PNA− (Fig. 2, A and B) and PNA+ (Fig. 2, C and D) MR cells by DIC microscopy and PNA staining (PNA-FITC). The PNA− fraction contained cells that were heterogeneous in morphology (Fig. 2A) but did not bind PNA (Fig. 2B). Less than 1% of all cells in this fraction had detectable PNA binding after MACS. The PNA+ fraction contained cells of which >95% were positive for PNA staining (Fig. 2D). The morphology of PNA binding cells could be viewed using DIC microscopy (Fig. 2C). PNA+ cells had high numbers of intracellular organelles, presumably mitochondria, small ovoid nuclei, and a somewhat flattened appearance. Staining of the PNA− and PNA+ cells with the vital dye calcein-AM demonstrated that >90% of the isolated cells were viable after the isolation procedure (data not shown).

FC was used to analyze the enrichment of the PNA cells using the MACS system. Using FC analysis, we were able to monitor relative size, complexity, and fluorescence intensity of cells according to changes in FLS, SLS, and fluorescence intensity (FL1), respectively. FC analysis of total gill cell populations isolated from a two-step gradient produced no noticeable foci and therefore little information could be gained from this analysis. With the use of a three-step gradient, gill cells collected from the 1.06–1.09 g/ml Percoll interface and before MACS were still heterogeneous in cell size and internal cell complexity as indicated by large scatter in the SLS vs. FLS plot (data not shown). Distinct cell populations could not be identified in the 1.06–1.09 g/ml cells before MACS separation, although fluorescence (FL1) analysis showed that there existed a population of highly fluorescent (PNA bound) cells within this population. Cells gating to this region were then monitored during subsequent flow cytometric analyses of the pre-MACS fraction (Fig. 3A), PNA− (Fig. 3B), and PNA+ (Fig. 3C) fractions. The PNA− fraction had very few highly fluorescent events gating to this region, suggesting a depletion of the cells from this region (Fig. 3B). Meanwhile, the number of highly fluorescent events in the PNA+ fraction increased well above the pre-MACS fraction, suggesting isolation and enrichment of the PNA+ cells.

Quantitative analysis of Mitotracker staining for each of the PNA+ and PNA− fractions was performed on populations of cells using a fluorescence plate reader and is expressed as relative fluorescence units (Fig. 4). Gill cells collected from the 1.06–1.09 g/ml fraction had significantly higher (~4-fold) relative fluorescence staining of Mitotracker Green than cells from the 1.03–1.06 g/ml fraction (Fig. 4). Separation of the mixed 1.06–1.09 g/ml MR fraction into both PNA+ and PNA− fractions using MACS demonstrated a small but significant difference in MR density between the PNA+ and PNA− fractions. However, both were still significantly higher than the 1.03–1.09 g/ml fraction, suggesting that two types of MR gill cells (from the 1.06–1.09 g/ml fraction), PNA+ and PNA−, are found in the freshwater fish gill. To further validate the findings that both fractions were indeed MR cells, we performed a Western blot for the presence of a con-
served mitochondria protein (Sco1) that is expressed in the inner mitochondria membrane. This antibody was raised against yeast Sco1 but detected a band at the appropriate size in the fish gill lysates, demonstrating heterologous cross-reactivity of this antibody. Figure 5A is a representative Western blot for gill cells fractionated by a three-step Percoll gradient (1.03, 1.06, and 1.09 g/ml) followed by separation of the 1.06–1.09 g/ml interface into PNA+ and PNA− fractions by MACS. PNA− and PNA+ fractions (1.06–1.09 g/ml) had Sco1 levels 23- to 26-fold higher than in cells from the 1.00–1.03 g/ml Percoll interface and ~4.5-fold higher than in cells from the 1.03–1.06 g/ml fraction (Fig. 5B), in good agreement with Mitotracker fluorescence of isolated gill cell fractions reported in Fig. 4. However, we did not observe a significant difference in Sco1 expression between PNA− and PNA+ gill cell fractions (Fig. 5B).

To definitively identify the cell types collected in the PNA− and PNA+ fractions, cells were isolated by MACS separation, fixed, and their ultrastructure examined by TEM (Fig. 6). PNA+ cells had features commonly identified in CCs, such as numerous mitochondria, an extensive network of intracellular tubes, and homogenous staining of chromatin (Fig. 6, A and B). In most cells, we could visualize the iron particles (~50 nm) conjugated to the anti-FITC microbeads (as required in MACS separation) bound to the plasma membrane of CCs alone. This can be seen as the particles located in the fuzzy coat that is seen on MR CCs (arrow in Fig. 6B). High-magnification images of these cells demonstrated a vesiculotubular network free from ribosomes, a classical identifying feature of MR chloride cells from the fish gill. Cells collected in the PNA− fraction were also rich in mitochondria (Fig. 6C), yet the remaining ultrastructural features in most of the cells present were more characteristic of PVCs. These features included irregularly shaped nuclei, dense chromatin staining throughout the nuclei, lack of a vesiculotubular network, and a plasma membrane forming microvilli-like projections. The plasma membranes lacked the fuzzy appearance produced by the 50-nm iron MACS particles and there was a noticeable absence of a vesiculotubular network (Fig. 6D). How-
ever, there were also cell types other than MR PVCs, present in this fraction, including those with dense core granules and a few contaminating RBCs.

Functional characterization of MR cells in the fish gill. We hypothesized that the MR cell subtypes in fish gills represent functionally distinct cells, similar to the functional differentiation of MR subtypes in the cortical collecting duct of mammalian kidney. To test this hypothesis, PNA− and PNA+ gill cells were isolated from normoxic and hypercapnic fish. We recently demonstrated the binding of PNA to a subtype of MR cell in the gill epithelium of freshwater fish (3). In the present study, a novel magnetic bead separation technique was used to enrich cells binding PNA from a heterogeneous mixture of isolated dispersed gill cells. The ultrastructure of PNA+ cells was characteristic of the MR CC, whereas PNA− cells had morphological features more typical of pavement cells, with the exception of having substantial numbers of mitochondria. Western blot analysis was used to monitor changes in protein expression of epithelial transport proteins in the MR CCs and MR pavement cells during respiratory acidosis and metabolic alkalosis. This study is the first to characterize two functionally distinct subtypes of MR cells in the gill epithelium of freshwater fish based on differential expression of epithelial transport proteins.

The presence of different subtypes of MR cells in the gill epithelium of freshwater fish has been reported numerous times. Pisam and colleagues (28, 30) proposed two distinct MR cellular subtypes in freshwater fish gill based on differences in ultrastructure and the staining characteristics of electrophilic stains used in TEM analysis. They termed these cell subtypes as α- and β-chloride cells. Further experiments demonstrated that both α- and β-cells were present in the gill epithelium of freshwater fish, and that β-CCs disappeared during seawater acclimation. Recently, Wong and Chan (40) also identified two types of MR cells in

DISCUSSION

We recently demonstrated the binding of PNA to a subtype of MR cell in the gill epithelium of freshwater fish (3). In the present study, a novel magnetic bead separation technique was used to enrich cells binding PNA from a heterogeneous mixture of isolated dispersed gill cells. The ultrastructure of PNA+ cells was characteristic of the MR CC, whereas PNA− cells had morphological features more typical of pavement cells, with the exception of having substantial numbers of mitochondria. Western blot analysis was used to monitor changes in protein expression of epithelial transport proteins in the MR CCs and MR pavement cells during respiratory acidosis and metabolic alkalosis. This study is the first to characterize two functionally distinct subtypes of MR cells in the gill epithelium of freshwater fish based on differential expression of epithelial transport proteins.

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the gills of freshwater-acclimated Japanese eel with discontinuous density centrifugation and FC. The criteria used for identification of cell types were differences in relative cell size, cell complexity, and autofluorescence. Goss et al. (3) recently showed that MR cells from the gill epithelium of rainbow trout also migrate exclusively to a high-density Percoll interface (1.06–1.09 g/ml) in a manner similar to those studied by Wong and Chan (40). However, unlike Wong and Chan (40), we had limited success in identifying subpopulations of MR cells using FC alone in rainbow trout. It is possible that differences in cell size, relative complexity, and autofluorescence are not distinct enough in the trout compared with the eel to differentiate the two MR cell populations. We showed through DIC microscopy and fluorescence imaging of a dispersed gill cell suspension that trout gill cells vary considerably in their size, internal complexity, and mitochondria content and these features likely make sorting by FC unfeasible in rainbow trout.

The subtyping of MR cells in transporting epithelia of other animals has been well documented in the literature (1, 20). One common method used to subtype MR cells involves the differential binding of PNA to the apical surfaces of some of these cells. PNA is a lectin that binds specifically to the terminal D-galactosyl residues of glycosylated proteins (18). LeHir et al. (15) first demonstrated that PNA could exclusively bind to the luminal membrane of intercalated cells in distal tubules and cortical collecting ducts of rabbit kidney. Recently, we (3) also demonstrated in situ PNA binding to the apical surface of the gill epithelium of rainbow trout. Cells staining strongly to PNA were located at the base of lamellae and in the interlamellar region of the filament toward the trailing edge of the filamental epithelium. The localization of PNA staining and the associated increase in staining after 6 days of cortisol injection strongly suggested that the PNA binding cell was an MR CC. This hypothesis was further supported by electron microscopy of dispersed gill cells. In the present study, fluorescence microscopy showed that MR cells always stained strongly with Mitotracker Green, a mitochondrial fluorescent dye, and showed expression of a conserved mitochondria membrane protein. However, only a small, yet significant, fraction stained strongly to PNA. At best, the percentage of PNA+ cells in a dispersed suspension could be enriched from ~2 to ~20% purity using Percoll density centrifugation alone, in agreement with the efforts of Perry and Walsh (25) in the toadfish. Because our objective is to functionally characterize the role of MR cell populations in acid/base regulation, we are required to work with relatively pure populations of each cell type. This meant we had to develop a novel technique for obtaining pure populations of PNA+ MR cells from a dispersed gill suspension.

We adopted a magnetic bead separation approach to enrich gill cells collected from a high-density Percoll centrifugation. Using this technique, we were able to separate MR gill cells into PNA+ and PNA− fractions, with the PNA+ fraction demonstrating >95% purity from an original fraction of <2%. Furthermore, TEM characterization of the PNA+ fraction definitively identifies it as the MR CC. The PNA− fraction was comprised primarily of MR cells as well, with characteristics more similar to pavement cells. However, there

![Image](https://example.com/image.png)
were also substantial numbers of other cells present in this fraction, including contaminating RBCs and cells with dense core granules in the cytoplasm. Development of a marker for the MR cells in the PNA⁻ fraction that can be used in our MACS isolation system would substantially enhance our ability to extract a more pure population. This would also allow for the in situ localization of the PNA⁻ MR cell subtype within the gill epithelium.

The existence of distinct subtypes of MR cells would allow for separation of the physiological functions, as has been found in the mammalian kidney (20). The differential staining of PNA to MR cells suggested that PNA⁻ and PNA⁺ cells likely had a distinct pattern of protein expression and that these proteins would confer certain functional properties with regard to acid/base regulation. Therefore, we explored whether PNA⁻ and PNA⁺ MR cells showed differential expression of two epithelial transport proteins, Na⁺-K⁺-ATPase and H⁺-ATPase, and tried to determine how the expression of these proteins was altered during acid/base disturbances. The Na⁺-K⁺-ATPase pump undoubtedly plays an important role in energizing both Na⁺ and Cl⁻ uptake across the gill epithelium of freshwater fish, and, therefore, its expression is expected to be high in cell types involved in ion and acid/base regulation (42).

If there is a functional separation of MR cell types into acid secreting and base secreting as has been found in the mammalian kidney (20), then we hypothesized that this might be reflected in changes in the relative expression of Na⁺-K⁺-ATPase expression between the two cell types. During acid-base and iono-regulatory disturbances, the relative activity, and hence expression of Na⁺-K⁺-ATPase of one cell type vs. another, might be altered. We found that hypercapnia resulted in an increase in the relative PNA⁻-to-PNA⁺ ratio of Na⁺-K⁺-ATPase from 0.3 to 0.8, which would be representative of increases in the relative activity of the PNA⁻ cell, decreases in activity of PNA⁺ cell type, or both. During hypercapnia, whole animal experiments (4, 12) revealed increases in H⁺ excretion and Na⁺ uptake during hypercapnia while Cl⁻ uptake and HCO₃⁻ secretion were shown to decrease. The changes in relative activity of Na⁺-K⁺-ATPase during hypercapnia are consistent with either an increase in activity in PNA⁻ cells, a decrease in activity of PNA⁺ cells, or both. During NaCl infusion there was also an unexpected increase in the Na⁺-K⁺-ATPase expression rat-

Fig. 7. Dispersed gill cells were harvested from the 1.06–1.09 g/ml Percoll fractions of fish exposed to 1% CO₂ (hypercapnia), 140 mM NaHCO₃ infusion, 140 mM NaCl infusion (infusion control), or untreated for 24 h. PNA⁻ and PNA⁺ fractions were obtained using magnetic cell sorting. Western blots (5 μg protein/lane) of cell fractions separated on 10% SDS-polyacrylamide gels and probed for an antibody against the Na⁺-K⁺-ATPase subunit. A: representative blot showing molecular mass markers at 52 and 83 kDa. B: values are means ± SE (n = 4–6) of the ratio of expression in PNA⁻ to PNA⁺ fractions. *Mean value significantly different from paired control.

Fig. 8. Dispersed gill cells were harvested from the 1.06–1.09 g/ml Percoll fractions of fish exposed to 1% CO₂ (hypercapnia), 140 mM NaHCO₃ infusion, 140 mM NaCl infusion (infusion control), or untreated for 24 h. PNA⁻ and PNA⁺ fractions were obtained using magnetic cell sorting. Western blots (5 μg protein/lane) of cell fractions separated on 10% SDS-polyacrylamide gels and probed for an antibody against a peptide in the 31-kDa subunit of bovine V-type ATPase. A: representative blot showing molecular mass markers at 28 and 52 kDa. B: values are means ± SE (n = 4–6 fish) of the ratio of expression in PNA⁻ to PNA⁺ fractions. *Mean value significantly different from paired control.
This change in ratio may be due to the overall reduction in required gill function for ionoregulation. NaCl infusion is known to decrease the CC fractional area exposed to water and measured $^{22}\text{Na}^+$ influx (6). This covering and reduction of expression in the MR CC cells (PNA$^-$) would account for the change in the ratio of Na$^+$-K$^+$-ATPase in the NaCl-infused fish.

The current model of Na$^+$ transport suggested that Na$^+$ transport across the apical surface of the gill epithelium of fish is electrogenically coupled to the H$^+$-ATPase transporter (22). Immunohistochemistry has shown elevated expression of H$^+$-ATPase in the gill epithelium of fish during respiratory acidosis. Cellular localization of the H$^+$-ATPase by Sullivan et al. (36) demonstrated expression of the 31-kDa subunit of the V-type H$^+$-ATPase exclusively in pavement cells on the lamellar epithelium of freshwater-acclimated rainbow trout. Furthermore, staining was noticeably increased in the lamellar epithelium of freshwater-acclimated rainbow trout. Additionally, immunocytochemical studies examining the distribution of plasma membrane vs. intracellular staining showed that the peripheral staining pattern of PNA on the membrane as would be required for an apical membrane transport across the apical surface of the gill epithelium remains controversial. The heterogeneity of cell types found on the gill epithelium has made characterization of transporters difficult to perform. Here we report a magnetic bead separation technique that demonstrates at least two functionally distinct populations of MR cells in the gill epithelium of freshwater rainbow trout. Our ability to enrich for specific MR cells should provide a means for advancing the molecular, cellular, and biochemical characterization of distinct MR cell subtypes.

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