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Scott D. Reid
Okanagan College

G. S. Hawkings
University of Alberta

F. Galvez
University of Alberta

G. G. Goss
University of Alberta

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Localization and characterization of phenamil-sensitive Na⁺ influx in isolated rainbow trout gill epithelial cells

Scott D. Reid^{1,2,*}, G. S. Hawkings², F. Galvez² and G. G. Goss²

¹*Dept of Biology, Okanagan University College, Kelowna, British Columbia, V1V 1V7, Canada and*

²*Dept of Biological Sciences, University of Alberta, Edmonton, Alberta, T5G 2E9, Canada*

*Author for correspondence (e-mail: sdreid@ouc.bc.ca)

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Summary

Percoll density-gradient separation, combined with peanut lectin agglutinin (PNA) binding and magnetic bead separation, was used to separate dispersed fish gill cells into sub-populations. Functional characterization of each of the sub-populations was performed to determine which displayed acid-activated phenamil- and bafilomycin-sensitive Na⁺ uptake. Analysis of the mechanism(s) of ²²Na⁺ influx was performed in control and acid-activated (addition of 10 mmol l⁻¹ propionic acid) cells using a variety of Na⁺ transport inhibitors (ouabain, phenamil, HOE-694 and bumetanide) and a V-type ATPase inhibitor (bafilomycin). We found that cells migrating to a 1.03–1.05 g ml⁻¹ Percoll interface [pavement cells (PVCs)] possessed the lowest rates of Na⁺ uptake and that influx was unchanged during either bafilomycin (10 nmol l⁻¹) treatment or internal acidification with addition of

propionic acid (10 mmol l⁻¹). Mitochondria-rich (MR) cells that migrated to the 1.05–1.09 g ml⁻¹ interface of the Percoll gradient demonstrated acidification-activated bafilomycin and phenamil-sensitive Na⁺ influx. Further separation of the MR fraction into PNA⁺ and PNA⁻ fractions using magnetic separation demonstrated that only the PNA⁻ cells (α -MR cells) demonstrated phenamil- and bafilomycin-sensitive acid-activated ²²Na⁺ uptake. We confirm the coupling of a V-type H⁺-ATPase with phenamil-sensitive Na⁺ uptake activity and conclude that high-density α -MR cells function in branchial Na⁺ uptake in freshwater fish.

Key words: mitochondria-rich cells, MR, chloride cells, CC, pavement cells, PVC, peanut lectin agglutinin, PNA, transport, fish, Na⁺ influx, density gradient.

Introduction

The gill of freshwater fish plays a key role in a variety of physiological processes such as gas transfer, ion regulation, acid–base balance and nitrogen excretion. Branchial ion regulation and acid–base balance are coupled events because freshwater fish modulate branchial ion uptake/loss in response to acid–base challenges (Cameron, 1976; Claiborne and Heisler, 1984, 1986; Perry et al., 1987). While there is general agreement on the mechanism (Cl⁻/HCO₃⁻-exchanger) and cellular location [chloride cells or mitochondria-rich cells (MR)] of branchial Cl⁻ uptake and base excretion (Foskett and Scheffey, 1982; Marshall, 1995; Perry, 1997), questions still remain regarding both the specific mechanism(s) for Na⁺ uptake and the cellular localization in the gills of freshwater fish. The current model proposes that Na⁺ uptake occurs through apical membrane epithelial Na⁺ channels (an eNaC homologue) that are electrogenically coupled to a proton pump (V-type H⁺-ATPase). This model is supported by pharmacological (bafilomycin-sensitive ATPase activity: Lin and Randall, 1991, 1993; thiocyanate inhibition: Morgan and Potts, 1995), morphological (Laurent et al., 1994; Lin et al., 1994; Sullivan et al., 1995) and molecular (Perry et al., 2000) studies. Attempts at cloning an eNaC homologue from fish gill

have been unsuccessful to date. Identification of the cell type responsible for Na⁺ uptake has also been elusive, with V-type H⁺-ATPase activity associated specifically with MR chloride cells (Lin and Randall, 1991), lamellar pavement cells (PVCs; Laurent et al., 1994; Sullivan et al., 1995) or both (Lin et al., 1994; Lin and Randall, 1995).

The primary goal of this study was to isolate pharmacologically and characterize the mechanism and site of Na⁺ uptake in isolated populations of gill epithelial cells. A pharmacological hallmark for eNaC activity is sensitivity to the 5'-substituted amiloride derivative phenamil (Kleyman and Cragoe, 1988; Alvarez et al., 2000). Percoll density-gradient separation, combined with peanut lectin agglutinin (PNA) binding and magnetic bead separation, was used to separate dispersed fish gill cells into sub-populations. We identify the PNA⁻ MR cell fraction (α -MR cell) as the site of acid-activated phenamil and bafilomycin-sensitive Na⁺ uptake in fish gill.

Materials and methods

Experimental animals and holding conditions

Freshwater rainbow trout (*Oncorhynchus mykiss* L.) of

either sex were obtained from Alberta Trout Growers Ltd, Tofield, AB, Canada and were held indoors in fiberglass tanks with flowing dechlorinated water. The water temperature was maintained at 15°C throughout. The fish were fed daily with commercial fish pellets. Photoperiod was maintained similar to the natural photoperiod in Edmonton, Alberta.

Isolation and fractionation of gill epithelial cells

Isolation and fractionation of gill epithelial cells was completed using a modification of techniques developed by Goss et al. (2001) and Galvez et al. (2002). Trout (210–325 g) were removed from the aquarium and killed by cephalic blow. The entire branchial basket was removed and rinsed quickly in dechlorinated tapwater to remove congealed external blood and mucus. Individual arches were isolated, lightly blotted and placed in a 50 ml polyethylene conical centrifuge tube (Falcon) containing approximately 20 ml ice-cold Cortland's saline: 143 mmol l⁻¹ NaCl, 5 mmol l⁻¹ KCl, 1.5 mmol l⁻¹ CaCl₂, 1 mmol l⁻¹ MgSO₄, 5 mmol l⁻¹ NaHCO₃, 5 mmol l⁻¹ NaH₂PO₄, 5 mmol l⁻¹ glucose, pH 7.8; osmolarity, 290 mosmol l⁻¹). The isolated arches were washed with Cortland's saline (4°C, 3×5 min), then re-suspended in 10 ml of 0.2 mg ml⁻¹ collagenase (Sigma Chemical Co., St Louis, MO, USA) and incubated for 20 min at 18°C with vigorous agitation (300 revs min⁻¹). After digestion, 10 ml of ice-cold phosphate-buffered saline (PBS; 137 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ KCl, 4.3 mmol l⁻¹ Na₂HPO₄, 1.4 mmol l⁻¹ NaH₂PO₄) was added to slow the collagenase digestion, and individual arches were gently scraped with a glass slide to remove the remaining gill epithelium. The resulting cell suspension was passed through a series of nylon meshes (gravity, 254 µm; vacuum, 94 µm) to remove large debris and isolate single cells. The resultant cell suspension was then transferred to a 50 ml centrifuge tube, diluted to a final volume of approximately 45 ml with PBS and centrifuged (500 g, 10 min, 4°C). The cell pellet was resuspended in 40 ml PBS and the centrifugation and resuspension were repeated. To remove contaminating red blood cells (RBCs), the cell pellet was resuspended in 7.5–10 ml of erythrocyte lysis buffer (154 mmol l⁻¹ NH₄Cl) for exactly 1 min. This technique is based on a standard technique for removing contaminating RBCs during white cell isolation. The cell lysis procedure is based on osmotically shocking suspended cells and using differences in rate of swelling to specifically eliminate RBCs. Those cell types that swell rapidly (RBCs) will burst, while those with a greater complement of ion transporters and the ability to generate a regulatory volume decrease (RVD) response (other cells) gain volume at a less hazardous rate. Exactly 1 min after resuspension in lysis buffer, the suspension was diluted to 45 ml with PBS (4°C) and centrifuged (500 g, 5 min, 4°C). The resultant cell pellet was washed (2×20 ml) and placed on ice for at least 45 min to allow the cells to recover. The cells were then centrifuged and resuspended in a final volume of 2 ml PBS. This procedure removed >90% of contaminating RBCs with little appreciable loss in other cell numbers. To separate the cells into various sub-populations, a modification of Galvez et al. (2002) was

used. The cell suspension was layered over a discontinuous Percoll density gradient (1.03 g ml⁻¹, 1.05 g ml⁻¹ or 1.09 g ml⁻¹ Percoll in PBS) in 15 ml conical centrifuge tubes (Falcon). The cell suspension was then centrifuged (45 min, 2000 g, 4°C) and the bands from each interface collected, washed twice in PBS and used for analysis as required.

Magnetic cell separation

A previous paper (Goss et al., 2001) has demonstrated that the 1.06–1.09 g ml⁻¹ interface of the Percoll gradient is made up of MR cells. However, within this fraction, there are two distinct populations: cells that bind the lectin PNA and those that do not. To separate these two fractions, we took advantage of this differential staining using a magnetic bead separation technique, as recently developed by Galvez et al. (2002). The addition of the erythrocyte-lysis technique slightly altered the migration pattern in these experiments such that the Percoll density gradient was altered to isolate MR cells from a 1.05–1.09 g ml⁻¹ interface. Cells from the 1.05–1.09 g ml⁻¹ interface were resuspended in either 1 ml or 2 ml of 40 µg ml⁻¹ PNA-FITC (fluorescein isothiocyanate) conjugate (20 min, 4°C with continuous mixing). As FITC is light sensitive, these and the following procedures were performed under minimal light conditions. After incubation, unbound PNA-FITC was removed by centrifugation (2×500 g, 5 min) and the cells resuspended in 1–2 ml of anti-FITC microbeads (10 µl microbead stock per ml PBS; Mitylnei Biotech, Auburn, CA, USA) and incubated (20 min, 4°C with continuous mixing). Unbound anti-FITC microbeads were removed by centrifugation (1×500 g, 5 min) and the cells resuspended in 1 ml or 2 ml PBS and applied to a rinsed positive-selection (LS+) separation column placed in a magnetic field. 30 µm pre-filters were attached to the top of the separation column. Following passage of the cell suspension through the column, the column was rinsed (3×3 ml degassed PBS) and the 9 ml cell suspension collected. As these cells were not bound by the PNA-FITC conjugate, we refer to this fraction as PNA-negative (PNA⁻). Cells that bound with PNA remained on the column until removed from the magnetic field. Once the column was removed from the magnetic field, 5 ml of degassed PBS was moved through the column using the plunger provided and the fraction was collected in a 15 ml conical centrifuge tube. This fraction is termed the PNA-positive (PNA⁺) fraction. The PNA⁻ and PNA⁺ fractions were centrifuged and the pellets resuspended in PBS. Cell concentrations were determined using duplicate 15 µl aliquots of suspension and directly counting cells with a hemocytometer. Cell viability was regularly assessed by Trypan blue exclusion. Concentrations were adjusted to that desired by further diluting (additional PBS) or concentrating (centrifugation and removal of supernatant) the cell suspension.

Na⁺ influx experiments

Accumulation of Na⁺ by isolated trout gill epithelial cells was based on the uptake of ²²Na⁺ by a fixed number of cells for a fixed period of time. The cell number and flux time were determined based on initial time course experiments using

crude suspensions of gill epithelial cells from a two-step (1.03–1.09 g ml⁻¹) discontinuous Percoll gradient.

To measure ²²Na⁺ uptake in cells from each fraction, aliquots were centrifuged (1000g, 5 min) and the pellet re-suspended in 1 ml flux solution (pH 7.8, 285 mosmol l⁻¹, 153 mmol l⁻¹ *N*-methyl-D-glucamine (NMDG)-Cl, 1 mmol l⁻¹ KCl, 1.8 mmol l⁻¹ CaCl₂, 1.1 mmol l⁻¹ MgCl₂, 5 mmol l⁻¹ Hepes, 15 mmol l⁻¹ NaCl) containing the desired combinations of inhibitors. Once re-suspended, 37 kBq ²²Na⁺ (1 μl ²²Na⁺ stock; NEN, Boston, MA, USA) was added to the wall of the centrifuge tube above the cell suspension and the suspension was mixed. After exactly 1 min, 200 μl (×3) of the cell suspension was removed and placed onto pre-wetted individual glass microfiber filter circles (Whatman GF/C, 2.4 mm diameter, 0.2 μm pore size) supported by small pieces of plastic mesh, contained within the barrel of 20 ml syringes connected to a vacuum line. The incubation solution was immediately removed by vacuum and the trapped cells rinsed (4×5 ml) with 154 mmol l⁻¹ NaCl saline. Preliminary tests of adding an aliquot of ²²Na⁺ in the absence of cells demonstrated that 2×5 ml rinses of the glass microfiber filters were sufficient to reduce ²²Na⁺ activity to background levels. This filter-vacuum system allowed for rapid (<10 s) washing of the cells and the removal of free ²²Na⁺. In parallel, two 100 μl aliquots of incubation saline were dispensed into individual 7 ml scintillation vials for later determination of specific activity. Washed filter disks were placed in 7 ml scintillation vials, and 5 ml scintillation cocktail (ACS; Amersham, Baie d'Urfe, QC, Canada) was added. ²²Na⁺ activity (c.p.m.) was determined (Beckman LC6200) and corrected for background activity (pre-washed filters in 5 ml scintillation cocktail).

To determine the mechanism contributing to overall Na⁺ uptake in the isolated cell populations, the flux saline for all conditions contained 50 μmol l⁻¹ ouabain to remove nonspecific Na⁺ efflux from cells due to the action of the Na⁺/K⁺-ATPase, 20 μmol l⁻¹ bumetanide to block the Na⁺/K⁺/2Cl⁻ co-transporter and 50 μmol l⁻¹ HOE-694 to block the endogenous basolateral Na⁺/H⁺-exchanger (NHE). Amiloride and other amiloride analogues could not be used to block the NHE due to their efficacy at also blocking eNaC family members. HOE-694 (10 μmol l⁻¹) is a selective NHE-1 inhibitor without effect on eNaC in mammalian systems (Scholtz et al., 1993; Woll et al., 1993; Le Grand et al., 1996; Loh et al., 1996). We demonstrate that HOE-694 is also effective in inhibiting NHE activity in epithelial cells isolated for trout gills.

Intracellular acidification and activation of the H⁺ excretion/Na⁺ uptake mechanism in the isolated cells was accomplished by replacement of NaCl with Na-propionate, which kept the extracellular [Na⁺] constant at 15 mmol l⁻¹ with no resulting changes in external specific activity (SA). Internal SA (based on a lowest predicted internal [Na⁺] of 2 mmol l⁻¹) did not exceed 5% of external SA, so correction for backflux was not performed. The involvement of H⁺-ATPase in ²²Na⁺ influx was determined by prior (1 min) incubation of the cells

with bafilomycin (10 nmol l⁻¹). The involvement of the purported fish gill eNaC homologue in ²²Na⁺ uptake was determined by prior (1 min) incubation of the cells with the Na⁺ channel blocker phenamil (10 μmol l⁻¹).

Calculations

Na⁺ influx (J_{in}^{Na} ; measured in nmol Na⁺ 10⁶ cells⁻¹) was calculated using the following equation:

$$J_{in}^{Na} = \frac{SA \times \text{sample counts}}{\text{cell numbers}}, \quad (1)$$

where sample counts represents the counts per minute (c.p.m.) of individual aliquots of washed cells trapped on the glass filters, SA represents the specific activity of the incubation saline of individual samples (expressed as nmol Na⁺ c.p.m.⁻¹), and cell number represents the number of gill cells in the individual aliquots. The calculated Na⁺ influx was expressed as a function of a standard number of cells (1×10⁶).

Na⁺ influx rate was calculated using equation 1 divided by the duration of the incubation and expressed as nmol Na⁺ min⁻¹ 10⁶ cells⁻¹.

Measurement of pHi in isolated MR cells

Cells were monitored for changes in intracellular pH (pHi) using the indicator dye BCECF-AM. Cells isolated from the Percoll gradient were placed in PBS containing BCECF-AM (5.0 μmol l⁻¹) for 45 min at 5°C. Cells were then washed, resuspended in PBS and placed on a cover slip and allowed to adhere for 15 min prior to examination. The cover slip was mounted in an imaging perfusion chamber and placed on an inverted microscope (Nikon Eclipse TM-300). Cell pHi was monitored by ratiometric imaging (measuring emission at 540 nm after excitation at 495 nm or 440 nm) of small populations of cells (3–5 cells per experiment) at 15 s intervals. The fluorescence microscope was equipped with epillumination *via* a xenon arc lamp (Lambda LS, Sutter Instruments, Novato, CA, USA). Cells were alternately excited at 495±5 nm and 440±10 nm using a dichroic 515 nm cut-on filter, and emitted light was measured using a 540±25 nm filter. Cells were visualized using Plan-Fluor objectives at 40× (0.6 N.A. air) or 100× (1.3 N.A. oil) magnification and images were digitally captured on a 12-bit CCD camera (Cooke SensiCam, Kelheim, Germany). Dye-incubated cells were imaged in the presence or absence of HOE-694 (50 μmol l⁻¹). *In situ* calibration of pHi at the end of each experiment was performed by perfusing through Hepes-buffered solutions of various pHi (pH 7.8, 7.5, 7.2, 6.9) in the continuous presence of nigericin (5 μmol l⁻¹) and monitoring the fluorescence ratio (f_{495}/f_{440}). The ratios were then used to construct a calibration curve to calculate pHi during the experimental run.

Chemicals

All drugs used were dissolved in dimethylsulfoxide (DMSO) and diluted to the required concentration with appropriate saline. The final concentration of DMSO was 0.01% in all ²²Na⁺ exposure salines. Bafilomycin A1

from *Streptomyces griseus*, bumetanide and ouabain were purchased from Sigma Chemical Co., phenamil (phenamil methanesulfate) was purchased from Research Biochemicals International (Natick, MA, USA), and HOE-694 (3-methylsulphonyl-4-peperidinobenzoyl, guanidine hydrochloride) was generously provided by Dr Lang of Hoeschst, Germany.

Statistical analysis

Difference between treatments was analyzed using two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) multiple means comparison test (JMP 3.1.6, SAS Institute Inc.). In all cases, the level of significance was set at $P < 0.05$.

Results

The mucocytes and other low density cells that failed to move through the 1.03 g ml^{-1} Percoll layer represented $70.70 \pm 4.53\%$ of the total cells collected from the gills. These cells were not used for any part of this study, as the presence of mucous confounded the experimental protocols. The remaining cells migrated to either the $1.03\text{--}1.05 \text{ g ml}^{-1}$ Percoll interface or the $1.05\text{--}1.09 \text{ g ml}^{-1}$ Percoll interface of a three-step discontinuous gradient. The cells collected at the $1.03\text{--}1.05 \text{ g ml}^{-1}$ and $1.05\text{--}1.09 \text{ g ml}^{-1}$ Percoll interfaces were found to be $22.40 \pm 3.99\%$ and $6.92 \pm 2.06\%$ of the total number of cells isolated, respectively (Fig. 1). The $1.03\text{--}1.05 \text{ g ml}^{-1}$ Percoll interface has been demonstrated recently to contain lamellar PVCs that do not have large numbers of mitochondria. The $1.05\text{--}1.09 \text{ g ml}^{-1}$ Percoll interface contains cells that can be classified as mitochondria-rich gill epithelial cells (Goss et al., 2001; Wong and Chan, 1999). In the $1.05\text{--}1.09 \text{ g ml}^{-1}$ fraction, however, there exist at least two sub-populations of MR cells based on differential lectin binding (Goss et al., 2001). Some cells migrating to this fraction bind the lectin PNA while others do not bind PNA. To isolate PNA-binding cells (PNA⁺) from non-PNA binding cells (PNA⁻), we employed a magnetic bead separation system to isolate PNA⁺ cells at $>95\%$ purity. Since the $1.05\text{--}1.09 \text{ g ml}^{-1}$ Percoll interface was the only fraction with appreciable PNA-binding cells, we only applied this fraction to the iron bead separation system. Of the total cells isolated from the gills, $6.09 \pm 1.83\%$ were PNA⁻ and $0.82 \pm 0.26\%$ were PNA⁺ (Fig. 1, insert). Therefore, the magnetic bead separation system accounted for a >100 -fold enrichment of PNA⁺ cells from that found in the total gill lysate.

Using a total gill cell population isolated from a $1.03\text{--}1.09 \text{ g ml}^{-1}$ two-step discontinuous Percoll gradient, we established the methodology for measurement of unidirectional $^{22}\text{Na}^+$ uptake. Preliminary experiments revealed measurable linear uptake of $^{22}\text{Na}^+$ for up to 1 min. We therefore employed a 1-min flux period for all measurements of $^{22}\text{Na}^+$ uptake. To measure Na^+ influx in isolated cells, we used bumetanide ($20 \mu\text{mol l}^{-1}$) as an antagonist of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC) and ouabain ($100 \mu\text{mol l}^{-1}$) as an antagonist for the Na^+/K^+ -ATPase; this reduced the background activity of these Na^+ transporters. However, to

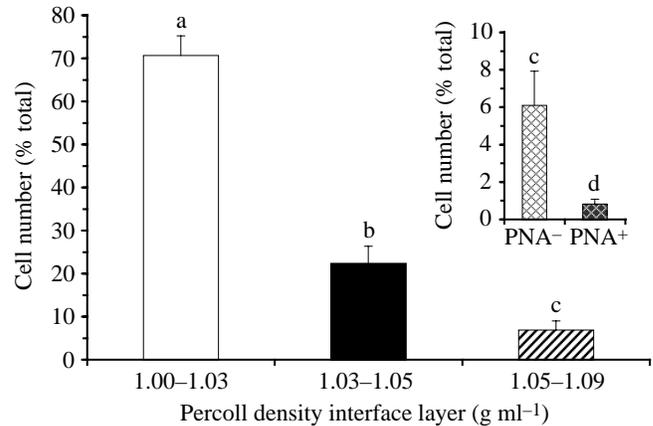


Fig. 1. Relative distribution of isolated gill epithelial cells. Cells were separated into different fractions using a discontinuous Percoll density gradient, peanut lectin agglutinin (PNA) binding and magnetic separation (see Materials and methods). Insert shows the numbers of the PNA⁻ and PNA⁺ cells found within the $1.05\text{--}1.09 \text{ g ml}^{-1}$ density interface layers expressed as a percentage of the total epithelial cells harvested. Data represent means + 1 S.E.M. ($N=4$). Means with different letters indicate that they are significantly different ($P \leq 0.05$).

measure influx through an Na^+ channel, inhibition of the gill cell Na^+/H^+ -exchanger (NHE) was also necessary. We were unable to use effective concentrations of amiloride or any of the amiloride derivatives to specifically block the NHE due to the confounding inhibitory effect of these compounds on the epithelial Na^+ channel (eNaC) at concentrations effective against the basolateral NHE. To resolve this problem, we used the NHE inhibitor HOE-694, a relatively new NHE-1-specific inhibitor (K_i approximately $1 \mu\text{mol l}^{-1}$) in other systems with no known inhibitory properties on the Na^+ channel (Sholtz et al., 1993). To ensure that HOE-694 was effective in inhibiting Na^+ uptake in fish gill cells, two approaches were used. First, $^{22}\text{Na}^+$ influx was determined in the presence and absence of a saturating concentration of $50 \mu\text{mol l}^{-1}$ HOE-694 (Fig. 2). In these experiments, control cells were found to have an Na^+ influx rate of $21 \pm 0.34 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$ in the absence of HOE-694, while in the presence of saturating concentrations of HOE-694, Na^+ influx rates were significantly reduced by 50% to $10.4 \pm 1.25 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$ (Fig. 2). Second, we measured pH_i by live cell imaging and examined the rate of recovery from addition of 10 mmol l^{-1} propionic acid to the bath in the presence and absence of HOE-694. While addition of propionic acid resulted in similar reductions in pH_i, the rate of recovery (dpH_i/dt) was reduced by 40% (from $0.731 \text{ pH units min}^{-1}$ to $0.41 \text{ pH units min}^{-1}$) in cells exposed to $50 \mu\text{mol l}^{-1}$ HOE-694 (Table 1). Higher concentrations ($100 \mu\text{mol l}^{-1}$) of HOE-694 did not further reduce dpH_i/dt. These results suggest that $50 \mu\text{mol l}^{-1}$ HOE-694, an NHE inhibitor, was effective at eliminating the NHE-mediated Na^+ influx in a suspension of mixed trout gill epithelial cells.

In gills, the transport rate for Na^+ under control conditions is approximately $200\text{--}300 \mu\text{mol kg}^{-1} \text{ h}^{-1}$. This Na^+ uptake is driven

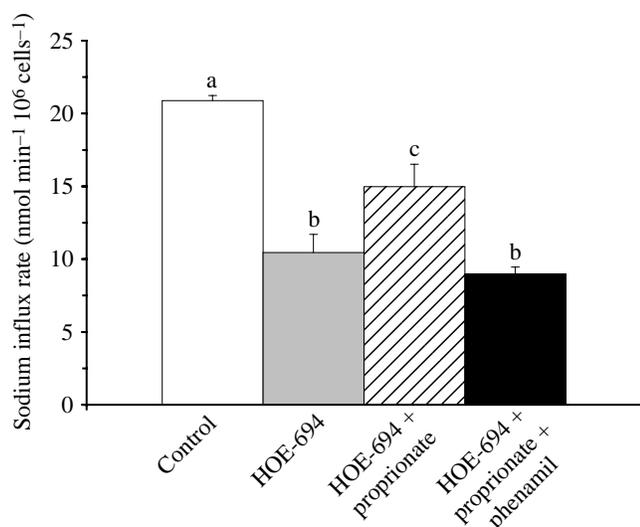


Fig. 2. The effect of the Na⁺/H⁺-exchange inhibitor HOE-694 alone or in combination with propionate and phenamil on Na⁺ influx rates of trout gill cells. Gill epithelial cells were collected at a 1.03–1.09 g ml⁻¹ Percoll density interface layer, as described in the Materials and methods, and Na⁺ influx rates were conducted in low (15 mmol l⁻¹) Na⁺ saline to which 50 μmol l⁻¹ ouabain and 20 μmol l⁻¹ bumetanide were added (see Materials and methods). The open histogram represents the Na⁺ influx rate from control (pH 7.8) gill cells in the absence of HOE-694. The shaded (gray) histogram represents the Na⁺ influx rate from cells incubated in the presence of 50 μmol l⁻¹ HOE-694. The hatched histogram represents the Na⁺ influx rate for control gill cells in the presence of HOE-694 and 10 mmol l⁻¹ Na⁺ propionate. The filled (black) histogram represents the Na⁺ influx rate for cells in the presence of HOE-694, propionate and 10 μmol l⁻¹ phenamil. Data represent means + 1 s.e.m. (N=4). Means with different letters indicate that they are significantly different (P<0.05).

by the constant production of metabolic acids in the body of the fish, requiring the gill ion-transporting cells to excrete H⁺ in exchange for Na⁺. However, in unstimulated cells isolated and maintained in a pH-buffered media, we hypothesized that the Na⁺ channel/H⁺ excretion would be relatively quiescent, and uptake through the Na⁺ channel would only become apparent during an event such as intracellular acidification and increased activity of the V-type H⁺-ATPase. If the uptake was present under acid-stimulated conditions and was sensitive to phenamil, this would indicate the presence of a phenamil-sensitive epithelial Na⁺ channel. To examine if acidification could increase the rate of ²²Na⁺ uptake, we measured uptake on isolated gill cells under control and acid-stimulated conditions. To induce an intracellular acidosis, 10 mmol l⁻¹ propionic acid was again added to the media approximately 1 min prior to flux measurement ([Na⁺] was kept constant). Addition of propionic acid resulted in a rapid decrease in pHi of approximately 0.2 pH units (see Table 1) and a significant 43% increase in Na⁺ influx from 10.5±1.25 nmol min⁻¹ 10⁶ cells⁻¹ to 15.0±1.54 nmol min⁻¹ 10⁶ cells⁻¹ (Fig. 2). If the noted increase in ²²Na⁺ influx were attributable to an increase in influx through

Table 1. Effect of HOE-694 on pHi recovery in isolated MR cells

Condition	ΔpHi	dpHi/dt (pH units min ⁻¹)
Control (N=8)	0.177±0.022	0.73±0.01
50 μmol l ⁻¹ HOE-694 (N=7)	0.168±0.007	0.41±0.006*

* indicates significantly different from control at P<0.05.

an Na⁺ channel, the pharmacological Na⁺ channel antagonist phenamil, which is ineffective against the NHEs at the concentration used (10 μmol l⁻¹), would prevent the increase. Fig. 2 shows that the increase in Na⁺ uptake noted during acidification was completely inhibited by the presence of the Na⁺ channel blocker phenamil.

The above experiments established that gill cells possessed an acid-stimulated, phenamil-sensitive increase in Na⁺ uptake. However, these experiments were conducted on a gill cell population isolated from a two-step gradient. Using a three-step Percoll density gradient separation as previously described (Goss et al., 2001; Wong and Chan, 1999), we were able to separate non-MR PVCs (1.03–1.05 g ml⁻¹ interface) from MR cells (1.05–1.09 g ml⁻¹ interface). It was found that the erythrocyte-lysis procedure used in isolating the MR cells tended to reduce the density of the MR cells by approximately 0.01 g ml⁻¹, resulting in MR cells migrating to a slightly less dense interface of the Percoll gradient. To obtain total yields, distribution patterns and similar populations of cells to that of previous studies (Galvez et al., 2002; Goss et al., 2001), the density layer that separated PVCs from MR cells was reduced from 1.06 g ml⁻¹ to 1.05 g ml⁻¹. We confirmed the appropriateness of these changes through independent testing of cell sub-population counts and mitochondria content (mitochondria fluorescence using the labeling dye Mitotracker).

This separation allowed us to further define the site of phenamil-sensitive Na⁺ uptake. Measurement of Na⁺ uptake in 1.03–1.05 g ml⁻¹-isolated PVCs under control-unstimulated conditions was 13.7±0.93 nmol min⁻¹ 10⁶ cells⁻¹. However, cells from this fraction demonstrated no propionate-stimulated increase in Na⁺ influx. Furthermore, application of phenamil or the V-type H⁺-ATPase inhibitor bafilomycin (2 μmol l⁻¹) was without effect in either the unstimulated or the propionic acid-stimulated cells (Fig. 3).

When Na⁺ uptake was measured in the 1.05–1.09 g ml⁻¹-isolated MR cells (Fig. 4), control rate was approximately 6-fold higher (68.3±7.32 nmol min⁻¹ 10⁶ cells⁻¹) than in the 1.03–1.05 g ml⁻¹ fraction (Fig. 3). The basal level of Na⁺ uptake was unaffected by phenamil treatment. However, addition of bafilomycin resulted in a significant decrease in Na⁺ influx, although this reduction was not further potentiated by the addition of phenamil (Fig. 4). Addition of propionic acid to the cells resulted in a 40% increase in Na⁺ influx from 68.3±7.32 nmol min⁻¹ 10⁶ cells⁻¹ to

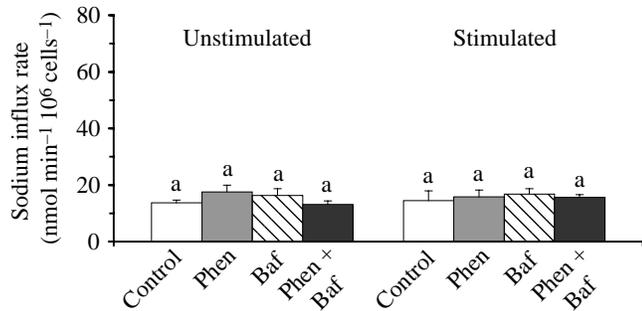


Fig. 3. Na^+ uptake in cells isolated from the 1.03–1.05 g ml^{-1} interface of the Percoll density gradient. Na^+ influx rates were conducted as described in Materials and methods. Na^+ influx rates were determined in the absence of any additional inhibitors (control; open histograms), $10 \mu\text{mol l}^{-1}$ phenamil (Phen; shaded histograms), 10nmol l^{-1} bafilomycin A1 (Baf; hatched histograms) or a combination of phenamil and bafilomycin (Phen + Baf; filled histograms) and in either the absence (unstimulated) or presence (stimulated) of 10mmol l^{-1} Na^+ propionate. Data represent means + 1 S.E.M. ($N=3$). Means with different letters indicate that they are significantly different ($P \leq 0.05$).

$96.0 \pm 11.0 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$ (Fig. 4). This increase was entirely phenamil-sensitive, as addition of phenamil returned the Na^+ influx to unstimulated values. Bafilomycin, which reduced basal Na^+ uptake in unstimulated cells, also reduced Na^+ uptake by a similar amount in propionic acid-stimulated cells. This reduction was, again, not further potentiated by phenamil, suggesting that a general reduction in Na^+ uptake

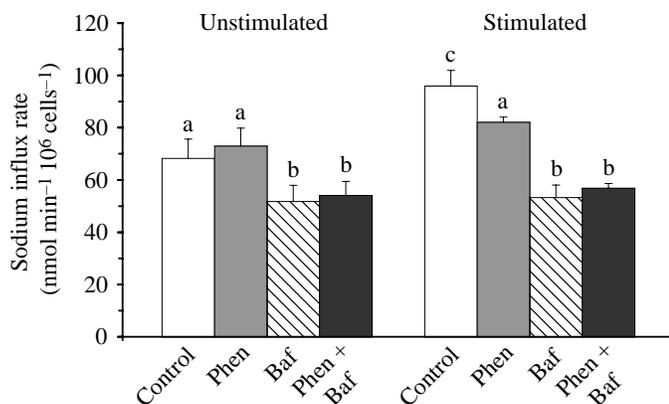


Fig. 4. Na^+ uptake in cells isolated from the 1.05–1.09 g ml^{-1} interface of the Percoll density gradient. Na^+ influx rates were conducted as described in Materials and methods. Na^+ flux rates were determined in the absence of any additional inhibitors (control; open histograms), $10 \mu\text{mol l}^{-1}$ phenamil (Phen; shaded histograms), 10nmol l^{-1} bafilomycin A1 (Baf; hatched histograms) or a combination of phenamil and bafilomycin (Phen + Baf; filled histograms) and in either the absence (unstimulated) or presence (stimulated) of 10mmol l^{-1} Na^+ propionate. Data represent means + 1 S.E.M. ($N=3$). Means with different letters indicate that they are significantly different ($P \leq 0.05$).

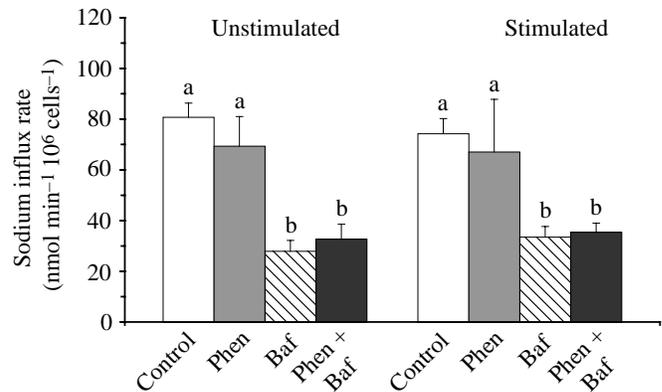


Fig. 5. Na^+ uptake in PNA^+ mitochondria-rich gill cells of rainbow trout. Gill epithelial cells were collected from the 1.05–1.09 g ml^{-1} Percoll density interface and further isolated using lectin binding and magnetic cell separation. Na^+ influx rates were conducted as described in Materials and methods. Na^+ influx rates were determined in the absence of any additional inhibitors (control; open histograms), $10 \mu\text{mol l}^{-1}$ phenamil (Phen; shaded histograms), 10nmol l^{-1} bafilomycin A1 (Baf; hatched histograms) or a combination of phenamil and bafilomycin (Phen + Baf; filled histograms) and in either the absence (unstimulated) or presence (stimulated) of 10mmol l^{-1} Na^+ propionate. Data represent means + 1 S.E.M. ($N=3$). Means with different letters indicate that they are significantly different ($P \leq 0.05$).

during bafilomycin treatment is not related to transport through the Na^+ channel.

Once we had demonstrated that there was a phenamil-sensitive component of acid-stimulated Na^+ uptake in MR cells, we used the magnetic bead separation technique to further isolate the phenamil-sensitive activity to a specific MR cell sub-type. Using this technique, we were able to isolate MR PNA^+ cells from MR PNA^- cells. The control rate of Na^+ uptake in the PNA^+ gill cells was similar to that found in the total MR gill cell fraction. Under control-unstimulated conditions, there was no significant phenamil sensitivity. However, bafilomycin reduced Na^+ uptake by almost 65% in PNA^+ cells, while addition of phenamil in the presence of bafilomycin did not result in a further reduction in Na^+ uptake. Intracellular acidification with propionic acid did not increase the rate of Na^+ uptake in PNA^+ cells nor did addition of phenamil affect the rate of uptake under acid-stimulated conditions. As was seen in the unstimulated cells, bafilomycin significantly reduced the basal rate of Na^+ uptake in propionic acid-stimulated cells, but this was not further reduced by phenamil (Fig. 5).

The PNA^- fraction of the MR cell layer represents the cells allowed to pass through the magnetic separation column while it was still in the magnet. We tested the rate of Na^+ uptake in these cells as well under both unstimulated and acid-stimulated conditions (Fig. 6). The control rate of Na^+ uptake in the PNA^- gill cells was similar to that found in both the MR gill cell fraction and the PNA^+ fraction. Additionally, similar to the PNA^+ cells, there was no significant phenamil sensitivity under

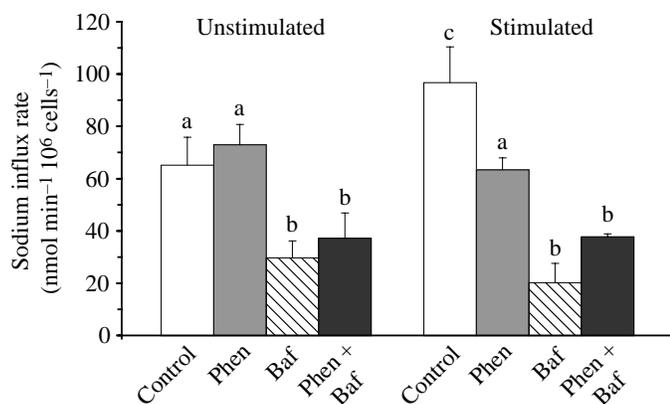


Fig. 6. Na⁺ uptake in PNA⁻ mitochondria-rich gill cells of rainbow trout. Gill epithelial cells were collected from the 1.05–1.09 g ml⁻¹ Percoll density interface and further isolated using lectin binding and magnetic cell separation. Na⁺ influx rates were conducted as described in Materials and methods. Na⁺ influx rates were determined in the absence of any additional inhibitors (control; open histograms), 10 μmol l⁻¹ phenamil (Phen; shaded histograms), 10 nmol l⁻¹ bafilomycin A1 (Baf; hatched histograms) or a combination of phenamil and bafilomycin (Phen + Baf; filled histograms) and in either the absence (unstimulated) or presence (stimulated) of 10 mmol l⁻¹ Na⁺ propionate. Data represent means + 1 s.e.m. (N=3). Means with different letters indicate that they are significantly different (P ≤ 0.05).

control-unstimulated conditions. Bafilomycin induced similar reductions in Na⁺ uptake (55%), while the addition of phenamil and bafilomycin did not result in a further reduction in Na⁺ uptake over that of bafilomycin-treated cells. Most significantly, however, unlike the PNA⁺ cells, intracellular acidification of PNA⁻ cells with propionic acid produced a significant increase in Na⁺ uptake that was completely blocked by phenamil, indicating the presence of phenamil-sensitive Na⁺ uptake in PNA⁻ cells (Fig. 6).

Discussion

The current model of branchial ion regulation in freshwater fish partitions anion exchange to the mitochondria-rich chloride cells (MR CCs) and cation transport to the respiratory or pavement cells (PVCs), although this latter point is the subject of considerable debate. Na⁺ uptake has been suggested to involve an electrically coupled Na⁺ channel and a V-type H⁺-ATPase. It remains unclear as to whether this Na⁺-uptake mechanism is located specifically within a sub-population of PVCs, in CCs or in both. The findings of this study suggest that bafilomycin-sensitive Na⁺ influx is specifically located in MR cells and not in traditional lower-density respiratory PVCs. The sub-population of MR cells is defined as PNA⁻ and possesses both V-type ATPase activity and acid-stimulated, phenamil-blocked Na⁺ influx. Recently, Galvez et al. (2002) reported that constitutive H⁺-ATPase activity was higher in PNA⁻ cells, relative to PNA⁺ cells, and that H⁺-ATPase levels were further elevated in the PNA⁻ cells in response to

hypercapnia. Considering the relative proportion of the PNA⁻ MR cells compared with the PNA⁺ MR cells in the mixed population of gill epithelial cells, it appears that the PNA⁻ cells are a strong candidate for the branchial epithelial location for the Na⁺ channel/V-type ATPase model of Na⁺ uptake in freshwater fish. Furthermore, Galvez et al. (2002) presented evidence that the morphology of these PNA⁻ cells was more consistent with being termed MR PVCs due to the presence of irregular nuclei, dense peripheral chromatin and a much less well developed vesiculotubular network.

One of the primary objectives of the present study was to isolate the location of the epithelial Na⁺ channel (eNaC) that has been purported to be electrically coupled to a V-type ATPase and to be responsible for branchial Na⁺ uptake in freshwater fish. The presence of an Na⁺ channel was confirmed following the identification of a phenamil-inhibitable acid-induced Na⁺ influx in only the cells that we have previously termed MR PVCs (Galvez et al., 2002).

The freshwater fish gill shares a number of common functional and morphological features with the inner medullary collecting duct (IMCD) of the mammalian kidney. Both epithelia function in ion regulation and acid/base regulation (Madsen and Tisher, 1984, 1986) and, importantly, have similar morphological and physiological features as well (Goss et al., 1997). The correlate of the respiratory PVC is termed the principal cell in the IMCD, while the MR cells of the IMCD are classified as intercalated cells (IC). Importantly, the latter have been subdivided into two functionally distinct cell types, α and β. The α-type (acid-excreting) IC is characterized as not binding PNA (LeHir et al., 1982), having apical H⁺-ATPase and basolateral Cl⁻/HCO₃⁻ exchange (Brown et al., 1988a,b), while the β-type (base-secreting) IC is characterized as being able to bind PNA, having apical Cl⁻/HCO₃⁻ exchange and basolateral H⁺-ATPase (Stetson and Steinmetz, 1985), i.e. a reversed polarity to that of the α-type cell. Given the morphological and physiological similarities, we suggest that the PNA⁻ MR cells are termed α-MR cells, to match the physiological characterization and nomenclature of the analogous α-IC found in the IMCD, while the PNA⁺ MR cells should also be renamed to match the similar PNA-binding characteristics found in the β-IC found in the IMCD. We will use the terms α-MR cell and β-MR cell throughout the remainder of the manuscript.

A significant concern with our methodology is the lack of proper orientation present in the cells when suspended in solution. MR cells exist as polarized cells with an apical and serosal solution. Ideally, our experiments would have been performed on cells cultured so as to allow for direct re-orientation of the MR cells with independent access to apical and basal surfaces. When a mixed suspension of gill epithelial cells are plated, the respiratory cells or PVCs will grow well in culture, while the MR cells will not, at least in a single seed protocol (Pärt and Wood, 1996). More recent publications have demonstrated successful growth of undefined MR cells in culture. However, their experimental evidence indicates that these preparations do not transport Na⁺ or Cl⁻ in a proper or similar manner to the fish

gill and therefore cannot, as yet, serve as a surrogate model for the fish gill culture (Fletcher et al., 2000; Kelly and Wood, 2001). As we were interested in characterizing the transport properties of each of the cell subtypes, our only choice was to complete these experiments *in vitro* using dispersed cells. We are currently trying (without success as yet) to incorporate gill epithelial cell culture and our sub-type separation into a viable model. Once this goal is achieved, it would be exciting to revisit these experiments using cells with the appropriate polarization and more environmentally relevant exposure media.

To isolate the movement of Na^+ through the Na^+ channel, we inhibited other routes of Na^+ using ouabain, an Na^+/K^+ -ATPase inhibitor, bumetanide, a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter inhibitor and HOE-694. HOE-694 is an Na^+/H^+ exchange (NHE) inhibitor that has been shown to be specific for NHE isoform 1 (NHE-1) in a variety of vertebrate tissues and cells (Scholtz et al., 1993; Woll et al., 1993; Le Grand et al., 1996; Loh et al., 1996; Bleich et al., 1998). Half-maximal inhibition of NHE activity is $10\ \mu\text{mol l}^{-1}$ and $20\ \mu\text{mol l}^{-1}$ in fibroblasts (Woll et al., 1993) and isolated rat atria (Le Grand et al., 1996), respectively. The present study represents the first application of HOE-694 in the study of ion transport in branchial epithelium of freshwater fish. Our findings suggest that HOE-694 is an effective NHE inhibitor in trout gill epithelial cells. In this study, $50\ \mu\text{mol l}^{-1}$ HOE-694 was found to inhibit Na^+ influx by almost 50% in a suspension of gill epithelial cells that contain PVCs, CCs and MR PVCs. The remaining movement of Na^+ could be due to the activity of other Na^+ -dependent transporters. Possible transporters include members of the $\text{Na}^+/\text{HCO}_3^-$ co-transport family or the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX) family. While there is only nominal HCO_3^- present in the bathing solution, it is likely that the effect of NBC would be minimized, but it cannot be discounted. NCX activity has been demonstrated to exist in CCs (Flik et al., 1985, 1995; Flik and Verbost, 1993; Li et al., 1997), while the existence of an NCX in the other cell types remains to be investigated.

The localization of the acid-stimulated Na^+ channel was based on the use of phenamil, an eNaC blocker. Phenamil was effective in reducing acid-stimulated Na^+ influx but only in α -MR (PNA^-) cells, suggesting that the route of Na^+ entry is *via* an eNaC-type Na^+ channel. These data do not support the existence of acid-sensing ion channels (ASICs) in gill epithelium. ASICs have been found in mammalian neuronal tissue (Bassilana et al., 1997; Lingueglia et al., 1997). However, ASIC Na^+ channels experience a decrease in Na^+ movement when exposed to reductions in pHi (Zeiske et al., 1999), an opposite response to that noted in the present study. We suggest an approximately 0.2 pH unit drop in pHi induced by propionic acid-simulated V-type H^+ -ATPase activity and an increased inward movement of Na^+ through the Na^+ channel to maintain charge balance.

Bafilomycin was extremely effective in reducing Na^+ influx under basal and acid-stimulated conditions, whether alone or in combination with phenamil. Fenwick et al. (1999) reported that the inhibitory effect of bafilomycin on the rate of Na^+ uptake was dose-dependent and seen at concentrations as low as $0.01\ \mu\text{mol l}^{-1}$. $10\ \mu\text{mol l}^{-1}$ bafilomycin reduced whole-body Na^+

influx by up to 90% and 70% in freshwater tilapia and carp, respectively (Fenwick et al., 1999). These data provide support for previous suggestions that Na^+ uptake in freshwater fish is associated with a proton-motive force created by a proton pump and provide indirect evidence for the major significance of this mechanism in the branchial uptake of Na^+ by freshwater fish. We found that bafilomycin ($2\ \mu\text{mol l}^{-1}$) reduced Na^+ uptake by 30–60% in our MR cells but not at all in the PVCs. The bafilomycin sensitivity was not significantly different between the PNA^+ and PNA^- MR cells, suggesting that both CCs and MR PVCs possess H^+ -ATPase. Caution must be exercised, however, with the use of bafilomycin and interpretation of net or unidirectional Na^+ movements, as bafilomycin inhibited the basal mechanisms of Na^+ influx probably *via* a change in membrane potential. Preliminary evidence from our lab using the membrane potential dye bis-oxynol demonstrates that addition of bafilomycin results in depolarization of the cell membrane potential. Evidence for a role for H^+ -ATPases in setting the membrane potential has resulted in a number of recent papers (Harvey et al., 1998; Wieczorak et al., 1999; Beyenbach, 2001) and it has been conclusively demonstrated for at least three types of MR cells with high plasma membrane H^+ -ATPase activity. In osteoclasts (Mattsson et al., 1993), frog skin MR cells (Ehrenfeld and Klein, 1997), MR cells of insect Malpighian tubules (Beyenbach et al., 2000) and even plant transporting cells (Sze et al., 1999), bafilomycin treatment significantly depolarizes these cells. This depolarization would reduce net driving forces for any Na^+ transport dependent on the electrochemical gradient and reduce overall Na^+ fluxes independently.

The β -MR cells (PNA^+) were found to be approximately 1% of the total cells collected, while the α -MR cells (PNA^-) represented approximately 6% of the cells collected from the gill. Pärt and Wood (1996) reported that bafilomycin had no effect on the pHi of primary cultured gill PVCs. However, as no MR cells were present in their study, this agrees with our present findings and those of others that V-type H^+ -ATPases are present only in MR cells and not in respiratory PVCs (Sullivan et al., 1995; Galvez et al., 2002). Only α -MR cells experienced increased rates of Na^+ influx in response to increased availability of intracellular H^+ and only this portion of the flux was phenamil-sensitive. We conclude that the α -MR cells are the main contributors to branchial Na^+ uptake in freshwater fish. Our identification of the Na^+ channel-containing cell type will allow for future studies to focus on elucidating the molecular identity and physiological characterization of this transport.

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