Comparative Biochemistry and Physiology, Part A 159 (2011) 234–241

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Mechanism of sodium uptake in PNA negative MR cells from rainbow trout, *Oncorhynchus mykiss* as revealed by silver and copper inhibition

Greg Goss [a,*], Kathleen Gilmour [b], Guy Hawkings [a], Jonathan H. Brumbach [a], Maily Huynh [a], Fernando Galvez [c]

[a] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6E 4W1
[b] Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5
[c] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, United States

ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 23 February 2011
Accepted 24 February 2011
Available online 3 March 2011

Keywords:
Oncorhynchus mykiss
Bafilomycin
Phenamil
Gill
Fish
Transport carbonic anhydrase
Sodium potassium ATPase

ABSTRACT

The rate of acid-stimulated and phenamil-sensitive sodium (Na^+) uptake was measured in three different cell lineages: pavement cells (PVC), total mitochondrion-rich (MR) cell populations, and peanut lectin agglutinin-negative mitochondrion-rich cells (PNA^- MR) isolated from the rainbow trout gill epithelium. Despite the presence of basal levels of Na^+ uptake in PVC, this transport was not enhanced by acidification, nor was it inhibited by independent treatment with bafilomycin (i.e., a V-type H^+-ATPase inhibitor), phenamil (i.e., a specific inhibitor of ENaC), or Ag (a specific inhibitor of active Na^+ transport in fish). In contrast, Na^+ uptake in PNA^- MR cells was increased by ~220% above basal levels following acidification of near 0.4 pH units in the presence of 1.0 mM external Na^+. Acid-stimulated Na^+ transport was entirely inhibited by both phenamil and bafilomycin. Silver (Ag) and copper (Cu), which are known to interfere with active Na^+ transport in fish, were also responsible for inhibiting acid stimulated Na^+ uptake in PNA^- MR cells, but by themselves had no effect on basal Na^+ transport. Thus, we demonstrate that Ag specifically prevented acid-stimulated Na^+ uptake in PNA^- MR cells in a dose-dependent manner. We also demonstrate rapid (<1 min) and significant inhibition of carbonic anhydrase (CA) by Ag in PNA^- MR cells, but not in PVC. These data lend further support to the idea of a PNA^- MR cell type as the primary site for Na^+ uptake in the freshwater (FW) gill phenotype of rainbow trout. Moreover, these findings provide support for the importance of intracellular protons in regulating the movement of Na^+ across the apical surface of the fish gill.

1. Introduction

There has been considerable debate in the literature regarding the cellular mechanism(s) of Na^+ uptake in FW fishes. Indeed, much of the literature is contradictory with respect to potential mechanisms and the cellular localization of transport pathways, and this is undoubtedly creating confusion. In one model of Na^+ uptake, an apical Na^+ channel along with H^+-ATPases is used in concert to take up Na^+ from the water when unfavorable electrochemical gradients exist (Parks et al., 2008), a mechanism similar to that seen in frog skin (reviewed by Harvey, 1992; Ehrenfeld and Klein, 1997; Ehrenfeld, 1998). In this model, the H^+-ATPase acts to create a favorable local electrochemical gradient for Na^+ entry through a phenamil-sensitive Na^+ channel located on the water-facing side of the gill epithelium (Avella and Bornancin, 1989; Lin and Randall, 1991; Perry, 1997). Recent alternative models of Na^+ uptake in FW fish have been proposed in which an apical isoform of the Na^+/H^+ exchanger, using either the NHE2 (Nawata et al., 2007; Nawata and Wood, 2008; Tsui et al., 2009) or NHE3b isoforms (Lin et al., 2008; Hwang and Lee, 2007; Hwang, 2009), works in concert with a H^+-ATPase and potentially RhCG ammonium transporters (Tsui et al., 2009). Presumably, NH_3 movement *via* the RhCG acts to alkalinize the apical boundary layer outside the fish, thereby promoting H^+ movement and allowing the NHE to function in lower Na^+ concentrations than might be predicted in the bulk solution. One significant criticism of this model is that substantial evidence concerning the ability of phenamil to inhibit Na^+ transport in whole organisms (Bury and Wood, 1999; Grosell and Wood, 2002; Rogers et al., 2004) and in isolated cells (Reid et al., 2003; Parks et al., 2007) is lacking. While NHEs from all other organisms are insensitive to the drug phenamil even at relatively high concentrations (Kleyman and Cragoe, 1988; Goss et al., 2001), it is possible that fish NHE2 and/or NHE3 are phenamil-sensitive; however, this remains to be determined.

Techniques for isolating and characterizing specific populations of gill cells based on differences in density have been developed (Wong and Chan, 1999; Goss et al., 2001; Galvez et al., 2002; Parks et al., 2007). We used these techniques to separate gill cells into two major fractions; the pavement cell (PVC) fraction, which migrates to a lighter density (1.03–1.05 g/L interface) compared to the mitochondrion-rich (MR) cell fraction (1.05–1.09 g/L interface). We have also demonstrated that a specific fraction of MR cells is peanut lectin agglutinin positive (PNA^+),

* Corresponding author at: University of Alberta, Edmonton, Alberta, Canada T5G 2E9. Tel.: +1 780 492 2381; fax: +1 780 492 2381.
E-mail address: greg.goss@ualberta.ca (G. Goss).

1095-6433/$ – see front matter
doi:10.1016/j.cbpa.2011.02.025

with the remainder of the MR cell fraction termed PNA^- (Goss et al., 2001). Subsequently, we developed a magnetic separation technique for isolating a relative pure PNA^+ MR cell population from the PNA^- MR cell fraction (Galvez et al., 2002). The PNA^- MR cells showed higher V-type H^+-ATPase expression relative to the PNA^+ MR cells following the induction of respiratory acidosis in fish. Reid et al. (2003) corroborated this finding by localizing acid-stimulated and phenamil-sensitive Na^+ influx to the PNA^- cell type while neither PNA^+ cells nor the PVC fraction demonstrated acid-stimulated and phenamil-sensitive Na^+ uptake.

Waterborne silver (Ag) and copper (Cu) are highly toxic to FW fish with acute lethal concentrations (LC_{50}) ranging from 25 to 175 μg/L for Cu (Smith et al., 2001) and 5–70 μg/L for Ag (Wood et al., 1996; Webb and Wood, 1998). In both cases, these metals interfere with Na^+ homeostasis by inhibiting the normal physiological Na^+ uptake mechanisms of the gill epithelium (Morgan et al., 2004b; Grosell and Wood, 2002). Therefore, we used these metals as probes to investigate the mechanisms of Na^+ transport in fish gills. The inhibition of Na^+ uptake by these metals is most commonly associated with the specific impairment of the Na^+/K^+ ATPase enzyme on the basolateral membrane (Lauren and McDonald, 1987a, 1987b; Morgan et al., 1997; Grosell and Wood, 2002; Morgan et al., 2004a; Bury and Wood, 1999), the inhibition of carbonic anhydrase (CA) (Morgan et al., 2004b; Soyut et al., 2008; Soyut and Beydemir, 2008), or the inhibition/blockade of a Na^+ channel due to the metal's slow permeation co-efficient through the Na^+ transport mechanism (Bury et al., 1999; Morgan et al., 2004a), although inhibition of the V-type ATPase could also interfere with Na^+ uptake.

The ionic forms of these transition metals have a much larger ionic radius than that of Na^+ when fully hydrated (Bell et al., 2002), and a lower permeation coefficient through the channel in isolated membrane preparations. In essence, the presence of waterborne Ag or Cu should result in an effective blockade of the channel (i.e. the presence of Ag or Cu should act to competitively reduce Na^+ transport), leading to immediate reductions in the rate of Na^+ uptake. However, movement of these metals into cells and subsequent inhibition of either Na^+/K^+ ATPase or CA would also indirectly reduce Na^+ counter transport either by raising intracellular Na^+ levels by inhibition of Na^+/K^+ ATPase, or *via* a decrease in the supply of H^+ to the apical H^+-ATPase in the case of CA. It is also worth noting that NHE function is not inhibited by relevant concentrations of Ag or Cu when tested in mammalian model culture systems.

The objectives of the present study were threefold. First, we assessed the cellular localization of Na^+ uptake inhibition by Ag and Cu by performing fluxes similar to those reported by Reid et al. (2003), but at external ion concentrations more characteristic of fresh water. We have previously suggested that the PNA^- MR cell fraction is the primary cell type responsible for Na^+ influx at the gill. Therefore, PNA^- MR cells were isolated by first removing PNA^+ MR cells from the total MR fraction using the MACS cell separation technique (Galvez et al., 2002). The remaining PNA^- fraction was then tested for Na^+ uptake and the ability of Ag and Cu to inhibit this Na^+ uptake. Second, the roles of Na^+ channels and H^+-ATPase in Na^+ uptake in isolated cells were investigated using pharmacological blockade with phenamil and bafilomycin, respectively. Third, we used Cu and Ag inhibition of Na^+ uptake to provide further insight into the mechanisms of Na^+ transport. Moreover, these data will also provide insight into possible mechanisms of Na^+ uptake inhibition by Ag and Cu. Finally, isolated cells were also exposed to Cd^{2+}, a treatment that served as a negative control because Cd^{2+} was predicted, based on previous studies, to have no effect on Na^+ uptake in whole fish.

2. Material and methods

2.1. Experimental animals

Adult rainbow trout (~200 g) (*Oncorhynchus mykiss*) from Alberta Trout Growers were maintained in 450 L fibreglass tanks supplied with aerated, dechlorinated Edmonton tap water (hardness 160 ppm as $CaCO_3$, total alkalinity 120 mg/L; pH 8.2; $[Na^+] = 0.3$ mM) under flow-through conditions. 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.

2.2. Materials

The magnetic cell separation system and streptavidin microbeads used for MACS separation were from Miltynei Biotech (Auburn, CA, USA). Scintillation cocktail was obtained from ACS (Amersham, Baie d'Urfe, QC, Canada). Radioactive stock $^{22}Na^+$ was ordered through NEN (Boston, MA, USA). All other reagents used in the protocol were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.3. Cell isolation 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 arches and placed in ice-cold Cortland's saline (in mM: 143 NaCl, 5.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 5.0 $NaHCO_3$, 3.0 NaH_2PO_4, 5.0 glucose; pH 7.8). Gills were digested in 0.2 mg/mL collagenase (type 1A) 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 Na_2HPO_4, 1.4 NaH_2PO_4; pH 7.8) and centrifuged at 500 × *g* for 10 min at 4 °C. The absence of Ca^{2+} 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, resuspended in 2 mL PBS, placed on a 1.09 g/mL Percoll solution, and centrifuged for 10 min at 500 × *g* (4 °C) to separate a substantial portion of the contaminating red blood cells (RBCs) from the gill cell suspension. The gill cells remained on top of the 1.09 g/mL Percoll solution, whereas most RBCs pelleted to the bottom of the tube. The gill cells were then collected from the Percoll and pelleted (1000 × *g* for 5 min), after which the pellet was resuspended in 1 mL of RBC lysis buffer (in mM: 154 NH_4Cl, 10 HEPES, 0.1 Na_2·EDTA, pH 7.8) for exactly 45 s to remove any remaining red cells from the cell isolate. Lysis was stopped by addition of a large volume (45 mL) of PBS. The gill cells were again centrifuged for 10 min at 500 × *g*, resuspended in 2 mL PBS, layered over a three-step gradient consisting of 1.03, 1.05, and 1.09 g/mL Percoll (2 mL each) and centrifuged at 2000 × *g* (45 min). Mucous cells and cellular debris from the top of the Percoll gradient were discarded. Cells from the 1.03–1.05 (PVC fraction) and 1.05–1.09 g/mL (total MR cell fraction) Percoll interfaces were collected independently, washed once in ~10 vol PBS, centrifuged (2000 × *g*; 10 min), and resuspended in 1.0–2.0 mL PBS (Goss et al., 2001). When appropriate, the MR cell fraction was also depleted of PNA^+ MR cells using a magnetic cell separation protocol. Briefly, gill cells collected from the 1.05–1.09 g/mL Percoll interface were incubated in 40 μg/mL PNA-biotin and rotated continuously for 20 min at 4 °C. Cells were then incubated in a solution of streptavidin covalently coupled to 50 nm iron particles (10 μL streptavidin-microbeads/mL PBS) for 20 min at 4 °C with continuous mixing. Cells were washed once to remove unbound streptavidin microbeads and were then 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^+, Miltynei held within a magnetic field). Cells with PNA-biotin and streptavidin microbeads bound are retained in the column as long as the magnetic field is present. The column was rinsed with 3 consecutive washes (9 mL total) of PBS buffer. The cells passing through the column in the presence of the magnetic field were collected and termed the PNA^- fraction. The PNA^- MR cells were