Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water

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Compared with terrestrial animals, aquatic fish have to cope with more challenging osmotic gradients in aquatic environments. Teleost fish have efficient osmoregulatory mechanisms to maintain their body fluid homeostasis. In hypotonic environments, they take up Na⁺, Cl⁻, and Ca²⁺ from the ambient water by specialized organs (gills and skin). Mitochondrion-rich cells (MRCs), a group of specialized ionocytes in the epithelium of the gills, are generally thought to play a critical role in transporting ions across the epithelium. The mechanisms underlying the ionic uptake by MRCs have been investigated for several decades in several model species, including salmon, trout, eel, tilapia, and killifish; however, the molecular mechanisms of Na⁺ uptake have been controversial and unclear for a long time (10).

Na⁺ uptake by gills of freshwater (FW) fish being linked to NH₄⁺ excretion was first proposed by August Krogh in the 1930s. Since then, many studies have been carried out to test Krogh’s hypothesis of Na⁺/NH₄⁺ exchange in fish gills, and a number of studies have produced conflicting conclusions (3). For example, several studies reported that the amount of NH₄⁺ excreted is close to the amount of Na⁺ taken up by fish gills (21, 26, 27, 33), and NH₄⁺ loading may stimulate Na⁺ uptake (27, 31), supporting the presence of a Na⁺/NH₄⁺ exchange pathway in fish gills. On the other hand, several studies reported that Na⁺ excretion and Na⁺ uptake are not tightly coupled, and nonionic diffusion of NH₃ plays a major role in branchial ammonia excretion (16, 30–32). In the 1970s, several landmark studies proposed that Na⁺ was, in fact, exchanged for H⁺ instead of NH₄⁺, and suggested that an amiloride-sensitive Na⁺/H⁺ exchanger (NHE) is involved in Na⁺ uptake by fish gills (14, 16). Avella and Bornancin (1) also reported that Na⁺ is exchanged for H⁺, but they questioned the thermodynamics of NHE in Na⁺ uptake in fish living in FW. Alternatively, an apical V-type H⁺-ATPase electrochemically linked to a Na⁺ channel that drives Na⁺ uptake was suggested. Since then, the two pathways through an NHE- or H⁺-ATPase-coupled Na⁺ channel have become dominant models for Na⁺ uptake in FW fish and have been tested for several decades.

Because of their suitability for genetic manipulation and the availability of genomic databases, the zebrafish model has been used to investigate the molecular mechanism of ionocytes in lower vertebrates (10). Lin et al. (19) established an in vivo zebrafish model that combines electrophysiological (scanning ion-selective electrode technique, SIET) and molecular approaches to demonstrate that a novel H⁺-ATPase-rich cell (HR cell, a subtype of MR cells) in gills and larval skin is an acid-secreting cell. Subsequent studies used sodium green labeling to show that HR cells are sites of Na⁺ uptake (2, 7). Yan et al. (36) cloned and identified an NHE3b (slc9a3b) in HR cells of zebrafish gills and found that nhe3b mRNA expression was induced by low-Na⁺ (L-Na⁺) water, suggesting that Na⁺ uptake in HR cells is via NHE3b. In contrast, they found that H⁺-ATPase (atp6v0c) expression was not induced but suppressed in L-Na⁺ water. In other species, including the Osorezan dace (Tríbolodon hakonensis) (4), rainbow trout (Oncorhynchus mykiss) (12), and tilapia (Oreochromis mossambicus) (5), NHE2/3 were identified in their MR cells and were also suggested to be involved in Na⁺ uptake. Although those molecular identifications support the role of NHE isoforms in branchial Na⁺ uptake, the driving force of NHE remains an unresolved question (25). Just as Avella and Bornancin (1) previously questioned the driving force of NHE, Parks et al. (25) also emphasized the thermodynamic constraints that might prevent the electroneutral NHE from functioning in FW environments. However, recent studies on the ammonia transporters, Rhesus glycoproteins (Rh pro-
they conduct nonionic ammonia (13, 15). Several isoforms of Rh proteins (Rhag, Rhbg, Rhcg1, and Rhcg2) were identified from fish gills, including rainbow trout (24), puffer fish (Takifugu rubripes) (23), killifish (Kryptolebias marmoratus) (9), medaka (35), and zebrafish (22). In zebrafish larvae, Rhcg1 was cloned and localized to apical membranes of HR cells, suggesting that HR cells are also involved in ammonia excretion (22). Using morpholino gene knockdown and SIET probing, Shih et al. (29) provided strong loss-of-function evidence for the function of Rhcg1 in ammonia excretion by HR cells and also demonstrated that H\textsuperscript{+}-ATPase in apical membranes of HR cells generates an acidic layer to drive nonionic NH\textsubscript{3} diffusion through Rhcg1. The presence of Na\textsuperscript{+} uptake and NH\textsubscript{4}\textsuperscript{+} excretion in HR cells of zebrafish led us to reconsider whether Krogh’s Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange is conducted by a coupling function of NHE3b and Rhcg1. Recently, the Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange mechanism was also investigated in cultured gill cells of trout, and it was suggested that Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange is mediated by a putative protein complex, including an Rh protein, the NHE, H\textsuperscript{+}-ATPase, and an unidentified Na\textsuperscript{+} channel (34). Using medaka larvae as a new model, we demonstrated that Na\textsuperscript{+} uptake and NH\textsubscript{4}\textsuperscript{+} excretion in medaka larvae were accomplished by the same MR cells, and there is a tight linkage between Na\textsuperscript{+} uptake and NH\textsubscript{4}\textsuperscript{+} excretion by those cells (35). An NHE (slc9a3) and two Rh proteins (Rhcg1 and Rhbg) were colocalized to MR cells of medaka larvae, and their mRNA expressions were induced by L-Na water, suggesting that a functional association of NHE3 and Rhcg1 plays a critical role in the NH\textsubscript{4}\textsuperscript{+}-dependent Na\textsuperscript{+} uptake mechanism. That study demonstrated the presence of a Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange in MR cells of medaka and also provided a new explanation for the driving force of the NHE.

However, to provide more solid evidence of the involvement of NHE3 and Rhcg1 in the NH\textsubscript{4}\textsuperscript{+}-dependent Na\textsuperscript{+} uptake mechanism, a loss-of-function approach is required. Therefore, the zebrafish model, which is suitable for a genetic knockdown study, was used in this study. In addition, both NHE3b and Rhcg1 have been localized to the apical membrane of HR cells, which play a critical role in Na\textsuperscript{+} uptake (36) and ammonia excretion (29). In this study, we attempted to examine whether the Na\textsuperscript{+} uptake by zebrafish is NH\textsubscript{4}\textsuperscript{+} dependent, and more specifically to test whether NHE3b and Rhcg1 are involved in the Na\textsuperscript{+} uptake mechanism with emphasis on low-Na\textsuperscript{+} situation, which was previously reported to stimulate NHE3b expression and function (36).

Table 1. Specific primer sets for the real-time quantitative PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward/Reverse</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhcg1</td>
<td>R</td>
<td>5’-TGCCCAACTGTCGAGGTTG-3’</td>
</tr>
<tr>
<td>nhe3b</td>
<td>R</td>
<td>5’-AGGAGTAGACGGAGGGAATATT-3’</td>
</tr>
<tr>
<td>atp6o1a</td>
<td>R</td>
<td>5’-ACGGAGCAGGAGGCTATGGC-3’</td>
</tr>
<tr>
<td>rpl13a</td>
<td>R</td>
<td>5’-CCGAGTGGGAGTCTTCCGATATG-3’</td>
</tr>
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Fig. 1. A: measurement of ionic gradients at yolk sac surface of a 4-day postfertilization (dpf) zebrafish larva. The ion-selective probe was moved to location “Y” (10–20 μm away from the yolk sac membrane) to record the yolk sac ionic concentrations ([Ion]Y); then the probe was immediately moved 10 mm away to location “B” to record the background concentration ([Ion]B). To calculate ionic gradients, the concentration at “Y” was subtracted by the concentration at back-ground. B–D: ionic gradients (Δ[NH\textsubscript{4}\textsuperscript{+}], Δ[Na\textsuperscript{+}], and Δ[H\textsuperscript{+}]) at the yolk sac surface of larvae acclimated to normal water (NW), low-Na\textsuperscript{+} water (L-Na), high ammonium water (H-Amm), or L-Na- H-Amm water. Data are presented as means ± SE (n = 10 larvae). Different letters indicate a significant difference (by one-way ANOVA and Tukey’s comparison, P < 0.05).
MATERIALS AND METHODS

Experimental animals. Adult zebrafish (AB strain) were reared in circulating tap water at 28°C with a photoperiod of 14 h of light/10 h of dark. Fertilized eggs were incubated in normal water (NW) or different artificial FWs for specific experiments. During acclimation experiments, fish were starved, and the media were changed daily to guarantee optimal water quality. The experimental protocols were approved (no. 95013) by the National Taiwan Normal University Animal Care and Utilization Committee.

Preparation of NW, L-Na, and H-Amm water and acclimation. All of the incubating solutions were prepared with double-deionized water supplemented with various salts (Sigma-Aldrich, St. Louis, MO). NW contained (in mM) 0.5 NaCl, 0.2 CaSO4, 0.2 MgSO4, 0.16 KH2PO4, and 0.16 K2HPO4 (pH 7.0); L-Na water contained (in mM) 0.005 NaCl, 0.25 MgCl2, 0.2 CaSO4, 0.16 KH2PO4, and 0.16 K2HPO4 (pH 7.0); H-Amm and L-Na-H-Amm waters were prepared by adding 2.5 mM (NH4)2SO4 to NW or L-Na water. Fertilized eggs were immediately transferred to the media after collection. The acclimated larvae were examined at 4 days postfertilization (dpf) in this study. No significant increases in mortality or developmental abnormalities were found in larvae acclimated to L-Na, H-Amm, or L-Na-H-Amm for 4 days. To examine the gene expression in gills, adult zebrafish were acclimated to L-Na for 7 days.

SIET. SIET was used to measure H+, Na+, and NH4+ activities at the surface of zebrafish larvae. Glass capillary tubes (no. TW 150–4; World Precision Instruments, Sarasota, FL) were pulled on a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, CA) into micropipettes with tip diameters of 3–4 μm. These were then baked at 120°C overnight and coated with dimethyl chlorosilane (Sigma-Aldrich) for 30 min. The micropipettes were backfilled with a 1-cm column of electrolytes and frontloaded with a 20–30-μm column of liquid ion-exchange cocktail (Sigma-Aldrich) to create an ion-selective microelectrode (probe). The following ionophore cocktails (and electrolytes) were used: NH4+/H+ ionophore I cocktail B (100 mM NH4Cl); Na+/H+ ionophore II cocktail A (100 mM NaCl); and H+/H+ ionophore I cocktail B (40 mM KH2PO4 and 15 mM K2HPO4; pH 7). The details of the system were described in previous reports (29, 35).

To calibrate the ion-selective probe, the Nernstian property of each...
microelectrode was measured by placing the microelectrode in a series of standard solutions (0.1, 1, and 10 mM NH4Cl for the NH4
probe; 0.1, 1, and 10 mM NaCl for the Na+ probe; and pH 6, 7, and 8 for the H+ probe). By plotting the voltage output of the probe against log [NH4\(^+\)], [Na\(^+\)], and [H\(^+\)] values, a linear regression yielded a Nernstian slope of 58.5 ± 0.4 (n = 10) for NH4\(^+\), 56.7 ± 0.5 (n = 10) for Na\(^+\), and 58.6 ± 0.8 (n = 10) for H\(^+\). According to technical documents published on the Sigma website (www.sigmaaldrich.com), the selectivity coefficients of the Fluka NH4\(^+\) ionophore I cocktail B is only four times more selective to NH4\(^+\) than to K\(^+\). To prevent interference from K\(^+\) in the medium, K\(^+\)-free recording medium [0.2 mM NaCl, 0.05 mM (NH4)2SO4, 0.2 mM CaSO4, 0.2 mM MgSO4, 0.16 mM NaH2PO4, 0.16 mM Na2HPO4, 300 \(\mu\)M MOPS buffer, and 0.3 mg/l Tricaine] was used when probing NH4\(^+\). In addition, because the calibration (Nernstian) slope of NH4\(^+\) gradually decays at concentrations below 0.1 mM, the NH4\(^+\) concentration in the recording medium was raised by adding 0.05 mM (NH4)2SO4 for a practical and precise calibration. In preliminary tests, according to technical documents published on the Sigma website (www.sigmaaldrich.com), the selectivity coefficients of the Fluka NH4\(^+\) ionophore I cocktail B is only four times more selective to NH4\(^+\) than to K\(^+\). To prevent interference from K\(^+\) in the medium, K\(^+\)-free recording medium [0.2 mM NaCl, 0.05 mM (NH4)2SO4, 0.2 mM CaSO4, 0.2 mM MgSO4, 0.16 mM NaH2PO4, 0.16 mM Na2HPO4, 300 \(\mu\)M MOPS buffer, and 0.3 mg/l Tricaine] was used when probing NH4\(^+\). In addition, because the calibration (Nernstian) slope of NH4\(^+\) gradually decays at concentrations below 0.1 mM, the NH4\(^+\) concentration in the recording medium was raised by adding 0.05 mM (NH4)2SO4 for a practical and precise calibration. In preliminary tests, according to technical documents published on the Sigma website (www.sigmaaldrich.com), the selectivity coefficients of the Fluka NH4\(^+\) ionophore I cocktail B is only four times more selective to NH4\(^+\) than to K\(^+\). To prevent interference from K\(^+\) in the medium, K\(^+\)-free recording medium [0.2 mM NaCl, 0.05 mM (NH4)2SO4, 0.2 mM CaSO4, 0.2 mM MgSO4, 0.16 mM NaH2PO4, 0.16 mM Na2HPO4, 300 \(\mu\)M MOPS buffer, and 0.3 mg/l Tricaine] was used when probing NH4\(^+\). In addition, because the calibration (Nernstian) slope of NH4\(^+\) gradually decays at concentrations below 0.1 mM, the NH4\(^+\) concentration in the recording medium was raised by adding 0.05 mM (NH4)2SO4 for a practical and precise calibration. In preliminary tests, the selectivity of the Fluka Na\(^+\) ionophore II cocktail A was about 10–16 times more selective to Na\(^+\) than to NH4\(^+\) (measured in 1–10 mM Na\(^+\) solution). Although slight underestimation of Na\(^+\) uptake could not be prevented (since inward Na\(^+\) signals could be weakened by outward NH4\(^+\) signals), it should not cause incorrect interpretation of the data.

Measurement of surface NH4\(^+\), Na\(^+\), and H\(^+\) gradients. The SIET was performed at room temperature (26–28°C) in a small plastic recording chamber filled with 1 ml of normal recording medium that contained NW, 300 \(\mu\)M MOPS buffer, and 0.3 mg/l ethyl 3-aminobenzoate methanesulfonate (Tricaine, Sigma-Aldrich). The pH of the recording media was adjusted to 7.0 by adding a NaOH or HCl solution. Different recording media (such as H-Amn and H-Na) were also used for different purposes. Before measurement, an anesthetized larva was positioned in the center of the chamber with its lateral side contacting the base of the chamber. After 3 min of waiting for signal stabilization, the ion-selective probe was moved to the target position (10–20 \(\mu\)m away from the yolk sac membrane, location “Y” in Fig. 1A) to record the ionic activities for 10 s; then the probe was immediately moved away (~1 cm, location “B” in Fig. 1A) to record the background for another 10 s. The averaged voltage (mV) from the serial recording in the 10 s was used to calculate the ionic concentration at target or background. To calculate ionic gradients, the concentration at target was subtracted by the concentration at background. In this study, \(\Delta[\text{NH}_4^+], \Delta[\text{Na}^+], \) and \(\Delta[\text{H}^+]\) were used to represent the measured \(\text{NH}_4^+\), \(\text{Na}^+\), and \(\text{H}^+\) gradients, respectively, between the targets (at the surface of larval skin) and background. Our previous study (19) showed that the measured ionic gradients vary with the distance between the probe tip and the larval skin; therefore, the probe’s location was maintained as consistent as possible with all samples. The noise of the system was usually less than 10 \(\mu\)V and was neglected when calculating the ionic gradients (the recorded voltage difference with larvae was usually 1–10 mV).

Acute effects of high-MOPS, high-NH4\(^+\), and high-Na\(^+\) (H-Na) media on ionic gradients. Ionic gradients (Na\(^+\) and NH4\(^+\)) of L-Na-acclimated larvae were recorded in four different recording media:

![Fig. 3. Real-time PCR analysis of nhe3b, Rhcg1, and atp6v1a expressions in whole larvae (A–C) and adult gills (D–F).](http://ajpregu.physiology.org/Downloadedfrom)
Normal (control), high-MOPS (H-MOPS), high-ammonium (H-Ammm), and high-Na⁺ (H-Na) recording media. The H-Ammm recording medium was prepared by adding 2.5 mM (NH₄)₂SO₄ to normal recording medium. The H-MOPS recording medium was prepared by adding 5 mM MOPS buffer to normal recording medium. The H-Na water was prepared by adding 5 mM Na₂SO₄ to normal recording medium. The pH of the four media was adjusted to pH 7.0. When probing NH₄⁺, K⁺ in the 4 recording media was free (KH₂PO₄ and K₂HPO₄ in the recording media were replaced by NaH₂PO₄ and NaH₂PO₄) and 0.05 mM (NH₄)₂SO₄ was added.

Effects of 5-ethylisopropyl amiloride on ionic gradients. The 5-ethylisopropyl amiloride (EIPA; Sigma) stock solutions were prepared by dissolving EIPA in DMSO (Sigma). A final concentration of 1 mM EIPA was applied to larvae for 10 min, according to a previous study (35). After treatment, the surface ionic gradients of larvae were immediately measured in normal recording medium.

Morpholino design and microinjection. Morpholino oligonucleotides (MO) were obtained from Gene Tools (Philomath, OR). The sequences of MO against Rhcg1 and nhe3b are Rhcg1 (5’-CATTT-GCCCATATCTACAGCTTGAG-3’) and nhe3b (5’-ATGAAAGAC-GCCATATCTACAGCTTGAG-3’). A standard control MO (5’-CCTCTTAC-CTCAGTACAATTTATA-3’) was also used as the control. The control MO provided by Gene Tools has no target and no significant biological activity. The MO solution was prepared with sterile water and contained 0.1% phenol red as a visualizing indicator. The MO solution was microinjected into the embryo at the 1–4-cell stage with an micropipette.

Preparation of total RNA. Zebrafish larvae or adult gills were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was purified following the manufacturer’s protocol. The total amount of RNA was determined by spectrophotometry (ND-1000, NanoDrop Technologies, Wilmington, DE), and the RNA quality was checked by running electrophoresis in RNA-denatured gels. All RNA pellets were stored at −20°C.

Real-time quantitative (q)PCR. Total RNA was extracted and reverse-transcribed from zebrafish larvae described above. The mRNA expression of target genes was measured by a qPCR with the Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). Primers for all genes were designed (Table 1) using Primer Premier software (vers. 5.0; Premier Biosoft International, Palo Alto, CA). PCRs contained 5 ng of complementary cDNA, 50 nM of each primer, and the LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a final volume of 10 μL. All qPCRs were performed as follows: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (the standard annealing temperature of all primers). PCR products were subjected to a melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with sterile water to determine levels of background and genomic DNA contaminations. The standard curve of each gene was confirmed to be in a linear range by dissolving EIPA in DMSO (Sigma). A final concentration of 1 mM

Statistical analysis. Data are expressed as the means ± SE. Values from each condition were analyzed using one-way ANOVA followed by Tukey’s pairwise comparisons. Student’s unpaired t-test (two-tailed) was used for simple comparisons of two means. In all cases, significance was accepted at a level of 0.05.

RESULTS

Na⁺, H⁺, and NH₄⁺ gradients at the yolk-sac surface of larvae acclimated to different waters. Surface ionic gradients of zebrafish larvae acclimated to NW, L-Na, H-Ammm, and

Fig. 4. Effects of Rhcg1 knockdown on Na⁺ uptake, NH₄⁺, and H⁺ excretion by zebrafish larvae. Δ[NH₄⁺] (A), Δ[Na⁺] (B) and Δ[H⁺] (C) at the yolk sac surface of wild-type (WT; 0 ng) and 1–4 ng Rhcg1 morpholino (MO)-injected larvae. The WT and MO-injected larvae were acclimated to L-Na water and measured at 4 dpf. Data are presented as means ± SE; n = 10 larvae. a,b Different letters indicate a significant difference (by one-way ANOVA and Tukey’s comparison, P < 0.05).
L-Na-H-Amm water for 4 days were measured with the SIET to determine ion excretion and uptake by larval skin. The yolk sac showed notable gradients of $\text{H}^+$, $\text{NH}_4^+$, and $\text{Na}^+$; therefore, we chose this location to compare ionic gradients (Fig. IA shows the location of probing and calculation of ionic gradients). Positive gradient values indicate secretion of ions by the yolk sac membrane; in contrast, negative values indicate uptake of ions. Results showed that $\text{H}^+$ and $\text{NH}_4^+$ were secreted by NW larvae; however, a $\text{Na}^+$ gradient was almost absent (Fig. 1, B–D). L-Na acclimation remarkably increased $\text{Na}^+$ gradients but not $\text{NH}_4^+$ gradients (Fig. 1, B and C). H-Amm acclimation increased $\text{NH}_4^+$ gradients but not $\text{Na}^+$ gradients (Fig. 1, B and C). There seemed to be no correlation between $\text{NH}_4^+$ excretion and $\text{Na}^+$ uptake. However, comparing the L-Na and L-Na-H-Amm groups, both $\text{NH}_4^+$ gradients and $\text{Na}^+$ gradients were induced by high $\text{NH}_4^+$ acclimation (Fig. 1, B and C). In addition, $\text{NH}_4^+$ and $\text{H}^+$ gradients were negatively correlated (Fig. 1, B and D), suggesting that the secreted $\text{H}^+$ was consumed by excreted $\text{NH}_3$ gas, as shown in our previous reports (29, 35). In this experiment, no significant differences were found in mortality or hatching rates among the four groups.

Acute effects of external medium on $\text{Na}^+$ and $\text{NH}_4^+$ gradients. To further test the linkage between $\text{Na}^+$ uptake and $\text{NH}_4^+$ excretion, larvae were subjected to acute changes in external media with H-MOPS (5 mM MOPS), H-Amm (5 mM $\text{NH}_4^+$), or H-Na (10 mM $\text{Na}^+$), and changes in $\text{Na}^+$ and $\text{NH}_4^+$ gradients were then determined by the SIET. L-Na-acclimated larvae were used because $\text{NH}_4^+$-dependent $\text{Na}^+$ uptake was found (Fig. 1C). In our previous study, the MOPS buffer was shown to effectively suppress $\text{H}^+$ accumulation and $\text{NH}_4^+$ excretion at the surface of larval skin (29). In this experiment, both the $\text{Na}^+$ and $\text{NH}_4^+$ gradients were suppressed by the H-MOPS buffer (Fig. 2, A and B). H-Amm water reversed the $\text{NH}_4^+$ gradient (values dropped from positive to negative; Fig. 2D) at the surface of larvae and almost completely eliminated the $\text{Na}^+$ gradient (Fig. 2C). In contrast, raising the external $\text{Na}^+$ level (H-Na water) increased the $\text{Na}^+$ gradient (Fig. 2E) and also increased the $\text{NH}_4^+$ gradient (Fig. 2F). Once again, this experiment revealed a tight linkage between the $\text{Na}^+$/$\text{NH}_4^+$ transport of zebrafish larvae.

Real-time qPCR of $\text{nhe3b}$, $\text{Rhcg1}$, and $\text{atp6v1a}$ in NW and L-Na zebrafish. To examine the expressions of genes involved in $\text{Na}^+$/$\text{NH}_4^+$ transport, a qPCR was applied to analyze transcripts of $\text{nhe3b}$, $\text{Rhcg1}$, and $\text{atp6v1a}$ in larvae and gills of adult zebrafish acclimated to NW or L-Na for 4 days (larvae) and 7 days (adult fish). Results showed that $\text{nhe3b}$ was significantly induced in L-Na larvae (Fig. 3A), whereas $\text{atp6v1a}$ was significantly downregulated in L-Na larvae (Fig. 3C). No significant change was found in $\text{Rhcg1}$ of whole larvae acclimated to L-Na water (Fig. 3B). In the gill of adult fish, both of $\text{nhe3b}$ and $\text{Rhcg1}$ were upregulated (Fig. 3, D and E), whereas $\text{atp6v1a}$ was significantly downregulated (Fig. 3F).

$\text{Na}^+$ and $\text{NH}_4^+$ transport in larvae with $\text{Rhcg1}$ gene knockdown. The effect of $\text{Rhcg1}$ knockdown on $\text{NH}_4^+$ excretion of zebrafish was reported in our previous study (29). In this experiment, we used the same approach to further investigate whether $\text{Na}^+$ uptake was also affected by $\text{Rhcg1}$ MOs. After being microinjected with $\text{Rhcg1}$ MOs, embryos were transferred to L-Na water and measured with SIET at 4 dpf. Dose-dependent decreases in the $\text{NH}_4^+$ gradient (Fig. 4A) and $\text{Na}^+$ (Fig. 4B) gradients were found in larvae with an $\text{Rhcg1}$ MO injection. A dose of 4 ng effectively decreased both the $\text{Na}^+$ and $\text{NH}_4^+$ gradients to significant levels (about 50% decreases). However, no significant change was found in $\text{H}^+$ gradient in $\text{Rhcg1}$ morphants (Fig. 4C). Although morphological changes in morphants (MO-injected larvae) were not obvious, the whole-body $\text{Na}^+$ content of morphants was significantly lower than that of control larvae (Fig. 5A).

$\text{Na}^+$ and $\text{NH}_4^+$ transport in larvae with EIPA treatment and $\text{nhe3b}$ gene knockdown. To test the role of NHE in ionic transport, 1 mM EIPA was applied to L-Na larvae for 10 min before measurements. As shown in Fig. 6, $\text{Na}^+$ gradients (uptake), $\text{NH}_4^+$ gradients (secretion), and $\text{H}^+$ gradients (secretion) were significantly suppressed by EIPA treatment. In addition, $\text{nhe3b}$ MO-injected embryos (4 dpf) were also examined. Results shows that knockdown of $\text{nhe3b}$ significantly decreased $\text{Na}^+$, $\text{NH}_4^+$, and $\text{H}^+$ gradients (Fig. 7) at the yolk-sac skin, and whole body $\text{Na}^+$ content (Fig. 5B).

**DISCUSSION**

Our previous studies with SIET have shown its advantages in investigating various ionic transports by ionocytes in the yolk-sac skin of fish embryos and larvae. Lin et al. (19) applied the SIET to identify HR cells as acid-secreting cells in the skin of zebrafish embryos. Shih et al. (29) further used the SIET to investigate the mechanism of ammonia excretion by HR cells. Horng et al. (6) used the SIET to analyze Cl$^-$ transport by FW-type MRCs and SW-type MRCs in tilapia larvae. Wu et al.

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**Fig. 5.** Whole-body Na$^+$ content in zebrafish larvae acclimated to L-Na with $\text{Rhcg1}$ or $\text{nhe3b}$ knockdown. Data are presented as means ± SE ($n = 6$). *Significant difference between the WT and MO (by Student’s t-test, $P < 0.05$).
used the SIET to demonstrate a NH₄⁺/H⁺-dependent Na⁺ uptake mechanism in MRCs of medaka larvae. Recently, Shen et al. (28) used it to demonstrate a functional plasticity of MRCs in medaka larvae. In this study, we further used the same approach to measure Na⁺, NH₄⁺, and H⁺ transport by zebrafish larvae. In zebrafish, we found that the Na⁺ signals from individual ionocytes (including HR cells and other subtypes) were not high enough to reveal their function in Na⁺ transport (signal-to-noise level is not good enough). Therefore, we only used SIET to measure ionic gradients at their yolk-sac surface but not individual ionocytes.

In our previous study on medaka larvae, a tight linkage between Na⁺ uptake and NH₃/NH₄⁺ excretion (NH₄⁺-dependent Na⁺ uptake) was found. L-Na acclimation induced compensatory Na⁺ uptake and also induced NH₃/NH₄⁺ excretion; H-Amm acclimation induced compensatory NH₃/NH₄⁺ excretion and also induced Na⁺ uptake (35). However, in the present study, L-Na acclimation induced compensatory Na⁺ uptake, but not NH₃ secretions (Fig. 1, B and C); H-Amm acclimation increased NH₃ secretions but not Na⁺ uptake (Fig. 1, B and C). A linkage between NH₃ excretion and Na⁺ uptake seemed to be absent or insignificant. However, both NH₃ secretion and Na⁺ uptake were induced by H-Amm acclimation when the Na⁺ level was low (compare L-Na and L-Na-H-Amm in Fig.

Fig. 6. Effects of 5-ethylisopropyl amiloride (EIPA) (1 mM) on Na⁺ uptake and NH₄⁺ excretion by zebrafish larvae. ∆[NH₄⁺] (A), ∆[Na⁺] (B), and ∆[H⁺] (C) at the yolk sac surface of L-Na larvae. The same concentration of solvent (DMSO) was added to L-Na water as a control. Data are presented as means ± SE; n = 10 larvae. *Significant difference between control and EIPA groups (by Student’s t-test, P < 0.05).

Fig. 7. Effects of nhe3b knockdown on Na⁺ uptake and NH₄⁺ excretion by zebrafish larvae. ∆[NH₄⁺] (A), ∆[Na⁺] (B), and ∆[H⁺] (C) at the yolk sac surface of wild-type (WT) and 4 ng NHE3b MO-injected larvae. The WT and MO-injected larvae were acclimated to L-Na water and measured at 4 days dpf. Data are presented as means ± SE; n = 10 larvae. *Significant difference between WT and MO (by Student’s t-test, P < 0.05).
1. B and C), suggesting that low-Na\(^+\) water can induce NH\(_4\)\(^+\)-dependent Na\(^+\) uptake. Since, more than one Na\(^+\) uptake mechanism by zebrafish has been proposed, the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake is probably not the dominant mechanism in normal Na\(^+\) water. The purpose of this study is to examine the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake pathway; therefore, we did most experiments with L-Na\(^+\)-induced larvae.

In addition to the above data, other data also support the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake in L-Na water. Acutely raising the external NH\(_4\)\(^+\) level blocked NH\(_3\) excretion and Na\(^+\) uptake; in contrast, raising the external Na\(^+\) level enhanced Na\(^+\) uptake and NH\(_4\)\(^+\) excretion. The addition of MOPS buffer to the medium, which was previously shown to decrease H\(^+\) accumulation and NH\(_4\)\(^+\) excretion at the skin surface, also suppressed Na\(^+\) uptake. The result of MOPS addition is interesting. MOPS buffer is supposed to diminish extracellular H\(^+\) and consequently increase Na\(^+\) uptake via NHE due to the favorable H\(^+\) gradient. However, the fact that MOPS decreased both Na\(^+\) and NH\(_4\)\(^+\) gradient supports the hypothesis that Na\(^+\) uptake is dependent on NH\(_4\)\(^+\) excretion.

As mentioned in the introduction, a coupling function of NHE3b and Rhcg1 in the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake was hypothesized and tested. We applied NHE inhibitor (EIPA) to the larvae and found that both Na\(^+\) uptake and NH\(_4\)\(^+\) excretion were suppressed. Genetic knockdown of Rhcg1 in zebrafish embryos was reported in our previous study, and we concluded that Rhcg1 on HR cells is critical for ammonia excretion (29).

In this study, we used the same MO to knock down Rhcg1 translation and further found that Rhcg1 MO suppressed both Na\(^+\) uptake and NH\(_4\)\(^+\) excretion (Fig. 4). In addition, nhe3b MO was also applied to the embryos and found that both Na\(^+\) uptake and NH\(_4\)\(^+\) excretion by larval skin were significantly suppressed, which is consistent with the effect of EIPA (Fig. 5). Taken together, the loss-of-function evidence supports that NHE3b and Rhcg1 in HR cells are involved in the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake.

We also examined the mRNA levels of related genes in whole larvae and gills from adult zebrafish acclimated to L-Na water. Results showed that nhe3b was upregulated, whereas atp6v1a was downregulated in L-Na larva. This result consolidates a previous report that showed nhe3b was upregulated and atp6v1a (encodes another subunit of H\(^+\)-ATPase) was downregulated in gills of adult zebrafish acclimated to L-Na water (36). However, we found that the Rhcg1 expression of larvae was not induced by L-Na acclimation (Fig. 3B). This result is inconsistent with a previous report, which showed that Rhcg1 mRNA in zebrafish larvae was induced by diluted FW (22). This inconsistency might be due to differences in the ionic composition of water or developmental stage of larvae. Because the mRNA level in whole larvae cannot represent that in the larval skin, we also examined the mRNA level in gills from adult individuals. Rhcg1 expression in gills was significantly induced after L-Na acclimation, supporting that Rhcg1 is involved in the NH\(_4\)\(^+\)-dependent Na\(^+\) uptake mechanism.

In our previous study on medaka, a new model of Na\(^+\)/NH\(_4\)\(^+\) exchange in the apical membrane of MR cells was proposed. Although the case of zebrafish is not totally identical to that of medaka, a similar model is suggested to elucidate Na\(^+\)/NH\(_4\)\(^+\) exchange in HR cells of zebrafish. In this model (Fig. 8), Rhcg1 and NHE3 function together to achieve Na\(^+\)/NH\(_4\)\(^+\) exchange, as we proposed in medaka. Rhcg1 deprotonates intracellular NH\(_4\)\(^+\) and conducts nonionic NH\(_3\). The dissociated H\(^+\) then provides a chemical gradient for the exchange of Na\(^+\) via NHE3b. In addition to the association of Rhcg1 and NHE3b, H\(^+\)-ATPase is involved in pumping H\(^+\) out of the apical membrane, which facilitates nonionic NH\(_3\) diffusion [by an acid-trapping mechanism, (29)] and, in turn, facilitates the deprotonation of intracellular NH\(_4\)\(^+\). However, the coupling of Rhcg1 and NHE3b seems to be more critical in terms of Na\(^+\) uptake. When the external Na\(^+\) level is low, the expression of NHE3b is upregulated to compensate the Na\(^+\) uptake; in contrast, the expression of H\(^+\)-ATPase is downregulated. Although the existence of H\(^+\)-ATPase promotes NH\(_4\)\(^+\) excretion, it does not favor the accumulation of H\(^+\) inside cells, which is required to drive the NHE. Therefore, to compensate for Na\(^+\) uptake in L-Na water, the expression of H\(^+\)-ATPase needs to be downregulated. The major role of H\(^+\)-ATPase seems to be in acid secretion and ammonia excretion, not in Na\(^+\) uptake, as suggested in our previous studies (7, 29). To cope with an extremely acid environment (pH 4), a larger apical membrane with abundant H\(^+\)-ATPase was induced in HR cells (8).

In a very recent study (17), a similar mechanism of Rhcg1 and NHE3b coupling was also reported in zebrafish under an acidified situation. In Kumai and Perry’s study (17), Rhcg1 or nhe3b knockdown decreased zebrafish Na\(^+\) uptake in acidic water, but not in normal water. The NHE3-specific inhibitor, EIPA, also decreased Na\(^+\) uptake in acidic water but showed no effect in normal water. However, the acidic environment was previously reported to suppress the expression and function of NHE3b in zebrafish gills (36). EIPA was previously...
found to suppress Na\(^+\) uptake in zebrafish embryos by either \(^{22}\text{Na}\) radioisotope tracer or sodium green fluorescence (2). More experiments are needed to clarify these inconsistent results.

In addition to the coupling of NHE3b and Rhcg1 in HR cells, previous studies on zebrafish also suggested that the maintenance of transcellular pH gradient of HR cells is necessary for absorbing Na\(^+\). Carbonic anhydrase (CA15a), anion exchanger (AE1, slc4a1b), and Na\(^+\) pump (apta1a1a.5) were found to be induced by L-Na\(^+\) acclimation in zebrafish (18, 20). Knockdown of CA15a (20) and AE1 (18) suppressed Na\(^+\) uptake by zebrafish embryos. Taken together, a model for HR cells is proposed (10, 11). In the apical membranes of HR cells, NHE3b and H\(^+\)-ATPase transport H\(^+\) out of the cells, and this H\(^+\) combines with environmental HCO\(_3\)\(^-\) to generate CO\(_2\) by CA15a. Then, CO\(_2\) enters HR cells and is hydrated by cytosolic carbonic anhydrase (CA2) to form H\(^+\) and HCO\(_3\)\(^-\). Basolateral AE1b extrudes cytosolic HCO\(_3\)\(^-\) out of HR cells, and this also provides an intracellular pH gradient favorable for the apical NHE3b to achieve the apical Na\(^+\) uptake. Basolateral NKA is responsible for generating low intracellular Na\(^+\) and negative membrane potential.

In addition to the proposed NH\(_2\)\(^+\)-dependent Na\(^+\) uptake pathway, we also agree with the existence of other pathways for Na\(^+\) uptake in zebrafish. A recent study on zebrafish with a double in situ hybridization indicated that Na\(^+\)-HCO\(_3\) cotransporter (NBC, slc4a4b) mRNA was coexpressed with the Na\(^+\)-Cl\(^-\) cotransporter (NCC, slc12a10.2) in another subtype of ionocytes (NCC cells), suggesting that a possible coordination of apical NCC and basolateral NBC in Na\(^+\) uptake exists (18). In addition, a preliminary experiment on zebrafish demonstrated that knockdown of gcm2, a transcriptional factor involved in the differentiation of HR cells, resulted in the disappearance of HR cells, but also caused a compensatory increase in both the number of NCC cells and Na\(^+\) uptake function in the skin of zebrafish embryos (11). Therefore, both HR cells and NCC cells seem to be responsible for the Na\(^+\) uptake of zebrafish. However, the NH\(_2\)\(^+\)-dependent Na\(^+\) uptake by HR cells is probably a dominant pathway when ambient Na\(^+\) is low.

**Perspectives and Significance**

The role of NCC cells in Na\(^+\) uptake is not well known. How do HR cells and NCC cells coordinate Na\(^+\) uptake is still an open question. This issue needs to be further investigated. In addition, the NH\(_2\)\(^+\)-dependent Na\(^+\) uptake mechanism seems to occur in extremely harsh water (pH 4 or L-Na water) in zebrafish, but it occurs in normal water in euryhaline medaka. In medaka, other Na\(^+\) uptake mechanisms have not been reported. Whether it does not have an alternative Na\(^+\) uptake pathway needs to be investigated. By comparing zebrafish and medaka, we might be able to reveal functional differences between stenohaline and euryhaline teleosts.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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