Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (Oryzias latipes) larvae

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Wu SC, Horng JL, Liu ST, Hwang PP, Wen ZH, Lin CS, Lin LY. Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (Oryzias latipes) larvae. Am J Physiol Cell Physiol 298: C237–C250, 2010. First published November 25, 2009; doi:10.1152/ajpcell.00373.2009.—In this study, a scanning ion-selective electrode technique (SIET) was applied to measure H⁺, Na⁺, and NH₄⁺ gradients and apparent fluxes at specific cells on the skin of medaka larvae. Na⁺ uptake and NH₄⁺/NH₃ excretion were detected at most mitochondrion-rich cells (MRCs). H⁺ probing at MRCs revealed two groups of MRCs, i.e., acid-secreting and base-secreting MRCs. Treatment with EIPA (100 µM) blocked 35% of the NH₄⁺/NH₃ secretion and 54% of the Na⁺ uptake, suggesting that the Na⁺/H⁺ exchanger (NHE) is involved in Na⁺ and NH₄⁺/NH₃ transport. Low-Na⁺ water (<0.001 mM) or high-NH₄⁺ (5 mM) acclimation simultaneously increased Na⁺ uptake and NH₄⁺/NH₃ excretion but decreased or even reversed the H⁺ gradient at the skin and MRCs. The correlation between NH₄⁺ production and H⁺ consumption at the skin surface suggests that MRCs excrete nonionic NH₃ (base) by an acid-trapping mechanism. Raising the external NH₄⁺ significantly blocked NH₄⁺/NH₃ excretion and Na⁺ uptake. In contrast, raising the acidity of the water (pH 7 to pH 6) enhanced NH₄⁺/NH₃ excretion and Na⁺ uptake by MRCs. In situ hybridization and real-time PCR showed that the mRNAs of the Na⁺/H⁺ exchanger (slc9a3) and Rhesus glycoproteins (Rhcg1 and Rhbg) were colocalized in MRCs of medaka, and their expressions were induced by low-Na⁺ acclimation. This study suggests a novel Na⁺/NH₄⁺ exchange pathway in apical membranes of MRCs, in which a coupled NHE and Rh glycoprotein is involved and the Rh glycoprotein may drive the NHE by generating H⁺ gradients across apical membranes of MRCs.

Na⁺/H⁺ exchanger; Rhesus glycoprotein; osmoregulation; gills; ionocytes

IN 1938, AUGUST KROGH PIONEERED the study of Na⁺/Cl⁻ uptake in freshwater (FW) fish and suggested that Na⁺ is exchanged for NH₄⁺ at fish gills. Since then, many studies have been carried out to investigate the mechanisms involved in Na⁺ uptake and NH₄⁺ excretion. However, this Na⁺/NH₄⁺ exchange mechanism has been controversial for a long time, and many studies produced conflicting conclusions (7).

Several studies reported that the amount of NH₄⁺ excreted is close to the amount of Na⁺ taken up in intact salmonids or in an isolated head preparation (24, 30, 35, 47). In addition, NH₄⁺ loading was found to stimulate Na⁺ uptake (35, 45). These observations supported the presence of a Na⁺/NH₄⁺ exchange pathway in fish gills. On the other hand, several studies also suggested that NH₄⁺ excretion and Na⁺ uptake are at best only loosely coupled and that nonionic diffusion of NH₃ plays a major role in ammonia excretion (20, 44–46). Kerstetter and colleagues (18) proposed that Na⁺ was in fact exchanged for H⁺ as opposed to NH₄⁺. A Na⁺/H⁺ exchanger (NHE) was further suggested to be a player in Na⁺/H⁺ exchange based on the inhibition of Na⁺ uptake by amiloride (20). Avella and Bornancin (1) also suggested that Na⁺ is exchanged for H⁺ instead of NH₄⁺, but NHE could not function in Na⁺ uptake in fish living in extremely dilute environments. They proposed that an apical v-type H⁺-ATPase electrochemically linked to a Na⁺ channel drives Na⁺ uptake from fresh water. 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 nearly two decades.

Several convincing lines of evidence have been proposed to support the H⁺-ATPase-dependent Na⁺ uptake pathway, although identification of the Na⁺ channel has not been successful (13, 14). Recently, we 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 (HRC) in gills and embryonic skin is an acid-secreting cell (22). Subsequent studies further suggested that Na⁺ uptake is mediated by HRCs (6, 12). On the other hand, Na⁺ uptake through the NHE has been questioned for a long time due to the unresolved driving force (29). However, recently accumulating evidence has shown its significant role in Na⁺ uptake. The first molecular cloning and functional study of the NHE in FW fish was from the Japanese Osorezan dace (Tribolodon hakonensis), which lives in an acidic pH of 3.5 (10). Interestingly, the extremely acidic water supposedly did not favor driving of the NHE, but the expression of the NHE was induced by acid acclimation, suggesting that the NHE plays a critical role in acid secretion and Na⁺ uptake (10). Recently, Yan et al. (49) cloned and identified zNHE3 in the HRC of zebrafish gills. Low-Na⁺ (L-Na⁺) acclimation induced the mRNA expression of zNHE3 but suppressed that of H⁺-ATPase. Ivanis et al. (16) used homologous antibodies to localize NHE2/NHE3 in the apical membrane of MRCs in rainbow trout (Oncorhynchus mykiss). In tilapia (Oreochromis mossambicus), a homologous antibody also labeled NHE3 in the apical membrane of a subtype of MRCs (11). Those studies all suggested that the NHE is involved in Na⁺ uptake in FW fishes. Early studies suggested that Na⁺/NH₄⁺ exchange might occur through the NHE, hence the NH₄⁺ gradient could drive
Na\(^+\) uptake (43). However, as mentioned above, several studies indicated that nonionic NH\(_3\) is the major form of ammonia excreted in gills through an acid-trapping mechanism. Recently, studies on Rhesus glycoproteins (members of the Amt/MEP/Rh superfamily) suggested that Rh proteins function as a NH\(_3\) channel for NH\(_3\)/NH\(_4\)\(^+\) excretion (17, 19, 32, 50). Several isoforms of Rh proteins (Rhog, Rhbg, Rhcg1, and Rhcg2) were also identified from fish gills (26, 27). Using a zebrafish model and morpholino gene knockdown, we demonstrated that the apical isofrom, Rhcg1, is involved in ammonia excretion by HRCs, and also suggested that H\(^+\) -ATPase generates an acidic layer to drive nonionic NH\(_3\) diffusion through Rhcg1 (37). That was the first loss-of-function study of Rh proteins conducted in an intact animal. The finding of Rh proteins consolidated the acid-trapping mechanism of NH\(_3\) excretion (41).

Although the Na\(^+\)/NH\(_4\)\(^+\) exchange pathway through the NHE is unlikely, it is still unclear how to link the pathway for NH\(_4\)\(^+\) excretion and Na\(^+\) uptake in fish gills. The presence of Na\(^+\) uptake and NH\(_4\)\(^+\) excretion in the same cell (HRCs) of zebrafish led us to reconsider other possible mechanisms for Na\(^+\)/NH\(_4\)\(^+\) exchange. Recently, Tsui and colleagues (40) examined the same question using cultured trout gill cells and suggested that Na\(^+\)/NH\(_4\)\(^+\) exchange is mediated by a putative protein complex including an Rh protein, the NHE, H\(^+\)-ATPase, and an unidentified Na\(^+\) channel. That model was further elaborated by Wright and Wood (48). However, it is still a premature model and needs more-direct evidence to support it. Recently, we used the SIET to detect Na\(^+\) uptake by HRCs of zebrafish larvae and tried to figure out whether Na\(^+\)/NH\(_4\)\(^+\) exchange occurs in HRCs. However, we found that the Na\(^+\) current detected at HRCs was too small to conduct functional assays. Alternatively, we detected significant Na\(^+\) influx at MRCs of another model fish, Japanese medaka (Oryzias latipes). Therefore, we established a new medaka model in this study to examine the mechanism of Na\(^+\) uptake and NH\(_3\)/NH\(_4\)\(^+\) excretion. Using the SIET, we attempted to measure Na\(^+\), H\(^+\), and NH\(_3\)/NH\(_4\)\(^+\) transport at the skin and individual MRCs of medaka larvae, and to determine the mechanism for Na\(^+\) and NH\(_3\)/NH\(_4\)\(^+\) transport. In this study, medaka larvae were acclimated to L-Na or high-NH\(_4\)\(^+\) (H-Amm) water to induce their mechanisms for Na\(^+\) uptake and NH\(_3\)/NH\(_4\)\(^+\) excretion. By probing three ionic gradients (Na\(^+\), H\(^+\), and NH\(_3\)), a linkage between Na\(^+\) uptake and nonionic NH\(_3\) excretion was found in the skin and individual MRCs. Furthermore, pharmacological and molecular data [in situ hybridization and real-time quantitative PCR (qPCR)] suggested that NHE3 (slc9a3) and Rh glycoproteins (Rhbg and Rhcg1) are involved in the NH\(_3\)/NH\(_4\)\(^+\) -dependent Na\(^+\) uptake pathway of MRCs.

**MATERIALS AND METHODS**

*Experimental animals.* Mature Japanese medaka (Oryzias latipes) were reared in circulating tap water at 27°C with a photoperiod of 14 h of light/12 h of dark. The females spawned every day, and the fertilized egg clusters were collected from the belly of females and rinsed with running tap water to remove the sludge and separate the clusters into single eggs. The eggs were incubated in normal water (NW; see below) or different artificial fresh waters for specific experiments. Embryos usually hatched at 7–8 days postfertilization (dpf), and the newly hatched larvae were used for the following experiments. During the experiments, the larvae were not fed, and the media were changed daily to guarantee optimal water quality. The experimental protocols were approved (no. S95013) by the National Taiwan Normal University Animal Care and Utilization Committee.

*Preparation of normal, L-Na, and high-Amm water and acclimation.* All of the incubating solutions were prepared by adding various salts (Sigma-Aldrich, St. Louis, MO) to the reddistilled water. The NW contained (in mM) 0.5 NaCl, 0.2 CaSO\(_4\), 0.2 MgSO\(_4\), 0.16 KH\(_2\)PO\(_4\), and 0.16 K\(_2\)HPO\(_4\) (pH 7.0). The L-Na water contained 0.25 MgCl\(_2\), 0.2 CaSO\(_4\), 0.16 KH\(_2\)PO\(_4\), and 0.16 K\(_2\)HPO\(_4\) (pH 6.8). The high-Amm (5 mM) water was prepared by adding 2.5 mM (NH\(_4\))\(_2\)SO\(_4\) to NW. Fertilized eggs were incubated in normal water for the first 2 days and then sorted into different acclimation groups for 5 more days. Incubation solutions were replenished daily during the acclimation. No significant delay in hatching was found in larvae acclimated to L-Na or H-Amm water.

**Scanning ion-selective electrode technique.** The SIET was used to measure H\(^+\), Na\(^+\), and NH\(_3\)/NH\(_4\)\(^+\) activities and fluxes at the surface of medaka 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 vapor-sanitized with dimethyl chlorosilane (Sigma-Aldrich) for 30 min. The micropipettes were backfilled with a 1-cm column of electrolytes and frontloaded with a 20- to 30-μm column of liquid ion exchanger cocktail (Sigma-Aldrich) to create an ion-selective microelectrode (probe). The following ionophore cocktails (and electrolytes) were used: H\(^+\) ionophore I cocktail B (40 mM KH\(_2\)PO\(_4\) and 15 mM K\(_2\)HPO\(_4\); pH 7); Na\(^+\) ionophore II cocktail A (100 mM NaCl); and NH\(_4\)\(^+\) ionophore I cocktail B (100 mM NH\(_4\)Cl). The details of the system were described in a previous report (22). To calibrate the ion-selective probe, the Nernstian property of each microelectrode was measured by placing the microelectrode in a series of standard solutions (pH 6, 7, and 8 for the H\(^+\) probe; 0.1, 1, and 10 mM NaCl for the Na\(^+\) probe; and 0.1, 1, and 10 mM NH\(_4\)Cl for the NH\(_3\) probe). By plotting the voltage output of the probe against log [H\(^+\)], [Na\(^+\)], and [NH\(_3\)] values, a linear regression yielded a Nernstian slope of 58.6 ± 0.6 (n = 10) for H\(^+\), 57.3 ± 0.7 (n = 10) for Na\(^+\), and 58.2 ± 0.8 (n = 10) for NH\(_3\).
microvolts was measured by probing orthogonally to the surface at 10-μm intervals. The "single-spot recording" was performed at the surface of an MRC or a keratinocyte (KC) for five replicates, and the median of the repeats was used for calculating the ion flux of the cell. In addition, a "line-scan recording" was made by probing a series of spots along a line (40 μm with nine spots) across the surface of MRCs and adjacent KCs. At every spot, the voltage difference in microvolts was measured by probing orthogonally to the surface at a 10-μm distance.

The calculation of ionic flux was shown in previous reports (4, 37, 38). Voltage differences obtained from the ASET software were converted into a concentration (activity) gradient using the following equation:

\[ \Delta C = C_b \times 10^{(\Delta V/6) - \Delta X} \] (1)

where \( \Delta C \) (μmol · 1⁻¹ · cm⁻³) is the concentration gradient between the two points, \( C_b \) (μmol/l) is the background ion concentration, \( \Delta V \) (μV) is the voltage gradient obtained from ASET, and \( S \) is the Nernst slope of the electrode. The concentration gradient was subsequently converted into ionic flux using Fick’s law of diffusion in the following equation:

\[ J = D(\Delta C)/\Delta X \] (2)

where \( J \) (pmol · cm⁻² · s⁻¹) is the net flux of the ion, \( D \) is the diffusion coefficient of the ion (9.37 × 10⁻⁵ cm²/s for H⁺; 1.55 × 10⁻⁵ cm²/s for Na⁺; and 2.09 × 10⁻³ cm²/s for NH₄⁺), \( \Delta C \) (pmol · 1⁻¹ · cm⁻³) is the concentration gradient; and \( \Delta X \) (cm) is the distance between the two points.

When probing H⁺ flux in a buffered medium, several reports adjusted the H⁺ flux for the buffering capacity of the medium (4, 8, 39). However, it is difficult to know the true buffering capacity, which is determined not only by additional buffers but also by other factors such as NH₃, CO₂, and HCO₃⁻ in the medium. Therefore, we did not calculate the buffering capacity but present the data as “apparent H⁺ flux” in this study, as we did with the presentation of NH₄⁺ flux. The SIET measures NH₄⁺ gradients at the cell surface. However, as we suggested in a previous study (37) and this study, MRCs mainly excrete NH₃ out of cells, and then the NH₃ is trapped as NH₄⁺ by external H⁺. Therefore, it is proper to present our data as the “apparent NH₄⁺ flux”.

**Treatment with inhibitors.** The inhibitors EIPA, bafilomycin A1, and phenamil were obtained from Sigma-Aldrich. Stock solutions were prepared by dissolving them into dimethyl sulfoxide (DMSO, Sigma-Aldrich). The final concentration of DMSO in working solutions (including control group) was 0.1%, and its effect on ion transport was absent in a preliminary test. Ten larvae were incubated in 1 ml medium with inhibitors for 10 min. After incubation, the larvae were rinsed in inhibitor-free medium and then transferred to the recording medium that did not contain inhibitors. The inhibitors were not added to the recording media to prevent any alteration of the properties of the electrodes.

**Sequential measurement of ionic fluxes at individual MRCs and KCs.** A MRC was randomly chosen from a larva and sequentially measured with a specific electrode for 30–40 s. After the recording, the recording medium was completely withdrawn and replaced by a new medium with high ammonium or low pH. The same recorded MRC was further recorded for another 30–40 s after the change of medium. The image of the recorded MRC was grabbed to indentify the same MRC. Before every recording at MRC, we moved the electrode slightly around the apical surface of MRC to find out the hot spot (highest signal), which ensured that the change of signal was not due to spatial drift.

**Molecular cloning of medaka slc9a3.** Partial open reading frames of medaka slc9a3 homologs obtained from the genome were carefully confirmed by the expressed sequence tag database. Specific primers were designed (Tables 1 and 2) for cloning and the RT-PCR analysis.

**PCR products thus obtained were subcloned into a pGEM-T vector (Promega, Madison, WI), and the nucleotide sequences were determined with an ABI 377 sequencer (Applied Biosystems, Warrington, UK). Sequence analysis was conducted with the BLASTx program (National Center for Biotechnology Information).**

**Table 1. Specific primer sets for the in situ hybridization probe**

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>slc9a3</td>
<td>5'-GCCCTGCTTCTGCTGTTGCG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGGGACAGCGATCCACCCAAAA-3'</td>
</tr>
<tr>
<td>Rhbg</td>
<td>5'-ACGAATTCTTGTACGGACCCA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGGCGTGAAGTCTCCGTCC-3'</td>
</tr>
<tr>
<td>Rhgj1</td>
<td>5'-CGTGTACGCTTCCAGCACT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CACCGACGATACCCTTGGTA-3'</td>
</tr>
</tbody>
</table>

**In situ hybridization and immunohistochemistry.** Medaka larvae were anesthetized on ice and fixed with 4% paraformaldehyde in a phosphate-buffered saline (PBS) solution (1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na₂HPO₄, and 0.002 mM KH₂PO₄; pH 7.4) at 4°C overnight. Afterward, the samples were washed with diethylpyrocarbonate (DEPC)-PBS (PBS with 0.1% Tween-20) several times (for 10 min each) and treated with proteinase K (10 mg/ml). After a brief rinse with PBST, embryos were fixed with 4% paraformaldehyde for another 20 min. After PBST washing, the samples were incubated with hybridization buffer (HyB, 50% formamide, 5 x SSC, and 0.1% Tween 20) at 65°C for 5 min and with HyB containing 500 μg/ml yeast tRNA at 65°C for 4 h before hybridization. After an overnight hybridization with 100 ng/ml DIG-labeled antisense or sense RNA probes, embryos were serially washed with 50% formamide-2 x SSC (at 65°C for 20 min), 2 x SSC (at 65°C for 10 min), 0.2 x SSC (at 65°C for 30 min, 2 times), and PBST at room temperature for 10 min. Afterward, the embryos were immunoreacted with an alkaline phosphatase-coupled anti-DIG antibody (1:8,000) and then treated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for the alkaline phosphatase reaction. Fluorescence in situ hybridization (sle9a3) was conducted with a commercial kit (TSA Plus Fluorescence Systems; Perkin-Elmer). Hybridization signals detected by the DIG-labeled RNA probes were amplified through fluorescein-tyramide signal amplification (TSA), while cyanine 3-TSA was used for the dinitrophenol-labeled probes.

**For double staining of the target gene’s in situ hybridization and Na⁺-K⁺-ATPase immunohistochemistry, the in situ-hybridized samples were subjected to immunohistochemistry. After being washed with PBS, samples were incubated with 3% bovine serum albumin and 5% normal goat serum for 30 min to block nonspecific binding. Samples were then incubated overnight at 4°C with an α-monoclonal antibody against the α-subunit of the avian Na⁺ pump (Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA). After being rinsed with PBS for 20 min, samples were further incubated in goat anti-rabbit immunoglobulin G (IgG) conjugated with FITC or goat anti-mouse IgG conjugated with Texas Red (Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h at room temperature.
Finally, samples were washed twice with PBST for 5 min each and then were stored in PBS at 4°C in a dark box for further examination and analysis. Images were obtained by a fluorescence microscope (Axioplan 2 Imaging, Carl Zeiss Oberkochen, Germany).

Preparation of total RNA. Medaka 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 qPCR. Total RNA was extracted and reverse-transcribed from the adult gills of medaka as described above. The mRNA expression of target genes was measured by qPCR with the Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). Primers for all genes were designed (Table 2) using Primer Premier software (version 5.0; PREMIER Biosoft International, Palo Alto, CA). PCRs contained 3.2 ng of cDNA, 50 nM of each primer, and the LightCycler 480 SYBR Green 1 Master (Roche) in a final volume of 10 μL. All qPCRs were performed as follows: 1 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 contamination. The standard curve of each gene was confirmed to be in a linear range with ribosomal protein L7 (rpl7) as an internal control.

Statistical analysis. Data are expressed as means ± SE (n, number of larvae or MRCs). Values from each condition were analyzed using one-way analysis of variance followed by Tukey’s pairwise comparisons. Student’s unpaired t-test (two-tailed) was used when appropriate for simple comparisons of two means. A χ²-squared test was used to compare the difference in the cell ratio between treatments (Fig. 4D). Significance was set at α level of 0.05.

RESULTS

Na⁺, H⁺, and NH₄⁺ gradients at yolk sac surface of larvae acclimated to NW or L-Na water. Surface ionic gradients of 7-dpf larvae acclimated to NW or L-Na water (larvae were acclimated to L-Na for 5 days) were measured with the SIET to determine the ionic excretion or uptake by larval skin. Since the yolk sac showed notable gradients of H⁺, NH₄⁺, and Na⁺, we chose this location to compare ionic gradients (Fig. 1A; the arrow indicates the location of the probe). Results showed that H⁺ and NH₄⁺ were secreted in NW larvae; however, Na⁺ gradient was almost absent (Fig. 1, B–D). L-Na acclimation remarkably increased Na⁺ uptake (negative gradient of Na⁺) and also increased NH₄⁺ excretion (positive gradient of NH₄⁺). Notably, L-Na acclimation decreased the H⁺ gradient, indicating that H⁺ secretion was suppressed or that base secretion was enhanced. In this experiment, no significant difference was found in mortality or hatching rates between the two groups.

Effects of inhibitors on ionic gradients. To test whether the NHE and V-type H⁺-ATPase are involved in H⁺ secretion, EIPA (a NHE inhibitor) and bafilomycin A1 (a H⁺-ATPase inhibitor) were applied to larvae by preimmersion for 10 min. Bafilomycin A1 (100 μM) did not significantly affect ∆[H⁺] at the larval skin (Fig. 2A). However, the ∆[H⁺] was inhibited by approximately 22% and 57% by 100 μM and 1,000 μM EIPA, respectively (Fig. 2A). The dosage of 1,000 μM seemed too high for the larvae, since ~30% of the larvae were feeble or died after the treatment. Thus, we chose the dosage of 100 μM EIPA for the following experiments. L-Na-acclimated larvae which showed higher Na⁺ and NH₄⁺ gradients were used to test the effect of EIPA on Na⁺ and NH₄⁺ gradients and also in-
secretion by 35% (Fig. 2B) and Na\(^+\) uptake by 54% (Fig. 2C). However, phenamil of 10 μM did not significantly block Na\(^+\) uptake, suggesting that epithelial Na\(^+\) channel is not involved in Na\(^+\) uptake of medaka larvae (Fig. 2C).

**Ionic gradients at MRCs and KCs.** Under the microscope, MRCs in the skin could be identified by their oval shape and apical opening (membrane) (Fig. 3A). Using the SIET, ionic gradients at specific cells were determined by probing voltage differences at specific cells. Serial probing over the apical opening of MRCs and adjacent KCs is shown in Fig. 3A (the dashed line indicates a 40-μm probing route). The peak of voltage differences appeared right at the apical opening of MRCs, and the differences gradually decreased as the probe was moved away, revealing that MRCs are “hot spots” of Na\(^+\) uptake and NH\(_3\)/NH\(_4\)\(^+\) excretion (Fig. 3, C and D). Interestingly, contrary H\(^+\) signals (H\(^+\) sink and H\(^+\) source) were found when probing over the MRCs, indicating that one group of MRCs secretes acid but another group secretes base (Fig. 3B). These two groups of MRCs were referred to as MRC\(^-\) (acid secreting) and MRC\(^+\) (base secreting).

**Apparent H\(^+\), Na\(^+\), and NH\(_4\)\(^+\) fluxes at MRCs and KCs in larvae acclimated to L-Na or H-Amm.** L-Na acclimation was shown to decrease the H\(^+\) gradient and increase the NH\(_4\)\(^+\) gradient in the above experiment (Fig. 1). We further examined whether the apparent H\(^+\) flux at MRCs and KCs was also affected by L-Na acclimation. Results showed that the apparent H\(^+\) flux at MRC\(^+\) was about 16-fold higher than that at adjacent KCs in the normal group. Low-Na acclimation significantly suppressed and reversed apparent H\(^+\) flux at KCs but did not significantly affect apparent H\(^+\) flux at either MRC\(^+\) or MRC\(^-\) (Fig. 4, A and B). However, the ratio of MRC\(^+\) and MRC\(^-\) remarkably changed. L-Na acclimation induced more MRC\(^-\) (Fig. 4D) and consequently decreased the H\(^+\) gradient measured at the skin as shown in Fig. 1B. The MRC\(^+\)/MRC\(^-\) ratio was calculated by pooling all recorded cells from different individuals. Fewer than 10 MRCs were usually recorded in the same individual.

To test whether the base secretion by MRCs is associated with NH\(_3\)/NH\(_4\)\(^+\) excretion, we examined larvae acclimated to H-Amm (5 mM NH\(_4\)\(^+\)) by which NH\(_3\)/NH\(_4\)\(^+\) excretion is supposed to be stimulated. H-Amm significantly decreased the number of MRC\(^+\) and increased the number of MRC\(^-\) (Fig. 4D). Moreover, H\(^+\) secretion was significantly suppressed at MRC\(^+\), and the apparent H\(^+\) flux became more negative at KCs (Fig. 4, A and C).

Since we were unable to discriminate between MRC\(^+\) and MRC\(^-\) with a microscope, we only compared ungrouped MRCs and KCs in Na\(^+\) and apparent NH\(_4\)\(^+\) fluxes. Apparent NH\(_4\)\(^+\) fluxes at both KCs and MRCs were positive, which indicates excretion of NH\(_3\)/NH\(_4\)\(^+\) by these cells (Fig. 5A). By pooling all data together, 91% and 85% of the recorded MRCs (only MRCs with a clear opening) exhibited significant Na\(^+\) uptake and NH\(_3\)/NH\(_4\)\(^+\) excretion, respectively. The apparent NH\(_4\)\(^+\) flux at MRCs was approximately 11- to 13-fold higher than that at adjacent KCs in the three groups. Both L-Na and H-Amm acclimation induced NH\(_3\)/NH\(_4\)\(^+\) excretion at MRCs (Fig. 5A). Na\(^+\) uptake (a negative Na\(^+\) flux) was detected at MRCs in normal larvae; in contrast, outflow of Na\(^+\) (a positive flux) was detected at KCs (Fig. 5B). Both L-Na and H-Amm acclimation significantly increased the Na\(^+\) influx at MRCs by 60%. The outflow of Na\(^+\) at KCs significantly decreased to nearly zero in L-Na and H-Amm larvae (Fig. 5B).

Negligible background values (noise signals) recorded by probing in medium without larvae were 0.004 ± 0.005, −0.03 ± 0.62, and 2.8 ± 5.1 pmol · cm\(^{-2}\) · s\(^{-1}\) (n = 68, 24, and 37) using H\(^+\), NH\(_3\), and Na\(^+\) electrodes, respectively.

**Acute effects of external H-Amm on ionic gradients.** The above experiments showed a correlation between Na\(^+\) uptake and NH\(_3\)/NH\(_4\)\(^+\) excretion. If NH\(_3\)/NH\(_4\)\(^+\) excretion can drive Na\(^+\) uptake, a decrease in Na\(^+\) uptake by blocking NH\(_3\)/NH\(_4\)\(^+\) excretion would be expected. In this experiment, we transferred larvae to the recording medium with H-Amm and examined the effect of acute H-Amm exposure on the ionic gradients. Three groups of larvae which had been preacclimated to NW, L-Na, and H-Amm were compared. When measuring larvae in normal water, NH\(_3\)/NH\(_4\)\(^+\) excretion was higher in both the L-Na and H-Amm groups than in the normal group (Fig. 6, A–C, black bars). Na\(^+\) uptake was also higher in the L-Na and H-Amm groups (Fig. 6, D–F, black bars). In
contrast, the $H^+$ gradient was lower in the L-Na group and even negative in the H-Amm group (Fig. 6, G–I, black bars). However, when measuring larvae in H-Amm water, dramatic changes occurred. External H-Amm reversed the $NH_4^+$ gradient from positive to negative values in the NW group, and decreased the $NH_3^+$ gradient to nearly zero in the L-Na and H-Amm groups (Fig. 6, A–C, gray bars). Meanwhile, external H-Amm increased Na$^+$ outflow in the NW group and significantly blocked Na$^+$ uptake in the other two groups (Fig. 6, D–F, gray bars). In contrast to changes in the $NH_3^+$ gradient, external H-Amm increased $H^+$ gradients in the three groups (Fig. 6, G–I, gray bars).

**Acute effects of external H-Amm on ionic fluxes of specific cells.** To answer whether changes in the above experiment also take place in MRCs, we measured ionic fluxes at specific MRCs sequentially before and after H-Amm was applied. Figure 7A shows the apparent $NH_4^+$ flux at three MRCs (solid symbols) and three KCs (open symbols) from different individuals. H-Amm suppressed $NH_3^+/NH_4^+$ excretion at MRCs right after the change of medium (Fig. 7A). An inward Na$^+$ flux (Na$^+$ uptake) was recorded at MRCs before H-Amm exposure, but it was dramatically abolished or even reversed to an outward flux after H-Amm was applied (Fig. 7B, solid symbols). However, no significant change occurred at KCs (Fig. 7B, open symbols). Interestingly, we found that MRCs turned into MRCs$^+$ after H-Amm was applied by sequentially probing the apparent $H^+$ flux (Fig. 7C). Again, the KCs did not significantly change.

**Acute effects of external pH on ionic gradients and fluxes.** L-Na (pH 7)-acclimated larvae were measured in recording media with different pH values (pH 6, 7, and 8) to examine the effects of acute external pH on ionic gradients. Acidic water (pH 6) significantly enhanced the $NH_4^+$ and Na$^+$ gradients but reversed the $H^+$ gradient at the larval skin (Fig. 8). However, alkaline water (pH 8) did not cause significant changes. Similar effects were also detected when probing ionic fluxes at specific MRCs. The apparent $H^+$ flux at the three MRCs was positive but dramatically dropped to negative values when larvae were transferred from pH 7 to pH 6 water (Fig. 9C). Meanwhile, the Na$^+$ influx and apparent $NH_4^+$ efflux at MRCs were also enhanced by the acid water (Fig. 9, A and B). However, the ionic fluxes at KCs (Fig. 9; open symbols) were relatively low and remained stable before and after the pH change.

**External pH gradient at the apical surface of MRCs.** To reveal the $H^+$ gradient at the microenvironment above the apical membrane of an MRC, serial SIET probing was performed starting from ~2 μm above the apical membrane and gradually stepping away to >100 μm (Fig. 10A; open circles). A parallel probing which began from the surface of a KC was also recorded for comparison (Fig. 10A; solid circles). In pH 6 water, the pH exponentially dropped from 6.08 to 5.94 when probing 30 μm away from the MRC and remained relatively constant thereafter. The dropping of the pH revealed a microenvironment generated by alkaline secretion ($NH_3$ secretion is suggested) of the MRC. Beyond the microenvironment, an acidic layer of ~100 μm thickness was revealed. The acidic layer might be generated by acid secretion from KCs, since the pH measured at the KC was relatively constant and lower than the background pH. In addition, it was noted that the acid layer was absent or unremarkable in the yolk sac area when measuring the larvae in pH 6 water (data not shown). Therefore, the present data were recorded in the trunk area adjacent to the yolk sac. As shown above (Fig. 8), $NH_3^+/NH_4^+$ excretion at the yolk sac was enhanced, and the $H^+$ gradient was simultaneously suppressed when probing the larvae in pH 6 water, suggesting that the enhanced $NH_3$ excretion consumed $H^+$ and diminished the acid layer.
By using a noninvasive electrophysiological technique, the SIET, this study provides direct and convincing evidence for Na⁺ uptake and NH₃/NH₄⁺ excretion by MRCs in intact medaka larvae. As mentioned in the Introduction, molecular evidence including localization of H⁺-ATPase and the NHE in MRCs suggested that Na⁺ uptake in FW fishes is mediated by MRCs. In addition, Na⁺ uptake was found in a subtype of MRCs (PNA⁻) using isolated branchial cells of rainbow trout (31). In zebrafish embryos, Na⁺ accumulation was found in HRCs (a subtype of MRCs) using sodium green as an indicator (6). Those recent studies suggested that MRCs are major sites for Na⁺ uptake in FW fishes. In this study, over 90% of the MRCs recorded (only MRCs with a clear apical opening were recorded) exhibited significant Na⁺ uptake consolidating previous findings. In contrast to MRCs, Na⁺ fluxes recorded at KCs were outward fluxes, suggesting that Na⁺ was lost passively from KCs (Figs. 3D and 5B). Active Na⁺ uptake by MRCs apparently balanced the loss and thus made the Na⁺ gradient at the skin surface very small (Fig. 1D). L-Na acclimation significantly induced compensatory Na⁺ uptake at MRCs (Fig. 5B) which usually exhibited larger apical membranes (data not shown).

Interestingly, 85% MRCs recorded in medaka larvae also excreted a significant level of NH₃/NH₄⁺, suggesting that both Na⁺ uptake and NH₃/NH₄⁺ excretion are conducted by the same MRCs. Although it was tricky to record Na⁺ and NH₃ fluxes at the same cells, we did it successfully and recorded both Na⁺ and NH₃ fluxes at a few MRCs (data not shown). In zebrafish larvae, we found that NH₃/NH₄⁺ was highly excreted by HRCs (a subtype of MRCs) which also secrete a large amount of H⁺ (37). Actually, we did not detect base-secreting genes' transcripts were higher in L-Na fish than in NW fish (Fig. 12), suggesting that they are involved in Na⁺ uptake by medaka.

**DISCUSSION**

Whole mount in situ hybridization of slc9a3, Rhcg1, and Rhbg. The major signals of the three genes, slc9a3, Rhcg1, and Rhbg, were observed in developing gills (Fig. 11A, arrowhead; Fig. 11, C and E, insets, arrows) and yolk sac skin (Fig. 11, A, C, and E, arrows) of newly hatched larvae. The salt-and-pepper pattern of the signals in the skin suggests that those genes are located in MRCs. Using Na⁺-K⁺-ATPase (NKA) immunostaining as a marker of MRCs, the three genes were further demonstrated to be mainly expressed in MRCs (the in situ signals were mostly colocalized with NKA signals, Fig. 11, B, D, and E). The signals of the three genes were higher in L-Na larvae than in NW larvae; therefore in situ hybridization was done on L-Na larvae.

Real-time qPCR of slc9a3, Rhcg1, and Rhbg. Real-time qPCR was used to examine the transcripts of the three genes, slc9a3, Rhcg1, and Rhbg, in the gills of adult medaka acclimated to NW or L-Na water for 5 days. Results showed that the three genes' transcripts were higher in L-Na fish than in NW fish (Fig. 12), suggesting that they are involved in Na⁺ uptake by medaka.
MRCs in zebrafish. In medaka, although most MRCs excreted NH$_3$/NH$_4^+$, a large portion of MRCs (MRC$^-$) was found to secrete base instead of acid. These base-secreting MRCs seemed to be associated with Na$^+$ uptake and NH$_3$/NH$_4^+$ excretion, since they could be induced by either L-Na or H-Amm acclimation. Precisely, the terms “acid secreting” and “base secreting” does not mean that a cell only secretes acid or base, but more acid or base. In the case of medaka, we suggest that MRC$^-$ secretes more base than acid and thus it turns out to be a base-secreting cell.

So, what is the base secreted by MRCs? We suggest that nonionic NH$_3$ is the dominant base which diffuses out of MRCs and titrates H$^+$ outside of MRCs. This acid-trapping mechanism for NH$_3$/NH$_4^+$ excretion was previously demonstrated in zebrafish larvae (37). Our evidence presented in this study also supports this mechanism. NH$_4^+$ loading (H-Amm acclimation) increased the number of MRCs$^-$, enhanced the NH$_4^+$ gradient, and also alkalinized the skin surface (Fig. 6). In contrast, acutely transferring the larvae to high-NH$_4^+$ water suppressed NH$_4^+$ excretion and raised the H$^+$ gradient at the skin and also at individual MRCs (Figs. 6 and 7). The reverse correlation between NH$_4^+$ and H$^+$ accumulation at the skin surface suggests that nonionic NH$_3$ is excreted, and the more NH$_3$ that is excreted, the more H$^+$ that is titrated. In MRCs$^-$, the amount of H$^+$ secreted is suggested to be less than that of nonionic NH$_3$ secreted which caused an overall base-secretion. In contrast, MRCs$^+$ may simply represent lower NH$_3$-excreting and/or higher H$^+$-secreting cells. Interestingly, in situ hybridization data (Fig. 11) showed that the mRNA of NHE3 (slc9a3) and Rh glycoproteins (Rhcg1 and Rhbg) was expressed in a major group of MRCs which did not have detectable H$^+$-ATPase (atp6v1a, data not shown). In addition, the fact that bafilomycin A1 did not block H$^+$ secretion from larval skin also suggests that H$^+$-ATPase is not a major player of H$^+$ secretion in medaka. In zebrafish, HRCs have both
H\textsuperscript{+}-ATPase and NHE in the apical membrane (49). The differential expression of H\textsuperscript{+}-ATPase may explain why these two cases differ. Moreover, we surprisingly found that the NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion by individual MRCs was much higher in medaka than in zebrafish (80 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} in the present study vs. 6 pmol · cm\textsuperscript{-2} · s\textsuperscript{-1} in Ref. 37), suggesting a much higher level of base (NH\textsubscript{3}) secreted by MRCs of medaka than that of zebrafish.

Another major finding of this study is the tight linkage between Na\textsuperscript{+} uptake and NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion. EIPA at 100 \textmu M inhibited both Na\textsuperscript{+} uptake and NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion, although the levels were not the same. L-Na acclimation induced compensatory Na\textsuperscript{+} uptake and also induced NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion. Once again, H-Amm acclimation induced compensatory NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion and also increased Na\textsuperscript{+} uptake. Acutely raising the external NH\textsubscript{4}\textsuperscript{+} significantly blocked NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion and Na\textsuperscript{+} uptake. In contrast, acutely raising the acidity of the water (pH 7 to pH 6) enhanced Na\textsuperscript{+} uptake and NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion. These data revealed a tight correlation between Na\textsuperscript{+} uptake and NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion not only by the SIET probing at the yolk sac skin but also at individual MRCs. This finding seems to support Krogh’s hypothesis of Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange in fish gills (43). However, we suggest that Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange is not simply mediated by the NHE. As mentioned in the Introduction, early studies suggested that NH\textsubscript{4}\textsuperscript{+} may take the place of H\textsuperscript{+} in the NHE and cause Na\textsuperscript{+}/NH\textsubscript{4}\textsuperscript{+} exchange; however, this suggestion has been argued in several studies (43). If ionic NH\textsubscript{4}\textsuperscript{+} is transported by the NHE or other proteins such as Rh glycoproteins, alkalization of the surfaces of larval skin or MRCs should not occur. Our data apparently do not support this possibility. In addition, accumulating reports have suggested that Rh glycoproteins conduct nonionic NH\textsubscript{3} instead of NH\textsubscript{4}\textsuperscript{+} (17, 19, 32, 50). The identification of Rhbg and Rhcg1 mRNA in MRCs of medaka also supports that NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} excretion is mediated by acid-trapping mechanism.

In other fish species, Rhcg1 is located in the apical membrane of MRCs in zebrafish (25) and pufferfish (26), whereas Rhbg is located in the basolateral membrane of pavement cells in pufferfish (26) and an unidentified cell in the skin of zebrafish larvae (2). In mammalian kidneys, Rhcg and Rhbg are generally located in the apical and basolateral membranes,
respectively. This study clearly showed that the two Rh isoforms are colocalized in MRCs of medaka larvae. It is quite possible that Rhcg1 and Rhbg are also the apical and basolateral isoforms, respectively.

In zebrafish, Rhcg1 mRNA expression was found to be induced by diluted fresh water, suggesting that Rhcg1 is also involved in ion uptake of freshwater teleost (25). In rainbow trout, Rhcg2 mRNA was also upregulated in cultured branchial cells pretreated with L-Na medium (40). In this study, we found that L-Na acclimation simultaneously induced mRNA expressions of slc9a3, Rhcg1, and Rhbg in the gills of medaka. Interestingly, the increased levels of slc9a3 and Rhcg1 were similar, suggesting a synergistic function of the two genes in Na\(^+\) uptake. Taking the SIET and molecular data together, we proposed a novel model to explain this Na\(^+\)/NH\(_4\)\(^+\) exchange in the apical membrane of MRCs (Fig. 13). In this model, the two major players, NHE3 and Rhcg1, are functionally and probably physically associated. The Rh glycoprotein deprotonates NH\(_4\)\(^+\) inside of MRCs and then facilitates nonionic NH\(_3\) diffusion down a chemical gradient generated by external acid trapping (17, 19, 32, 50). The H\(^+\) dissociated from intracellular NH\(_3\) may accumulate beneath the apical membrane and provides an effective H\(^+\) gradient to drive Na\(^+\) uptake via the NHE. The NHE and Rh glycoprotein might form a complex and thus function as a Na\(^+\)/NH\(_4\)\(^+\) exchanger. A physical association of the NHE and Rh proteins has not been reported, however, RhAG was found to associate with anion exchanger and carbonic anhydrase in mammalian red blood cells (3).

As mentioned in the Introduction, the driving force of the NHE in gills of FW has been debated for a long time (29). The present model provides a reasonable explanation for the driving force of the NHE and also for the (Na\(^+\)/NH\(_4\)\(^+\)) exchange pathway. We also suggest that this model might not only work in medaka but also in other FW fishes. In the special case of Osorezan dace, which lives in an extremely acidic (pH 3.5) lake, NHE3 was located in the apical membrane of MRCs, and its mRNA expression in gills was higher in individuals reared in pH 3.5 than in individuals reared in pH 7 (10). The NHE3 seems to be unfavorable to work in extremely acid water, if considering the thermodynamics of the NHE. The authors suggested that Na\(^+\)/NH\(_4\)\(^+\) exchange might be a possible mechanism. It is quite possible that the dace also uses a mechanism similar to our model for Na\(^+\) uptake. In a very recent report, an interesting model was proposed to explain the Na\(^+\)/NH\(_4\)\(^+\) exchange mechanism in rainbow trout gills (40). Through a large protein complex which contains H\(^+\)-ATPase, Na\(^+\) channel, NHE and Rh glycoproteins, Na\(^+\) uptake and NH\(_4\)\(^+\) excretion are linked. However, in the case of medaka, our data suggest that H\(^+\)-ATPase and putative epithelial Na\(^+\) channel (ENaC) are not involved in Na\(^+\) uptake and NH\(_4\)\(^+\) excretion. In this concern, medaka would be a simpler model for investigating the Na\(^+\)/NH\(_4\)\(^+\) exchange in fish.

External acidification is generally thought to suppress Na\(^+\) uptake based on earlier studies on ion transport in frog skin. In 1949, Ussing proposed this notion for isolated frog skin, and Schoffeniels (36) and Romeu et al. (34) also found that Na\(^+\) uptake was greatly suppressed because the skin external pH fell below 4 on in vitro or in vivo frog skin. Thereafter, several studies on goldfish (23) and rainbow trout (1, 47) also supported this finding. Current models for Na\(^+\) uptake in apical membranes of MRCs including NHE- or H-ATPase-dependent pathway are basically derived from this early finding. However, several studies showed conflicting results. For example, external alkalization did not cause a higher Na\(^+\) influx in rainbow trout (47); Na\(^+\) influx was stimulated by external acidification and negatively correlated to water pH (35). Recently, Na\(^+\) accumulation monitored by sodium green was stimulated by acidic water in HRCs of zebrafish embryos (9). In the present study, increases in Na\(^+\) uptake of the yolk sac skin (Fig. 8B) and individual MRCs (Fig. 9B) were observed in medaka larvae transferred from pH 7 to pH 6. This finding seems to be counter to the NHE pathway for Na\(^+\) uptake. However, in a comparison of apparent H\(^+\) and NH\(_4\)\(^+\) fluxes before and after the acid water transfer (Figs. 8 and 9), once again a reverse correlation between NH\(_4\)\(^+\) and H\(^+\) gradients suggests an

![Fig. 10. Spatial changes in pH in the microenvironment generated by a MRC on the skin of medaka larvae. A: an egress recording of pH was performed by a SIET probing from the apical membrane (~2 μm from the surface of the membrane) of an MRC (open symbols) or an adjacent KC (solid symbols) to the background (5,000 μm away from the skin). The larva was measured in pH 6 medium, and the recorded background pH was ~6.02 (dotted line). B: an illustration of the probing routes (dotted arrow) starting from an MRC or a KC. The data show that the MRC and adjacent KC are a proton sink and source, respectively.](http://ajpcell.physiology.org/)

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acid-trapping mechanism. If nonionic NH₃ excretion is associated with Na⁺/H⁺ exchange, acid water’s stimulation of Na⁺ uptake can reasonably be explained. Although the external low pH is theoretically unfavorable for Na⁺ uptake via the NHE, in contrast, it is favorable for nonionic NH₃ diffusion across apical membranes (37, 42). We suggest that external acid simultaneously promotes NH₃ excretion through Rh glycoproteins and intracellular H⁺ accumulation which in turn drives Na⁺ uptake via the NHE.

In this study, a microenvironment above the opening of MRCs was shown by probing H⁺ at serial points above the MRCs (Fig. 10). An acidic layer about 100 μm thick was detected surrounding the larvae, and it gradually decayed with distance from the skin surface. The acidic layer is probably formed by H⁺ secretion by MRC or CO₂ diffusion from KCs. However, at the surface of MRCs, the pH dramatically increased when approaching the apical membrane of MRCs, showing this MRC to be a base-secreting MRC (MRC⁻). The highest pH (6.08) was detected at a point ~2 μm away from the surface of the apical membrane of the MRC. A higher pH probably 6.1 or more can be expected if the probe could get closer to the apical membrane. Although, we are still unable to determine whether the H⁺ gradient can drive Na⁺ uptake before the intracellular or precisely the subapical pH is measured, this finding provides evidence for the existence of a microenvironment and the possibility of driving the NHE in FW. On the other side of the apical membrane (intracellular), a microenvironment with a pH lower than the cytosolic pH may exist. Future work using fluorescent pH indicators may be able to provide convincing evidence for the subapical microenvironment.

Since a specific inhibitor of the Rh glycoprotein is not available, genetic knockdown or knockout seemed to be
believed to pass through the branchial epithelium mainly in its gaseous form. It would be interesting to study whether or not the branchial Rh glycoprotein also facilitates CO₂ diffusion. Recently, Nawata and Wood (28) examined the effects of a 12-h exposure to external hypercapnia on Rh mRNA expression in the gills and skin of rainbow trout, and they found that CO₂ did not directly elicit changes in Rh mRNA transcription levels in the gills and skin. A dual function for gill and skin Rh proteins in CO₂ and ammonia transport was not evident from that study. The present study suggests that Rh proteins conduct NH₃ gas and which in turn titrates H⁺ at the surface of MRCs. If Rh proteins conduct much more CO₂ than NH₃, then NH₄⁺ excretion and acid trapping would unlikely to be seen at MRCs. However, we cannot rule out the possibility that Rh also has minor permeability to CO₂.

Zebrafish has been shown to be a powerful model for investigating ionic transport in fish (13). Medaka (O. latipes) is another genetic fish model with a genomic database that provides sufficient bioinformation. However, unlike zebrafish, medaka is a euryhaline teleost which resides in FW environments, such as ponds, marshes, and paddy fields (15). By comparing medaka and zebrafish, we might be able to reveal functional differences between euryhaline and stenohaline teleosts. Moreover, medaka might be a good model for investigating ionic regulation, acid-base balance, and ammonia excretion in hypertonic environments.

**Fig. 12.** Relative mRNA expression levels of slc9a3 (A), Rhcg1 (B), and Rhbg (C) in gills of adult medaka acclimated to NW or L-Na water for 5 days. Data are presented as means ± SE (number of fish is shown in parentheses). *Significant difference between NW and L-Na groups (Student’s t-test, \( P < 0.05 \)).

**Fig. 13.** Proposed model of “NH₄⁺/H⁺-dependent Na⁺ uptake” in MRCs of medaka larvae. The two key players, the Na⁺/H⁺ exchanger (NHE) and Rhesus glycoprotein (Rh), are suggested to be involved in the apical transport of Na⁺ and NH₄⁺. The Rh deprotonates NH₄⁺ inside the MRC and facilitates NH₃ gas diffusion down the gradient generated by the acid trapping of NH₃ outside the membrane. Rh-mediated NH₄⁺ excretion may generate a H⁺ gradient across the apical membrane to drive Na⁺ uptake through the NHE.
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DISCLOSURES
No conflicts of interest are declared by the author(s).

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