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Chloride transport in mitochondrion-rich cells of euryhaline tilapia
(Oreochromis mossambicus) larvae

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1Institute of Cellular and Organismic Biology, Academia Sinica, Taipei; 2Department of Life Science, National Taiwan Normal University, Taipei; and 3Department of Marine Biotechnology and Resources, Division of Marine Biotechnology, Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung, Taiwan

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Horng JL, Hwang PP, Shih TH, Wen ZH, Lin CS, Lin LY. Chloride transport in mitochondrion-rich cells of euryhaline tilapia (Oreochromis mossambicus) larvae. Am J Physiol Cell Physiol 297: C845–C854, 2009.—A noninvasive scanning ion-selective electrode technique (SIET) was applied to measure Cl− transport at individual mitochondrion-rich cells (MRCs) in the skin of euryhaline tilapia (Oreochromis mossambicus) larvae. In seawater (SW)-acclimated larvae, outward Cl− gradients (20–80 mM higher than the background) were measured at the surface, indicating a secretion of Cl− from the skin. By serial probing over the surface of MRCs and adjacent keratinocytes (KCs), a significant outward flux of Cl− was detected at the apical opening (membrane) of MRCs. Treatment with 100 μM ouabain or bumetanide inhibited the Cl− secretion by ~75%. In freshwater (FW)-acclimated larvae, a lower level of outward Cl− gradients (0.2−1 mM) was measured at the skin surface. Low-Cl− water (~0.005 mM) acclimation increased the apical Na+−Cl− cotransporter (NCC) immunoreactivity of MRCs in the larval skin. An inward flux of Cl− was detected when probing the exterior surface of a group of MRCs (convex-MRCs) that express the NCC. An NCC inhibitor (100 μM metolazone) reduced the flux by ~90%. This study provides direct and convincing evidence for Cl− transport by MRCs of SW- and FW-acclimated euryhaline tilapia and the involvement of an apical NCC in Cl− uptake of MRCs of FW-acclimated fish. 

Na+−Cl− cotransporter, osmoregulation; gills; ionocytes; ionoregulation

Euryhaline fish, such as tilapia (Oreochromis mossambicus), are able to maintain their internal osmolarity and ionic concentrations in waters with a wide range of salinities. Mitochondrion-rich cells (MRCs) in the gill epithelium play a critical role in regulating internal ionic concentrations, either by secreting excess internal Na+ (through a paracellular pathway) and Cl− in seawater or by taking up Na+ and Cl− from fresh water (8). The direct evidence for Cl− secretion by chloride cells (a former name of MRCs) was first reported by using vibrating probe in isolated opercular epithelium of seawater (SW)-acclimated teleost Sarotherodon mossambicus (9). In SW-acclimated tilapia larvae (O. mossambicus), MRCs in the yolk-sac skin were also demonstrated to be the sites for Cl− secretion by using a chloride test and X-ray microanalysis (20). The mechanism of NaCl secretion by MRCs in seawater (SW)-acclimated fish has been well elucidated, consisting primarily of the cooperative action of the basolateral active transporters Na+−K+−ATPase and Na+−K+−2Cl− cotransporter (NKCC) and an apical Cl− channel (7, 18). However, the ion-uptake mechanism by MRCs in freshwater (FW)-acclimated fish has been controversial over the past decade (7, 17, 18).

So far, at least two dominant models were proposed for the apical transport of Na+ in the gill epithelium: 1) an apical H+−ATPase electrically linked with Na+ absorption via the epithelial Na+−channel (ENaC) and 2) the electroneutral exchange of Na+ and H+ via an apical Na+/H+ exchanger (NHE). With the Cl− uptake mechanism, anion exchangers (Cl−/HCO3− exchangers) were suggested to drive apical Cl− uptake. However, these putative pathways for Na+−Cl− uptake (particular for Cl− uptake) have been debated (8, 17, 18).

Inhibitors of anion exchangers reduce Cl− uptake and produce metabolic alkalosis in FW fishes (1, 13, 32, 33). In situ hybridization with an oligonucleotide probe, complementary to rat anion exchanger (AE1) cDNA, indicated mRNA signals in both pavement cells (PVCs) and MRCs in trout gills (40). A polycylonal antibody against trout blood cell AE1 was used to localize the transporter in apical membranes of gill cells of tilapia and coho salmon (Oncorhynchus kisutch) (48, 49); however, the specificity of this antibody was questioned (43). Recently, the pufferfish (Tetraodon nigroviridis) AE1 protein was detected in the basolateral instead of the apical membrane of MRCs of FW-acclimated fish by an antibody raised against tilapia AE1, and its function was suggested to be linked with acid-base regulation in FW (42). In zebrafish, mRNA of an AE1 paralogue was localized in a group of ionocytes. However, the zAE1 mRNA expression was not affected by low-CI− water, suggesting that zAE1 might not be involved in Cl− uptake (17). Taken together, there is still no convincing evidence to demonstrate an AE isoform that is specifically expressed in the apical domain of MRCs or PVCs and which functions in Cl− uptake in FW fishes. Recently, other candidates for an apical Cl− transporter were reported. Hwang and colleagues (18) first reported that a gill-specific isofrom of Na+−Cl− cotransporter (zslc12a10.2) had been cloned and localized in a novel type of ionocyte in zebrafish. Knockdown of the zslc12a10.2 by the morpholino oligonucleotides and treatment of metolazone (a NCC-specific inhibitor) decreased Cl− uptake in zebrafish embryos, suggesting that the NCC is involved in Cl− uptake of zebrafish (47). In tilapia (O. mossambicus), Hiroi and colleagues (15) also cloned an orthologue of zslc12a10.2 and showed that the NCC was located in the apical membrane of a group of MRCs. These studies suggested a novel pathway for Cl− uptake in a specific type of MRCs. However, these studies did not perform functional assays on the NCC-expressed MRCs.

Although the mechanism of Cl− uptake in FW-acclimated teleosts is not well understood, MRCs instead of PVCs were
suggested to be the major pathway of Cl$^-$ uptake. Morphological evidence indicated a correlation between the size/density of MRCs and the Cl$^-$ uptake capability. Increases in the surface area and density of MRCs were found in salmon (12, 24) and killifish (21) acclimated to low-Cl$^-$ water. In gill and skin MRCs of tilapia, the apical surface area/density of wavy-convex MRCs was also found to increase in response to low-Cl$^-$ water, and the increase was correlated with the increase in whole fish $^{36}$Cl$^-$ influx (2, 26, 27). However, electrophysiological evidence demonstrating and analyzing Cl$^-$ uptake in individual MRCs has not yet been reported.

Using a noninvasive electrophysiological scanning ion-electrode technique (SIET) and molecular approaches, Lin et al. (25) identified H$^+$/ATPase-rich cells (HRCs; a subtype of MRCs) as acid-secreting cells in the skin of zebrafish embryos. Recently, the SIET was further applied to detect NH$_4^+$ flux in zebrafish larvae and revealed a mechanism of ammonia/ammonium excretion by HRCs (36). In this study, we attempted to use the SIET to detect Cl$^-$ fluxes at the skin surface of tilapia larvae and provide direct evidence for Cl$^-$ secretion and uptake by MRCs of SW- and FW-acclimated fish, respectively.

**MATERIALS AND METHODS**

**Animals and various media.** Mature adult Mozambique tilapia (*Oreochromis mossambicus*) were kept in circulating FW at 26−28°C under a photoperiod of 12−14 h of light. Fertilized eggs were retrieved from the mouths of females that had initiated mouth breeding. About 1 day before hatching, the eggs were acclimated to various media including normal water, low-Cl$^-$ water, or SW for specific experiments. The normal water contained (in mM) 0.5 NaCl, 0.2 MgSO$_4$, 0.2 CaSO$_4$, 0.005 KH$_2$PO$_4$, and 0.08 K$_2$HPO$_4$ (pH 7.0). In the low-Cl$^-$ water, the 0.5 mM NaCl was replaced by 0.25 mM Na$_2$SO$_4$. The seawater (30 ppt) was prepared from redistilled water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH). The temperature of the media was kept at 26−28°C. 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. 95013) by the National Taiwan Normal University Animal Care and Utilization Committee.

**Immunohistochemistry and image analysis.** Tilapia larvae were acclimated on ice and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C. After being rinsed with phosphate-buffered saline (PBS), the larvae were postfixed and permeabilized with 90% ethanol at −20°C for 10 min. After being washed with PBS, samples were incubated with 3% bovine serum albumin (BSA) and 5% normal goat serum for 30 min to block nonspecific binding. The larvae were then incubated overnight at 4°C with a polyclonal antibody against the tilapia NCC (diluted 1:1,000 with PBS). The antibody was provided by Dr. Hiroi (15). After being rinsed with PBS for 20 min, the larvae were further incubated in goat anti-rabbit immunoglobulin G (IgG) conjugated with FITC (diluted 1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature (26−28°C).

The period of time over which the image was captured, the photomultiplier tube gain, and the scanning rules of the confocal microscope were optimized before each experiment and maintained throughout the experiments to standardize the intensity of fluorescence among experiments. The confocal images were taken with 60/1.45 oil lenses and analyzed with ImageJ software (free software by NIH). The pixel intensities of the NCC-associated labeling in the apical pole of individual MRCs were summed as the NCC intensity of one MRC. Twenty-five and twenty-one MRCs from 4 L-Cl and 4 normal larvae were randomly selected for analysis. The mean of the 25 MRCs from L-Cl larvae was referred to 100% relative NCC abundance.

**SIET.** To measure Cl$^-$ activities and fluxes at the surface of tilapia larvae, Cl$^-$-selective microelectrodes were constructed. 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 3−5 μm. These glass micropipettes were baked at 120°C overnight and then vapor-silanized with dimethyl chlorosilane (Sigma-Aldrich, St. Louis, MO) for 30 min. Before use, 1 cm of the column of the micropipettes was backfilled with 100 mM NaCl. Then the micropipettes were frontloaded with a 20−30-μm column of liquid ion exchanger cocktail (chloride ionophore I-cocktail A; Sigma-Aldrich). The details of the system were described in a previous report (25).

**Measurement of surface Cl$^-$ gradients.** The SIET was performed at room temperature (26−28°C) in a small plastic recording chamber filled with 3 ml of “recording medium” that contained 0.5 mM NaCl (for FW-tilapia) or 500 mM NaCl (for SW-tilapia), 300 mM MOPS buffer (Sigma-Aldrich), and 0.2 mg/l ethyl 3-aminobenzoate (Tricaine, Sigma-Aldrich). The pH of the recording media was adjusted to 7.0 (FW) and 8.0 (SW) by adding NaOH or HNO$_3$ solutions. Before the measurement, an anesthetized embryo was positioned in the center of the chamber with its lateral side contacting the base of the chamber. To record the Cl$^-$ activities (concentrations) at the surface of a larva, the microelectrode was moved to the target position about 10−20 μm away from the skin. After a target point was recorded, the microelectrode was then moved away (−10 mm) to record the background. In this study, ΔCl$^-$ was used to represent the measured Cl$^-$ gradients between the point of interest (skin surface) and the background.

**Measurement of Cl$^-$ flux in MRCs and keratinocytes.** An anesthetized larva was laid laterally in the chamber for the SIET measurement. Under the microscope, the apical membrane of MRCs could be identified on its skin. To record the local Cl$^-$ flux at specific cells, the microelectrode was moved to a position about 2 μm above the surface of the cells. At every position, the voltage difference in microvolts was measured by probing orthogonally to the surface at 10-μm intervals. The calculation of Cl$^-$ flux was described in a previous report (73). Briefly, voltage gradients obtained from the ASET software were converted into a concentration gradient using the following equation:

$$\Delta C = C_b \times \frac{\Delta V}{S}$$

where ΔC is the concentration gradient between the two points measured (in μmol·cm$^{-1}$·cm$^{-3}$); $C_b$ is the background ion concentration, calculated as the average of the concentration at each point measured (in μmol/l); ΔV is the voltage gradient obtained from ASET (in μV); and $S$ is the Nernst slope of the microelectrode. The concentration gradient was subsequently converted into an ion flux using Fick’s law of diffusion in the following equation:

$$J = D(\Delta C)/\Delta X$$

where $J$ is the net flux of the ion (in pmol·cm$^{-2}$·s$^{-1}$); $D$ is the diffusion coefficient of the ion (2.03 × 10$^{-5}$ cm$^2$·s$^{-1}$ for Cl$^-$); ΔC is the concentration gradient (in pmol/cm$^2$); and ΔX is the distance between the two points measured (in cm).

**Calibration and selectivity of the microelectrode.** The Nernstian property of the Cl$^-$-selective microelectrode was measured by placing the microelectrode in a series of standard solutions (0.1, 1, 10, 100, and 1,000 mM NaCl). By plotting the voltage output of the microelectrode against the log Cl$^-$ concentrations, a linear regression yielded the Nernstian slope. The microelectrode’s selectivity to the primary Cl$^-$ ion and the interfering HCO$_3^-$ ion was determined with a simplification of the separate-solution method described by Umezawa et al. (45), by comparing measured voltages of 0.1−100 mM solutions of NaCl and NaHCO$_3$.

**Treatment with inhibitors and measurement of Cl$^-$ fluxes.** The inhibitors were purchased from Sigma-Aldrich. The stock solutions of...
the inhibitors were prepared by dissolving ouabain, bumetanide, 4,4′-disothiocyanatostilbene-2,2′-disulfonate (DIDS), or metolazone in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Larvae were exposed to a final concentration of 100 μM of the inhibitor (dissolved in normal water or SW with 0.1% DMSO) for 30 min. After treatment, the larvae were immediately measured using the SIET. The inhibitors were not added to the recording media to prevent any alteration of the properties of the electrodes. To determine the effect of the inhibitor on Cl− flux at individual MRCs, approximately three to five convex-MRCs were measured with the SIET in an individual. The duration of probing an individual was usually less than 10 min.

RESULTS

Calibration and selectivity of the Cl−-selective microelectrodes. The Nernstian response of the Cl−-selective microelectrodes was tested by placing the electrode in 0.1, 1, 10, 100, and 1,000 mM standard NaCl solutions. The recorded voltages were linear with respect to log [NaCl] (Fig. 1). The slope of the line was −56 ± 1 mV (n = 10), indicating that the Cl−-selective microelectrodes were ideal Nernstian electrodes. To evaluate the interference of HCO3− on the electrode selectivity, the voltage was recorded by placing the microelectrode in 0.1, 1, 10, and 100 mM standard NaHCO3 solutions (Fig. 1). The selectivity coefficients (log K) were determined by using the separate-solution method (45, Table 1). In 100 mM NaHCO3, the measured value of log K was −1.59, which is close to the value provided by Sigma-Aldrich (−1.5; measured by the same method). At lower concentrations (0.1–10 mM) of NaHCO3, the log K value was in the range of −0.82 to −1.17. I/K values represent the selectivity ratio of the microelectrode responding to Cl− over HCO3−. In 100 mM NaCl, the microelectrode was about 40-fold more sensitive to Cl− than to HCO3−; however, it was about 12–15-fold more sensitive in 1–10 mM NaCl. The addition of Tricaine and MOPS buffer (equal to the amount in the recording medium) to the standard NaCl solution did not significantly alter the voltages indicating that the microelectrode was not sensitive to them (data not shown).

Chloride flux at specific cell types on SW-acclimated larval skin. With the use of the Cl−-selective microelectrode, Cl− flux at specific cells was measured by probing Δ[Cl−] at 10-μm intervals perpendicular to the cell membrane. Under the microscope, MRCs in the trunk area (adjacent to yolk sac) could be identified by their oval shape and apical pit (Fig. 3A, arrows). Vital staining with fluorescent MitoTracker confirmed that the observed cells were MRCs (Fig. 3B). Serial probing over the apical surface (membrane) of MRCs and adjacent KCs is shown in Fig. 3C (with the dashed line indicating a 40-μm probing route). The SIET detected an outward flux of Cl− at KCs, and the flux gradually increased when probing toward the apical surface of MRCs (Fig. 3D). Negative values indicate an outward flux of Cl−. Figure 3E shows a comparison of Cl− flux by MRCs and KCs. The flux by MRCs was about fourfold higher than that by adjacent KCs.

Effects of ouabain and bumetanide on Cl− secretion. To demonstrate whether the Cl− efflux was generated by MRCs, ouabain or bumetanide was applied to respectively inhibit the Na+/K+-ATPase or Na+/K+-2Cl− cotransporter. SW-acclimated larvae were immersed in SW with 100 μM ouabain or 100 μM bumetanide for 30 min. After immersion, the larvae were immediately measured with the SIET. Results showed that both ouabain and bumetanide significantly blocked the Cl− secretion by MRCs (Fig. 4). No significant effect was shown in larvae treated with the DMSO control (Fig. 4).

Chloride gradients at the surface of FW-acclimated tilapia larvae. Tilapia larvae at 4 dph acclimated to normal FW were measured with the SIET. Δ[Cl−] values measured at the six spots (the same as in SW larvae, Fig. 5A) from five individuals are shown in Fig. 5B. Positive Δ[Cl−] values indicate an outward flux of Cl− from the larval skin. Δ[Cl−] at the surface of FW-acclimated larvae was much lower (0.2–0.6 mM at the yolk sac and pericardial cavity, Fig. 5B) than that of SW-acclimated ones. Similar to SW-acclimated individuals,

### Table 1. Selectivity coefficients (log K) of Cl−-selective electrodes determined by the separate-solution method

<table>
<thead>
<tr>
<th>Primary and Interfering Ions (Cl− and HCO3−)</th>
<th>Concentration, mM</th>
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<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Log K</td>
<td>−0.82</td>
</tr>
<tr>
<td>I/K</td>
<td>6.6</td>
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*Indicates values reported by Sigma-Aldrich.

![Fig. 1. Voltage-log concentration curves for determining the selectivity coefficient (using the separate-solution method) of primary Cl− ions against interfering HCO3− ions. The test solution contained 0.1–1,000 mM NaCl or 0.1–100 mM NaHCO3.](http://ajpcell.physiology.org/)

![Fig. 5A.](http://ajpcell.physiology.org/)
The Cl\(^-\) gradient (ΔCl\(^-\)) at the skin surface of saltwater (SW)-acclimated tilapia larvae. A: 6 spots measured with the Cl\(^-\)-selective electrode technique (SIET): pericardial cavity (1), anterior yolk-sac (2), lateral yolk-sac (3), posterior yolk-sac (4), cloaca (5), and tail (6). B: egestion occurred when probing at the cloaca; the arrow indicates the tip of the electrode. C: ΔCl\(^-\)] measured at 6 spots of 5 individuals. D: ΔCl\(^-\)] at the yolk sac (the average of 3 spots) increased with larval development from 2–4 days posthatching (dph). Data are presented as means ± SD. Different letters (a, b, c) indicate a significant difference (one-way ANOVA, Tukey’s comparison, P < 0.05).

DISCUSSION

With a noninvasive, electrophysiological technique (SIET), this study demonstrated Cl\(^-\) secretion by MRCs in SW-acclimated tilapia larvae and Cl\(^-\) uptake by a group of MRCs in FW-acclimated ones. For the first time, the Cl\(^-\) transport at individual MRCs was directly measured in intact animals, revealing the functional plasticity (Cl\(^-\) uptake and secretion) of MRCs in a euryhaline teleost tilapia. By analyzing the Cl\(^-\) flux at MRCs with inhibitor treatments and protein localization, an NCC-dependent Cl\(^-\) uptake pathway was demonstrated in MRCs of FW-acclimated fish.

During the past few decades, the Cl\(^-\)-secreting function of MRCs in SW fishes has been extensively studied. Foskett and Scheiffe (9) used a vibrating (voltage) probe to detect ion currents at MRCs in isolated opercular epithelium of a SW teleost, demonstrating the Cl\(^-\) secretion by MRCs. Many pharmacological and molecular approaches further revealed that the Cl\(^-\)-secreting mechanism consists of a basolateral Na-K-ATPase, a NKCC, and an apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^-\) channel (18, 29). In this study, we used the SIET to measure Cl\(^-\) flux at MRCs of SW-acclimated larvae and clearly showed an outward Cl\(^-\) flux at the apical openings (pits) of MRCs, indicating Cl\(^-\) secretion by MRCs. Treatment with Na-K-ATPase or
NKCC inhibitors (ouabain and bumetanide, respectively) significantly blocked the Cl$^{-}/H^+$ flux, which further demonstrated that the flux (secretion) is driven by these two key transporters. The SIET or similar techniques, such as “self-referencing ion-selective (SERIS) probe” and “microelectrode ion flux estimation (MIFE) technique” have been instrumental in detecting the very weak ion fluxes near single cells and tissues that arise as ions cross the plasma membrane through ion channels and transporters (5, 35, 36, 39). These techniques for $H^+/K^+$, $Cl^-/K^+$, $NH_4^+/K^+$, $Na^+/K^+$, and $Ca^{2+}/K^+$ have extensively been used to measure transmembrane ion fluxes near plant and animal cells (6, 11, 22, 30, 38, 41). However, applications of these techniques to intact animals are relatively few. Lin et al. (25), for the first time, applied the SIET to investigate $H^+$ secretion in the skin of zebrafish embryos and larvae and demonstrated a new in vivo system for studying vertebrate ion transport. A series of studies by Hwang’s group further revealed the advantage of this model system (4, 16, 28, 36, 50).

In teleosts, $Na^+/K^+$ and $Cl^{-}/K^+$ are the most important ions for maintaining body fluid osmolarity, and their regulation has been extensively examined. However, as mentioned in the introduction, the mechanisms for $Na^+$ and $Cl^-$ uptake in FW teleosts are still controversial. We also applied the SIET to detect $Na^+$ and $Cl^-$ fluxes at the surface of zebrafish larvae; however, the detected signals were not strong enough to reveal the function of specific types of skin ionocytes. Other species such as tilapia (O. mossambicus) and medaka (Oryzias latipes) were thus chosen for $Na^+/K^+$ and $Cl^{-}/K^+$ probing. Unlike zebrafish, tilapia is a euryhaline teleost and has been extensively used to investigate ion regulation in hypotonic and hypertonic environments (3, 19, 44, 46). By comparing tilapia and zebrafish, we might be able to reveal functional differences between euryhaline and stenohaline teleosts. In this study we focused on $Cl^{-}$ probing, since the $Na^+$ signal was found to be relatively small in MRCs of FW-acclimated fish.

When compared with FW-MRCs, the $Cl^{-}$ flux of SW-MRCs is extremely high, which shows its high transporting capability and is also reflected in its larger size, and higher abundances of mitochondria and transporters (8, 29). The high $Cl^{-}$ gradient at the surface of the yolk sac indicates a large amount of $Cl^{-}$

Fig. 3. Cl$^{-}$ fluxes at mitochondrion-rich cells (MRCs) and keratinocytes (KCs) of SW-acclimated tilapia larvae. A: under a microscope, MRCs with apical openings (indicated by arrows) were identified, and these cells were also labeled by MitoTracker (B). Inset C: “line scan” probing route (40 $\mu$m) over an apical opening of MRCs is shown. D: voltage differences of the line scan over the surface of an MRC is shown. Negative voltage differences indicate outward fluxes of Cl$^-$. E: Cl$^{-}$ fluxes at MRCs and KCs were compared. Data are presented as means ± SD. *Significant difference (Student’s t-test, $P$ < 0.05). Bracketed numbers are sample sizes.
concern in this study, since HCO₃⁻ was suggested to be exchanged with Cl⁻ in FW-MRCs. Moreover, CO₂ diffusion might also generate HCO₃⁻ gradients at the skin surface. However, applying an inhibitor of an anion exchanger (DIDS) did not significantly affect Cl⁻ influx at the convex-MRCs, suggesting that the anion exchanger is not a major player in Cl⁻ uptake by convex-MRCs.

Most importantly, when probing the Cl⁻ flux at the cellular level, an inward Cl⁻ flux was detected at the apical surface of a group of MRCs (convex-MRCs). To enhance the capability of Cl⁻ uptake, the larvae were acclimated to low-Cl⁻ water, and consequently more significant fluxes were detected at the convex-MRCs. In FW-acclimated tilapia larvae, MRCs were classified secretion, which is consistent with the high density of MRCs in this region (37). However, the high Cl⁻ gradient at the cloaca reflects the high Cl⁻ content in the guts. When probing at the cloaca, the Cl⁻ gradient varied a lot particularly when peristalsis of the gut and egestion occurred. When probing at SW-MRCs, we found that Cl⁻-secreting cells usually formed multicellular complexes or were accompanied by accessory cells as mentioned in the literature (34, 37).

In FW-acclimated larvae, it is interesting that the Cl⁻ activity at the surface was slightly higher than that at the background indicating that the net flux of Cl⁻ is outward. One would expect to see a net inward flux of Cl⁻, since larvae were shown to uptake Cl⁻ in FW (27). It is well known that FW fish lose internal ions (mostly Na⁺ and Cl⁻) by passive diffusion across the gill epithelium of adults and probably skin epithelium of larvae, and the loss is balanced by active uptake of NaCl in water and foods. Therefore, the outward flux of Cl⁻ by larval skin might have been due to the passive loss of Cl⁻ being higher than active Cl⁻ uptake. Interestingly, the outflow of Cl⁻ might not be through simply diffusion but by a facilitative maxi Cl⁻ channel identified in cultured pavement cells (31). In addition to Cl⁻, Na⁺ net flux measured by the SIET was also outward at the skin of larval tilapia (unpublished data). However, we cannot rule out the possibility that the probe also detected outward gradients of interfering ions (such as HCO₃⁻) instead of simply Cl⁻.

One of the drawbacks or limitations of ion-selective microelectrode-based techniques, including SIET, is the poor selectivity in detecting some ions including Na⁺ and Cl⁻. Commercially available liquid ion exchangers (LIXs) for preparing Na⁺- and Cl⁻-selective electrodes generally have poor selectivity coefficients and cannot specifically discriminate ions of interest and interference. For instance, the Na⁺-selective LIX is only approximately threefold more sensitive to Na⁺ than to K⁺; the Cl⁻-selective LIX (used in the present study) was ~12-fold more sensitive to Cl⁻ than to HCO₃⁻ when probing in medium containing ~1 mM Cl⁻. Therefore, when interfering ions are present in the medium, the data should be carefully interpreted. The removal of interfering ions from the medium is a practical way of removing the interference outside of cells, but the interference coming from inside cells cannot be avoided. Interference by HCO₃⁻ was a major

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**Fig. 4.** Effects of DMSO, ouabain (0.1 mM), and bumetanide (0.1 mM) on Cl⁻ fluxes by MRCs of SW-acclimated tilapia larvae. Data are presented as means ± SD. Different letters (a, b, c) indicate a significant difference (one-way ANOVA, Tukey’s comparison, \( P < 0.05 \)). Bracketed numbers are sample sizes.

**Fig. 5.** Cl⁻ gradient (\( \Delta [\text{Cl}⁻] \)) at the skin surface of fresh water (FW)-acclimated tilapia larvae. A: 6 spots measured with the Cl⁻-SIET: pericardial cavity (1), anterior yolk-sac (2), lateral yolk-sac (3), posterior yolk-sac (4), cloaca (5), and tail (6). B: \( \Delta [\text{Cl}⁻] \) measured at the 6 spots of 5 individuals. C: \( \Delta [\text{Cl}⁻] \) at the yolk sac (the average of 3 spots) decreased in larvae acclimated to low-Cl⁻ water. Bracketed numbers are sample sizes. *Significant difference (Student’s t-test, \( P < 0.05 \)).
into three groups, wavy convex, shallow basin, and deep hole, by their apical structures (18, 27). An increase in the apical size/density of convex-MRCs was found in tilapia larvae acclimated to low-Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+} water and was suggested to be associated with upregulation of 36Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+} uptake (27). Recently, different types of MRCs were identified in tilapia by the distribution of molecular makers including Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, NKCC, NCC, CFTR, and Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 (NHE3) (15). Inokuchi et al. (19) further suggested that the MRCs with convex surface are the NCC expressing MRCs and responsible for the Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+} uptake. The present study provides convincing evidence for this notion. Although, we cannot exclude the possibility that other types of MRCs also take up Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+}, their capabilities are apparently much lower. The confocal images (Fig. 6, C and D) showed that the basin type of MRCs also expressed NCC with lower abundance. NCC immunostaining of the apical membrane of MRCs further consolidated the SIET evidence. We found that the apical NCC abundance in low-Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+}-induced MRCs was also higher than that in MRCs without low-Cl\textsuperscript{-} induction. Most importantly, an NCC inhibitor (metolazone) significantly blocked the Cl\textsuperscript{-} influx in individual convex-MRCs, demonstrating the involvement of NCC in Cl\textsuperscript{-} uptake by convex-MRCs. NCC (SLC12A3) is a kidney-specific transporter in mammals and is mainly located in the apical membrane of distal convoluted cells for NaCl reabsorption (10, 14). In fish, the gill-specific NCC was suggested to be associated with Cl\textsuperscript{-}/H\textsubscript{11002}\textsuperscript{+} uptake of MRCs (15, 47), but the present study provided convincing evidence to support the NCC-dependent Cl\textsuperscript{-} uptake pathway in specific type of MRCs.

Although the involvement of NCC in Cl\textsuperscript{-} uptake of MRCs has been revealed, the driving force remains an open question (47). In general, the Na\textsuperscript{+}/Cl\textsuperscript{-} concentrations in FW are less than 1 mM and apparently not high enough to drive Na\textsuperscript{+}/Cl\textsuperscript{-} uptake through the NCC, unless the intracellular Na\textsuperscript{+}/Cl\textsuperscript{-} levels are much lower than external Na\textsuperscript{+}/Cl\textsuperscript{-} levels (15). However, we should not neglect the contribution of external microenvironment in which ionic concentrations are not equal to that in bulk water. In the present study and our unpublished
works, the SIET detected outward fluxes of Na\(^+\) and Cl\(^-\) from keratinocytes in FW-acclimated tilapia, medaka, and zebrafish larvae. The Na\(^+\)-Cl\(^-\) concentration at the surface of the tilapia skin is slightly higher than that in ambient water (\(0.4\) mM higher than the background when probing at the point about 10–20 \(\mu\)m away from the cells), and the concentration is supposed to increase exponentially when approaching the cell membrane (23). Therefore, we suggest that the passive outflow of Na\(^+\) and Cl\(^-\) from keratinocytes accumulates Na\(^+\)-Cl\(^-\) in the unstirred layer surrounding the larval skin, and this layer might have Na\(^+\)-Cl\(^-\) concentrations high enough to drive the Na\(^+\)-Cl\(^-\) cotransport. A model illustrating this suggestion is shown in Fig. 9.

In several studies, DIDS or SITS has been shown to inhibit \(^{36}\)Cl\(^-\) uptake in FW fishes, suggesting anion exchanger is involved in branchial Cl\(^-\) uptake (1, 13, 32, 33). Our finding...
seems to be inconsistent with these reports. However, the $^{36}$Cl$^-$ uptake experiments revealed the effect of inhibitors on Cl$^-$ uptake pathways in gills instead of specific cell types. In the present study, we analyzed the effect of DIDS on a specific group of MRCs (convex-MRCs) and found that the Cl$^-$ uptake pathway in convex-MRCs is not anion exchanger dependent. However, we cannot exclude the Cl$^-$/HCO$_3$ exchange pathway in other type of MRCs that might also take up Cl$^-$ with lower capability.

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