Cl^- Uptake Mechanism in Freshwater-Adapted Tilapia (*Oreochromis mossambicus*)

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ABSTRACT

In this study, the correlation between Cl^- influx in freshwater tilapia and various transporters or enzymes, the Cl^-/HCO_3^- exchanger, Na^+,K^+-ATPase, V-type H^+-ATPase, and carbonic anhydrase were examined. The inhibitors 2×10^{-4} M ouabain (a Na^+,K^+-ATPase inhibitor), 10^{-5} M NEM (a V-type H^+-ATPase inhibitor), 10^{-2} M ACTZ (acetazolamide, a carbonic anhydrase inhibitor), and 6×10^{-4} M DIDS (a Cl^-/HCO_3^- exchanger inhibitor) caused 40%, 60%–80%, 40%–60%, and 40%–60% reduction in Cl^- influx of freshwater tilapia, respectively. The inhibitor 2×10^{-4} M ouabain also caused 50%–65% inhibition in gill Na^+,K^+-ATPase activity. Western blot results showed that protein levels of gill Na^+,K^+-ATPase, V-type H^+-ATPase, and carbonic anhydrase in tilapia acclimated in low-Cl^- freshwater were significantly higher than those acclimated to high-Cl^- freshwater. Based on these data, we conclude that Na^+,K^+-ATPase, V-H^+-ATPase, the Cl^-/HCO_3^- exchanger, and carbonic anhydrase may be involved in the active Cl^- uptake mechanism in gills of freshwater-adapted tilapia.

Introduction

In freshwater or diluted media, fish face problems of passive and continuous loss of ions such as Na^+, Cl^-, and Ca^{2+}, but homeostasis of these ions in the body fluids is achieved and maintained by mechanisms for their active uptake. Gills, an important extrarenal organ, play a crucial role in the uptake of Na^+, Cl^-, and Ca^{2+} and in acid-base regulation (Perry 1997; Marshall 2002). In gill epithelia of freshwater teleosts, more than one type of mitochondria-rich (MR) cells has been identified, and these cells were suggested to be major sites for the regulation of ions (Pisam et al. 1987; Lee et al. 1996*a*, 1996*b*; Chang et al. 2001, 2003; Galvez et al. 2002). Studies in which the pH of the acclimation media was manipulated (Goss et al. 1992) and studies by using an x-ray microanalysis approach (Morgan et al. 1994) suggested that gill MR cells are responsible for Cl^- uptake in salmonids. In our previous artificial-freshwater acclimation experiments, a positive correlation between Cl^- uptake and the appearance of gill wavy-convex MR cells in tilapia was demonstrated (Chang et al. 2003).

Involvement of Cl^-/HCO_3^- exchanger has been documented to be involved in Cl^- uptake in fish gill MR cells. Studies of the effects of 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), an inhibitor of the Cl^-/HCO_3^- exchanger, and $NaHCO_3$ infusion (Perry and Randall 1981) suggested the possibility of the involvement of the Cl^-/HCO_3^- exchanger in Cl^- uptake in rainbow trout *Salmo gairdneri*. Molecular evidence for Cl^-/HCO_3^- exchanger mRNA was reported in the gill interlamella of rainbow trout *Oncorhynchus mykiss* with a metabolic alkalosis condition (Sullivan et al. 1996), and more recently the existence of the protein was also demonstrated in the apical region of MR cells in tilapia gills (Wilson et al. 2000). The current model for Cl^- uptake, in which Cl^- is absorbed in exchange of the excretion of HCO_3^- via the Cl^-/HCO_3^- exchanger, however, is oversimplified as stated by Perry (1997). Since the intracellular HCO_3^- concentration in MR cells may be insufficient to overcome the unfavorable Cl^- gradient (Perry 1997), the model seems to work only in the situation of metabolic alkalosis in which the intracellular HCO_3^- gradient is favorable to operate the Cl^-/HCO_3^- exchanger. The question we asked here is whether there are transporters or enzymes other than the Cl^-/HCO_3^- exchanger involved in the mechanism of Cl^- uptake in fish gill MR cells.

In the mammalian collecting duct and in amphibian skin, the Cl^-/HCO_3^- exchanger and H^+-ATPase or H^+,K^+-ATPase were demonstrated to carry out active Cl^- uptake (Jensen et al. 1997; Silver et al. 1998; Zhou et al. 1998). Na^+,K^+-ATPase is abundant in the tubular system of gill MR cells, and its role in ion uptake in freshwater teleosts has been proposed (Marshall 2002). H^+-ATPase was suggested to be associated with Na^+ uptake and acid excretion in the pavement cells of teleost gills (Perry 1997). However, localization of H^+-ATPase in pavement cells or MR cells of teleost gills is still being debated (Wilson et al. 2000; Katoh et al. 2003). Recently, bafilomycin, an inhibitor of H^+-ATPase, was found to inhibit Cl^- uptake in tilapia *Oreochromis mossambicus* (Fenwick et al. 1999). Mar-

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shall proposed that the situation of local acidification in the apex of an MR cell theoretically drives the Cl^-/HCO_3^- exchanger for Cl^- uptake (Marshall 2002). In addition, carbonic anhydrase was also suggested to be involved in the mechanism of Cl^- uptake in frog (*Rana temporaria*) skin (Kristensen 1972) and goldfish (*Carassius auratus*; Maetz and Romeu 1964). In this situation, it is important to evaluate the model for the Cl^- uptake across freshwater-adapted teleost gills by conducting an integrative study on the roles of several possible transporters or enzymes.

Based on the above information, we hypothesized that in addition to the Cl^-/HCO_3^- exchanger, Na^+,K^+-ATPase, H^+-ATPase, and carbonic anhydrase may be involved in Cl^- uptake in gills of freshwater-adapted fish. In this study, the effects of the inhibitors of these transporters and enzymes on Cl^- influx and the effects of environmental Cl^- levels on the expression of these transporters and enzyme in the gills of freshwater-adapted tilapia (*O. mossambicus*) were examined.

Material and Methods

Animals

Tilapia (*Oreochromis mossambicus*) at 0.3–0.7 g were obtained from laboratory stocks. All individuals were reared in 26°–28°C aerated local tap water (Table 1) with a photoperiod of 12L : 12D before the acclimation experiments. Fish were fed commercial pellets, but feeding was stopped 3 d before the sampling.

Experimental Design

Effects of Inhibitors on Cl^- Influx. Based on our hypothesis, treatments with inhibitors of the related transporters and enzymes should inhibit Cl^- influx in tilapia; therefore, the experiments were designed to examine the time-course inhibitory effects. In the preliminary experiments, 10^{-3}, 5×10^{-4}, 2×10^{-4}, 10^{-4}, and 10^{-5} M of ouabain (Na^+,K^+-ATPase inhibitor), 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M of NEM (N-ethylmaleimide,V-H^+-ATPase inhibitor), 6×10^{-4} and 10^{-4} M of DIDS (4,4′-Diisothio-cyanostilbene-2,2′-disulfonic acid, Cl^-/HCO_3^- exchanger inhibitor), as well as 10^{-2}, 10^{-3}, and 10^{-4} M of ACTZ (acetazolamide, a carbonic anhydrase inhibitor) were tested to see the effects on mortality, behavior, and Cl^- influx in tilapia. The inhibitors $2 \times 10^{-4} - 10^{-5}$ M of ouabain, $10^{-5} - 10^{-6}$ M of NEM, $6 \times 10^{-4} - 10^{-4}$ M of DIDS, and $10^{-2} - 10^{-4}$ M of ACTZ did not caused any mortality or abnormal behavior in fish within 3 h. Incubation with 2×10^{-4}, 10^{-5}, 6×10^{-4}, and 10^{-2} M of ouabain, NEN, DIDS, and ACTZ, respectively, significantly inhibited Cl^- influx; therefore, these concentrations were selected to examine the time-course inhibitory effects on tilapia Cl^- influx. Ouabain, NEM, DIDS, and ACTZ, respectively, were added to the $^{36}Cl^-$ tracer medium (see below) to give final concentrations of 2×10^{-4}, 10^{-5}, 6×10^{-4}, and 10^{-2} M. Each fish was transferred to the tracer medium for Cl^- influx measurement (see below), and water samples were collected at 0.5-h intervals for 2 h. The inhibitors were added to the tracer media at 0.5 h after transfer; therefore, the influx data at the first 0.5 h was a control, and the subsequent serial data with 0.5-h intervals up to 2 h were treatments.

Effects of Ouabain on Gill Na^+,K^+-ATPase Activity. The time-course effects of ambient ouabain on the gill Na^+,K^+-ATPase activity were examined to confirm that the exogenous ouabain reaches the target, the basolateral Na^+,K^+-ATPase. Tilapia gills were sampled for Na^+,K^+-ATPase activity assay at 0 (control), 0.5, 1, 1.5, and 2 h after addition of ouabain (final concentration 2×10^{-4} M) to the experimental media.

Effects of Ambient Cl^- on Expression of Transporters and Enzymes. In a previous study (Chang et al. 2003), it was demonstrated that acclimation to low ambient Cl^- causes an increase in Cl^- influx in tilapia. Based on our hypothesis, enhanced Cl^- influx should result from stimulation of expression of the related transporters and enzymes. Tilapia were acclimated to high-Na-high-Cl (H-Na-H-Cl) and high-Na-low-Cl (H-Na-L-Cl) artificial freshwater media, respectively, for 1 wk, and then gills were sampled for western blotting of Na^+,K^+-ATPase, V-H^+-ATPase, and carbonic anhydrase. According to Chang et al. (2003), low ambient Na^+ level would inhibit Cl^- influx; therefore, a high Na^+ level was designed in the artificial freshwater to diminish the effects of Na^+. In order to maintain the water quality, the acclimation medium was aerated with a filtered air pump and was changed every 2 d. Fish were fed commercial pellets 1 h immediately before the water changes every 2 d, but feeding was stopped 3 d before the sampling.

Table 1: Ionic compositions (mM) in the artificial freshwater

Medium	Na^+	Cl^-	Ca^{2+}	K^+	Mg^{2+}	pH
Local tap water	.295–.385	.045–.055	.176–.201	.038–.050	.140–.185	6.90–7.20
H-Na-L-Cl	10.161–10.781	.002–.012	.167–.175	.152–.176	.186–.207	6.75–6.87
H-Na-H-Cl	9.267–1.035	7.314–7.880	.186–.190	.148–.168	.176–.186	6.70–6.78

Cl^- Influx

Whole-body Cl^- influx was measured following Wood (1992) with some modifications. Tracer medium was prepared by adding appropriate amounts of $^{36}Cl^-$ (Amersham, Piscataway, N.J.) to local freshwater to give a final working specific activity of $^{36}Cl^-$, (2.1 to 2.6) × 10^5 cpm × μmol^{-1}. After rinsing briefly with freshwater, fish were transferred to plastic flux chambers with 20 mL of tracer media. The tracer medium in the flux chambers was gently aerated, and the water quality was confirmed to show no significant change during the period of incubation. In a preliminary experiment, it was demonstrated that the nonspecific surface binding was negligible; the accumulated radioisotope by the fish was linear over the first 6 h (sampling times: 1, 2, 3, 4, 5, and 6 h), indicating a negligible back flux of $^{36}Cl^-$ during the 6 h. Counting solution (Fluoran-safe Scintran, BDH, Poole, United Kingdom) was added to water samples (200 μL) from the $^{36}Cl^-$ media, and then radioactivity was determined in a beta counter (LS6500, Beckman, Fullerton, Calif.). Volumes of the tracer media were measured at each incubation time point.

Cl^- influx was calculated by the following formula:

$$J_i = \frac{[(Q_i \times V_i) - (Q_f \times V_f)]}{[1/2 \times (SA_i + SA_f) \times t \times W]},$$

where Q_i and Q_f (cpm mL^{-1}) refer to the initial and final radioactivities in the tracer media; V_i and V_f (mL) refer to the initial and final volumes of the tracer media; SA_i and SA_f are the initial and final specific activities (cpm × μmol^{-1}); t is the incubation time; and W (g) is the body weight of the fish. Since all water samples were in the same condition, no quenching effect needed to be calibrated.

Transporter Inhibitors

Ouabain (Na^+,K^+-ATPase inhibitor; Sigma, O3125, Saint Louis, Mo.), NEM (N-ethylmaleimide, *V*-H^+-ATPase inhibitor; Sigma, E1271), DIDS (4,4′-Diisothio-cyanostilbene-2,2′-disulfonic acid, Cl^-/HCO_3^- exchanger inhibitor; Sigma, D3514), and ACTZ (Acetazolamide, carbonic anhydrase inhibitor; Sigma, A6011) were used in the present study. Ouabain and NEM were directly dissolved in water, ACTZ was dissolved in 0.1N NaOH at first, and DIDS was directly dissolved in DMSO. The pH levels of all the working solutions of the inhibitors were appropriately adjusted to near that of the experimental media.

Preparation of Artificial Freshwater

Two kinds of artificial freshwater, H-Na-L-Cl and H-Na-H-Cl, were prepared by adding appropriate amounts of NaCl, Na_2SO_4, $MgSO_4$, K_2HPO_4, KH_2PO_4, and $CaSO_4$ to double-deionized water (Milli-RO60, Millipore, Billerica, Mass.). The ion concentrations of K^+, Ca^{2+}, and Mg^{2+} in the artificial freshwater were near the ranges of local freshwater (Table 1). The ionic compositions of the two media were confirmed by measuring Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations with an atomic absorption spectrophotometer (Hitachi Z-8000, Tokyo) and Cl^- with a spectrophotometer (Hitachi U-2000). The water temperature was maintained at 26°–28°C.

Homogenates and Preparation of Gill Epithelial Cells

Gills were excised and blotted dry. Gills from each individual fish yielded one sample. All subsequent operations were carried out in ice. Gills were cut into small pieces and then suspended in homogenization solution (100 mM imidazole-HCl buffer, pH 7.6; 5 mM Na_2EDTA; 200 mM sucrose; and 0.1% sodium deoxycholate). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a motorized Teflon pestle at 600 rpm for 20 strokes. The homogenate was centrifuged at 4°C and 13,000 *g* for 10 min, and the supernatant was stored at −80 °C. In order to prevent contamination of gill cells by blood cells, enrichment of the epithelial cells was carried out for all the western blotting. Gill epithelium was scraped off the underlying cartilage with a scalpel in dissociated buffer (2 mM Na_2EDTA and 1% Percoll in phosphate-buffered saline [PBS]) and stirred slowly at 4°C for 30 min. Dissociated gill cells were filtered through nylon gauze and then gently layered on a 20% Percoll discontinuous gradient. After centrifugation using swing rotors at 1,000 *g* and 4°C for 10 min, the white layer of epithelial cells was separated from the red layer of blood cells. Isolated gill epithelial cells were washed with PBS and centrifuged at 1,000 *g* at 4°C for 5 min; the pellet was finally subjected to homogenization as described above. Protein concentrations of the homogenates (whole tissues or isolated epithelial cells) were determined with the reagents of a Bio-Rad Protein Assay Kit using bovine serum albumin as a standard.

Antibodies

The primary antibody against the catalytic subunit of Na^+,K^+-ATPase used in the present study was the mouse monoclonal antibody α 5 raised against the α subunit of the avian sodium pump. A rabbit polyclonal antibody against H^+-ATPase was raised against a synthetic peptide corresponding to a sequence from the 31-kDa E-subunit of tilapia vacuolar H^+-ATPase (MALSDADVQKQ, GenBank accession number AY322478). The polyclonal antibody against the branchial carbonic anhydrase of flounder was developed by Sender et al. (1999). The specificity of α 5 antibody and carbonic anhydrase antibody to tilapia has been confirmed by cross-reacting with the recombinant proteins of tilapia Na^+,K^+-ATPase α 1 and carbonic anhydrase, respectively, which were synthesized via an expression system constructed with recombinant full-length cDNAs of tilapia Na^+,K^+-ATPase α 1 (accession number AF109408;

Feng et al. 2002) and carbonic anhydrase (accession number AY322477). The specificity of H^+-ATPase antibody was confirmed by using a competitive enzyme-linked immunosorbent assay.

Western Blotting

The gill or gill epithelial cell homogenate was subjected to polyacrylamide gel electrophoresis (PAGE) on 8 × 10-cm sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%) gels at 100 V for 2 h. Protein at 10 μg per well was loaded. After separation, proteins were transferred onto polyvinylidene difluoride membrane (Millipore) with a transfer electrophoresis unit (SE 600, Hoffer) at 100 V for 2 h. After blocking for 1.5 h in 5% nonfat dried milk, the blots were incubated with various primary antibodies: α 5 (2 h, diluted 1 : 5,000), H^+-ATPase (2 h, diluted 1 : 1,000), BIII-136 (2 h, diluted 1 : 500), and carbonic anhydrase (2 h, diluted 1 : 10,000), respectively. After incubation, the membrane was washed in PBST (phosphate buffer saline with 0.2% Tween 20) and reacted for 30 min with alkaline-phosphatase-conjugated secondary antibodies (goat antimouse IgG, Jackson, diluted 1 : 1,000; goat antirabbit IgG, Jackson, diluted 1 : 1000). Blots were extensively washed with PBST and then developed with 0.015% nitro-blue tetrazolium and 0.007% bromochloroindolyl phosphate in a reaction buffer containing 100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$ (pH 9.5). Tissue of rat kidney was also run as a positive control, and negative controls were conducted without primary antibodies (data not shown). Immunoblots were scanned and imported as JPG files into a commercial software package (Image-Pro Plus, Version 1.2, Media Cybernetics, Silver Spring, Md., 1994), and the results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

Na^+,K^+-ATPase Activity Assay

Gill Na^+,K^+-ATPase activity was determined as described by Hwang et al. (1988). Activity was assayed by adding the supernatant prepared as described above to the reaction mixture (500 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, 5 mM Na_2 ATP). The reaction was run at 37°C for 30 min and then stopped by addition of 200 μL of ice-cold 30% trichloroacetic acid. The inorganic phosphate concentration was determined by the method of Peterson (1978). The enzyme activity of Na,K-ATPase was defined as the difference between the inorganic phosphate liberated in the presence and absence of 5 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

Statistical Methods

All values are presented as the mean ± SE (N). The significant difference of Cl^- influxes or Na^+,K^+-ATPase activity among different incubation times was assessed by one-way ANOVA (Tukey's pairwise comparison). Student's t-test was used to assess the significant differences in the relative amounts of specific proteins as identified by westerns between the two groups.

Results

Effects of Inhibitors on Cl^- Influx

Addition of 2×10^{-4} M ouabain to the tracer medium resulted in a significant reduction in Cl^- influx in freshwater-adapted tilapia (Fig. 1*a*). Initially, a slight reduction (15%) in Cl^- influx was observed at 0.5 h. After a 1-h exposure, a significant decrease (27%) occurred, and then a maximum degree of about 38%–41% was reached from 1.5 h to the end of the incubation.

After exposure to 10^{-5} M NEM, whole-body Cl^- influx in tilapia was significantly inhibited (Fig. 1*b*). No significant change was found in Cl^- influx within the first 0.5 h of incubation, but thereafter a significant reduction (64%–82%) occurred.

Similar to ouabain and NEM, it was not until 1 h after incubation that 10^{-2} M ACTZ caused dramatic and evident decreases (52%–73%) in the Cl^- influx of tilapia (Fig. 1*c*). However, 6×10^{-4} M DIDS resulted in significant inhibition of the Cl^- influx of tilapia from the beginning of incubation, and inhibition was intensified, from 31% to 55%, following incubation (Fig. 1*d*).

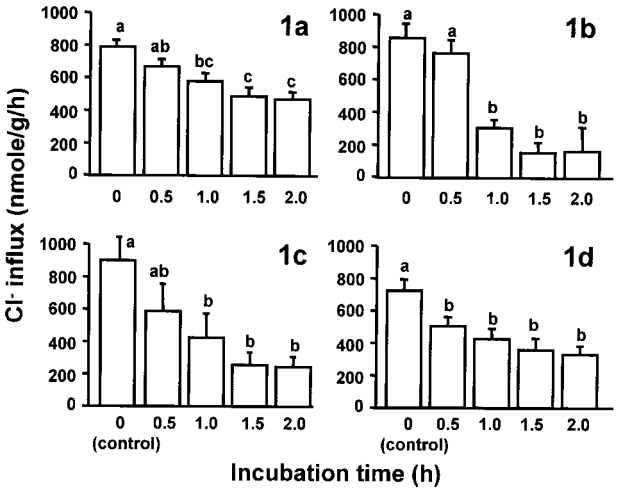

Figure 1. Effects of transporter or enzyme inhibitors on Cl^- uptake. Individual inhibitors resulted in significant reduction of Cl^- influx at different times of incubation. *a*, Ouabain; *b*, NEM; *c*, ACTZ; *d*, DIDS. Values were mean ± SE ($N = 6$). Different letters indicated significant differences among different sampling times (Tukey's pairwise comparison).

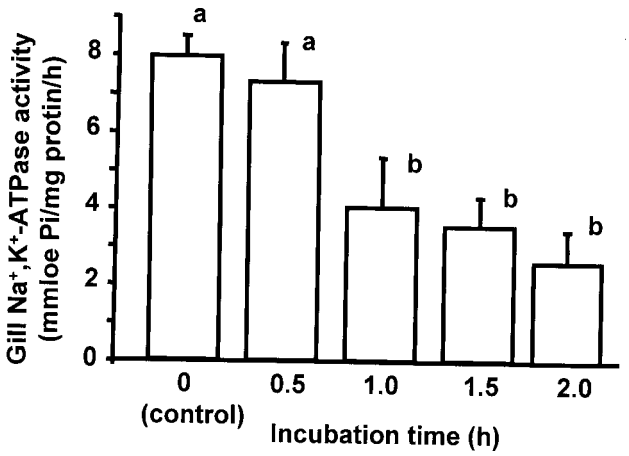

Figure 2. Effects of ambient ouabain (2×10^{-4} M) on gill Na^+,K^+-ATPase activity. Addition of ouabain to the ambient medium resulted in significant reduction of the activity at different times of treatment. Values were mean ± SE ($N = 4$). Different letters indicate significant differences among different sampling times (Tukey's pairwise comparison).

Effects of Ambient Ouabain on Gill Na^+,K^+-ATPase Acitivity

Addition of ouabain (final concentration 2×10^{-4} M) to the experimental media (local freshwater) caused evident inhibitions on gill Na^+,K^+-ATPase activity in tilapia (Fig. 2). Significant inhibition, near 50%, appeared at 0.5 h after the exposure to ouabain, and the inhibition increased to about 55%–65% but maintained the similar level till the end of the experiment. This indicated that ambient ouabain did reach the target, the basolateral Na^+,K^+-ATPase, in gills.

Effects of Ambient Cl^- on Expression of Transporters and Enzymes

Levels of gill Na^+,K^+-ATPase, V-H^+-ATPase, and carbonic anhydrase in the low-Cl^- group were approximately 2.6-, 2.4-, and 1.5-fold higher than those in the high-Cl^- group, respectively (Fig. 3).

Discussion

Major findings of the present study were that (1) inhibitors of Na^+,K^+-ATPase, V-H^+-ATPase, the Cl^-/HCO_3^- exchanger, and carbonic anhydrase provided significant inhibition on Cl^- uptake in tilapia (*Oreochromis mossambicus*), and (2) acclimation to low-Cl^- freshwater stimulated the protein expression of gill Na^+,K^+-ATPase, V-H^+-ATPase, and carbonic anhydrase in tilapia. Based on these data, we conclude that Na^+,K^+-ATPase, V-H^+-ATPase, the Cl^-/HCO_3^- exchanger, and carbonic anhydrase may be involved in the active Cl^- uptake mechanism in gills of freshwater-adapted tilapia.

As described above, most previous studies focused on functions of one or two transporters or enzymes in the Cl^- uptake mechanism of freshwater fish (Maetz and Romeu 1964; Kerstetter and Kirschner 1972; Perry and Randall 1981). This article is a more integrative study to elucidate the functional role of the various transporters and enzymes (Na^+,K^+-ATPase, V-H^+-ATPase, the Cl^-/HCO_3^- exchanger, and carbonic anhydrase) in freshwater fish. With regard to the molecular evidence of these transporters and enzymes, most previous studies provided data for protein immunolocalization (Sender et al. 1999; Wilson et al. 2000) or in situ mRNA hybridization (Sullivan et al. 1996). This article is the first to provide biochemical evidence for these

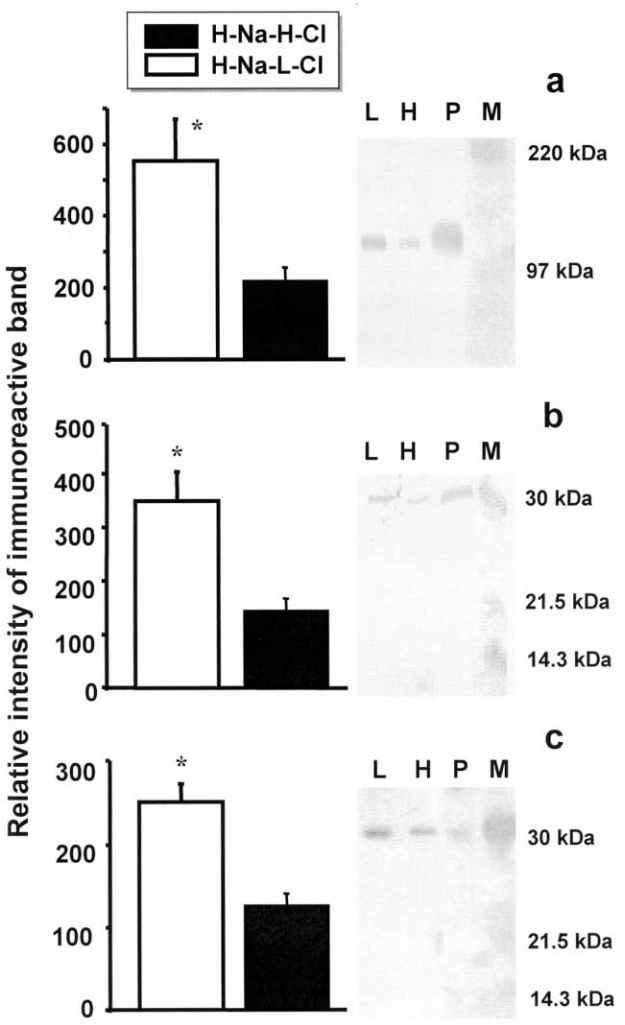

Figure 3. Relationships between ambient Cl^- and expression of transporters and enzymes. Low environmental Cl^- stimulated enhanced expression of transporters and enzymes. *a*, Na^+,K^+-ATPase; *b*, H^+-ATPase; *c*, carbonic anhydrase. *L*, H-Na-L-Cl; *H*, H-Na-H-Cl; *P*, positive control of rat kidney; *M*, molecular weigh marker. Values were mean ± SE ($N = 6$). An asterisk indicates significant differences from high-Cl^- group (Student's *t*-test, $P < 0.05$).

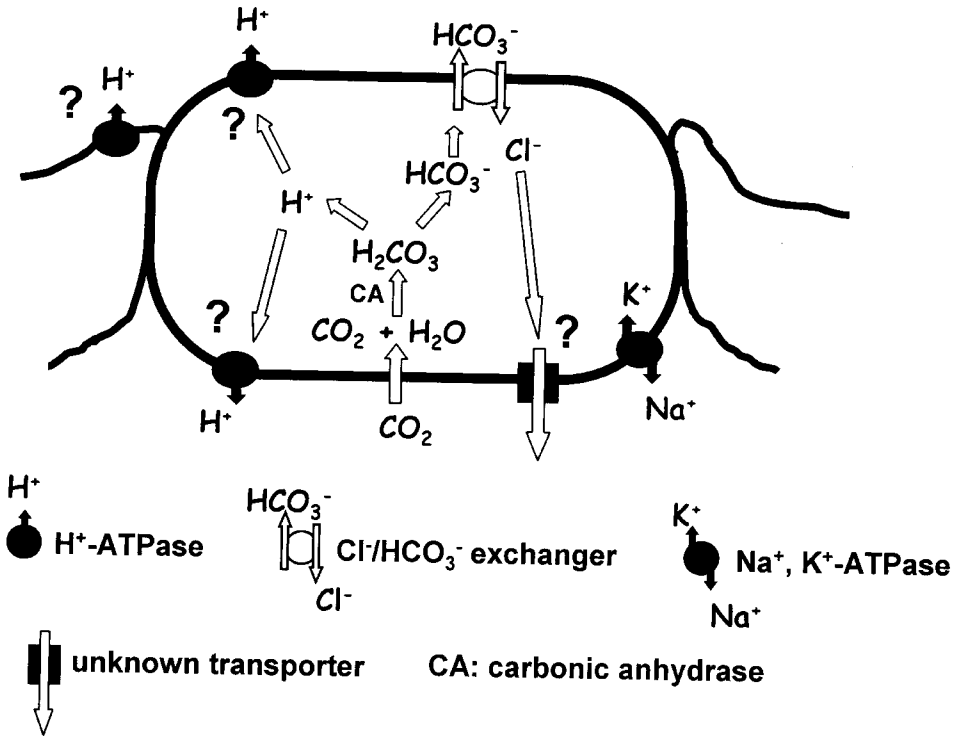

Figure 4. Proposed model for active Cl^- uptake in the freshwater fish gill (refer to the text). A question mark indicates the pathways to be clarified.

transporters and enzymes whose protein expressions are positively correlated with Cl^- uptake in fish.

Incubation with and injection of bicarbonate resulted in a reduction and an increase, respectively, of Cl^- influx in goldfish *Carassius auratus* (Maetz and Romeu 1964) and rainbow trout *Salmo gairdneri* (Kerstetter and Kirschner 1972). SITS, an inhibitor of the Cl^-/HCO_3^- exchanger, caused 66%–71% inhibition in the Cl^- influx of rainbow trout *S. gairdneri* (Perry and Randall 1981). Similarly, in the present study, DIDS, another inhibitor of the Cl^-/HCO_3^- exchanger, also caused about 55% inhibition in the Cl^- influx in tilapia. All these physiological data demonstrated the correlation between Cl^- influx and HCO_3^- efflux via the Cl^-/HCO_3^- exchanger in fish gills. Based on experiments of alkalosis and acidosis in rainbow trout *Oncorhynchus mykiss* and brown bullhead *Ictalurus nebulosus*, Goss et al. (1992) indicated that the Cl^-/HCO_3^- exchanger is not only involved in acid-base balance but also carried out Cl^- uptake. They also proposed that increased Cl^- uptake should be accompanied by enhanced expression of the Cl^-/HCO_3^- exchanger that is required for acid-base regulation. To support this, a remarkable increase in Cl^-/HCO_3^- exchanger mRNA during metabolic alkalosis was reported in rainbow trout *O. mykiss* (Sullivan et al. 1996). Recently, Wilson et al. (2000) used an immunocytochemical approach to localize the protein of the Cl^-/HCO_3^- exchanger in gill MR cells in tilapia *O. mossambicus*. In our previous studies (Chang et al. 2001, 2003), acclimation to low environmental Cl^- resulted in enhanced Cl^- uptake in tilapia and goldfish *C. auratus*. Further study is needed to examine whether the protein expression of the Cl^-/HCO_3^- exchanger is stimulated in tilapia acclimated to low environmental Cl^-.

In the situation with metabolic alkalosis, the Cl^-/HCO_3^- exchanger was proposed to be driven to absorb Cl^- in exchange with the accumulated intracellular HCO_3^- in gill MR cells; however, in his review, Perry (1997) addressed the fact that the intracellular HCO_3^- gradient in gill MR cells may be insufficient to overcome an unfavorable Cl^- gradient. It has been demonstrated that active Cl^- uptake via the Cl^-/HCO_3^- exchanger is driven by H^+,K^+-ATPase in mammalian collecting ducts (Silver et al. 1998; Zhou et al. 1998) or by H^+-ATPase in amphibian skin (Larsen et al. 1992, 1996; Jensen et al. 1997). Based on this information, it is reasonable to propose that some other ATPase is required to provide the driving force to operate the Cl^-/HCO_3^- exchanger for active Cl^- uptake in gill MR cells of freshwater-adapted teleosts.

Based on morphological, physiological, and biochemical data, Na^+ uptake through apical Na^+ channels down the electrochemical gradient in part by H^+ secretion via the apical V-type H^+-ATPase has been proposed in gill pavement cells in rainbow trout *O. mykiss*, brown trout *Salmo trutta* L, and brown

bullhead *I. nebulosus* (Goss et al. 1992; Morgan et al. 1994). However, localization of V-type H^+-ATPase in fish gills is still being debated with occurrence in pavement cells only (Wilson et al. 2000), in MR cells only (Katoh et al. 2003), or in both MR cells and pavement cells (Wilson et al. 2000). In frog skin, V-type H^+-ATPase was localized in MR cells (Klein et al. 1997) and was demonstrated to be associated with the Cl^-/HCO_3^- exchanger for active uptake of Cl^- from diluted media in several electrophysiological studies (Larsen et al. 1992, 1996; Jensen et al. 1997). In a recent review, Marshall (2002) proposed that local acidification in the apical crypt of MR cells by H^+-ATPase theoretically will lower HCO3$^-$ activity at the apical surface sufficiently to drive the Cl^-/HCO_3^- exchanger for Cl^- uptake, but no substantial data was provided. However, there were some clues indicating the possibility of the function of V-type H^+-ATPase, other than for Na^+ uptake, in gill MR cells of freshwater-adapted fish. Bafilomycin, an inhibitor of V-type H^+-ATPase, reduced Cl^- uptake in tilapia larvae (Fenwick et al. 1999). In the present study, NEM, a commonly used inhibitor of V-type H^+-ATPase for assaying enzyme activity (Pedersen and Carafoli 1987), was also found to inhibit Cl^- influx in tilapia. Furthermore, the present study demonstrates that the protein expression of gill V-type H^+-ATPase in tilapia is stimulated by acclimation to low ambient Cl^-, which was previously reported to induce an evident increase in Cl^- influx in tilapia (Chang et al. 2001). All these imply the possibility that V-type H^+-ATPase is involved in the Cl^- uptake across fish gills.

In teleosts, Na^+,K^+-ATPase in gill MR cells is another important transporter that plays a crucial role in ion- and osmoregulation balance. It has been well documented that Na^+,K^+-ATPase provides the major driving force for Cl^- secretion in gill MR cells of seawater-adapted teleosts (Evans et al. 1999; Marshall 2002); Na^+,K^+-ATPase provides the transmembrane Na^+gradient to drive Cl^- into the cell via the basolateral Na^+,K^+, 2 Cl^- cotransporter, and then the Cl^- exits the cell via the apical CFTR-type anion channels. A role was proposed for Na^+,K^+-ATPase in Na^+ or Cl^- uptake in gills of freshwater-adapted teleosts (McCormick 1995); however, there was no substantial evidence for this inference until now. Acclimation to ambient low Cl^- resulted in significant increases in both Cl^- influx (Chang et al. 2001, 2003) and protein expression of gill Na^+,K^+-ATPase (this study) in tilapia, indicating the positive correlation between Cl^- influx and the Na^+,K^+-ATPase protein in freshwater-adapted fish gills. Furthermore, treatment with ouabain, an inhibitor of Na^+,K^+-ATPase, caused an evident decline in Cl^- uptake in tilapia. Taking all these into account, Na^+,K^+-ATPase may be involved in the mechanism for Cl^- uptake in freshwater-adapted fish gills.

In the models for the active Cl^- uptake in amphibian skin and mammalian collecting ducts, HCO_3^- (for Cl^-/HCO_3^- exchanger) and H^+ (for H^+,K^+-ATPase or V-type H^+-ATPase) are fueled by carbonic anhydrase. In teleosts, two different carbonic anhydrase isoenzymes, erythrocyte and gill epithelia types, were identified (Rahim et al. 1988), and the gill epithelia type of carbonic anhydrase was localized in both MR cells and pavement cells in the gills of salamander (Lewinson et al. 1987) and flounder (Sender et al. 1999). The gill epithelia type of carbonic anhydrase hydrates CO_2 to yield HCO_3^- and $H^+$for the Cl^-/HCO_3^- exchanger and the $Na^+/H^+(NH_4^+)$ exchanger, suggesting the possibility that the enzyme participates in ion regulation in gills of teleosts (Sender et al. 1999). Treatment with ACTZ, an inhibitor of carbonic anhydrase, resulted in a significant reduction in Cl^- uptake in freshwater goldfish *C. auratus* (Maetz and Romeu 1964). The present study also indicates similar results in freshwater-adapted tilapia. Moreover, a positive correlation between carbonic anhydrase protein expression and Cl^- influx was also derived from the present study and previous data (Chang et al. 2003). Taken together, carbonic anhydrase may be involved in Cl^- uptake in fish gills via yielding HCO_3^- and H^+ for the Cl^-/HCO_3^- exchanger and H^+-ATPase, respectively.

The current model for the active Cl^- uptake in gill of freshwater-adapted teleosts is more clear based on several lines of evidence from the present study and other previous studies: (1) the effects of the inhibitors of Na^+,K^+-ATPase, V-H^+-ATPase, the Cl^-/HCO_3^- exchanger and carbonic anhydrase on Cl^- influx; (2) the positive correlation between Cl^- influx and protein expression of these transporters and enzymes in freshwater-adapted fish gill; and (3) localization of the protein or mRNA of these transporters and enzymes in gill cells. In this model (Fig. 4), CO_2 is hydrated by carbonic anhydrase to yield HCO_3^- and H^+. HCO_3^- is exchanged via the Cl^-/HCO_3^- exchanger for the Cl^- uptake. H^+ is pumped out of MR cells via the apical V-H^+-ATPase as proposed in the toad skin (Larsen et al. 1996) or via the basolateral V-H^+-ATPase as proposed by Piermarini and Evans (2001), but convincing molecular evidence in fish gill MR cells is still lacking. The apical V-H^+-ATPase may contribute to Cl^-/HCO_3^- exchange; the extruded protons could act to decrease the HCO_3^- activity in the apex of gill MR cells and thus provide sufficient HCO_3^- gradient for the Cl^-/HCO_3^- exchanger. The basolateral Na^+,K^+-ATPase creates the electrochemical gradient that fuels Cl^- efflux across the basolateral membrane and thus completes the Cl^- uptake mechanisms.

For the proposed model of active Cl^- uptake in gill MR cells of freshwater-adapted fish, there are still many unclear and controversial points remaining to be answered. The inhibitor experiments were conducted in vivo, and those lipid-soluble inhibitors might pass across epithelia. Some of the inhibitor concentrations used in the present experiments were different from those in previous in vivo or in vitro studies (Maetz and Romeu 1964; Fenwick et al. 1999). This may be due to the differences between species in the membrane structure and thus the permeability to the inhibitors. In the time-course experiments of the inhibitors, significant inhibition occurred within the initial 0.5–1 h of inhibitor treatments. The main target of this initial inhibition is probably the gill epithelial cells because

the inhibitors first enter the gill cells before they approach other cells or tissues. However, the possibility of indirect effects of those inhibitors through unknown and perhaps unspecific pathways cannot be excluded. For example, the effect of acetazolamide on Cl^- influx may be the result of buffering effects and/or inhibition of red blood cell carbonic anhydrase activity with a concomitant reduction in CO_2. Therefore, in vitro experiments (isolated operculum) with inhibitors should be conducted to confirm the present results concerning the effects of the inhibitors. Results concerning the localization of several ion transporters in fish gills are inconsistent among different species (Wilson et al. 2000; Katoh et al. 2003). In contrast to the present study, gill Cl^- uptake was not influenced by the inhibitor of carbonic anhydrase in rainbow trout *S. gairdneri* (Kerstetter and Kirschner 1972). The reasons for these conflicting data may be due to differences in the specificity of the antibody used or in the environment and the species studied. The model should be tested in a species other than tilapia. Na^+,K^+-ATPase, V-H^+-ATPase, and carbonic anhydras have been proposed to involve in Na^+ uptake in fish gills (Evans et al. 1999; Marshall 2002); therefore, the inhibitors may also affect the gill Na^+ uptake, although no measurement was conducted in the present study.

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