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Phenotypic Changes in Mitochondrion-Rich Cells and Responses of Na^+/K^+-ATPase in Gills of Tilapia Exposed to Deionized Water

Cheng-Hao Tang[1†], Il-Chi Chang[2†], Chia-Huang Chen[1], Tsung-Han Lee[1*] and Pung-Pung Hwang[3]

[1]*Department of Life Sciences, National Chung-Hsing University, Taichung. 402 Taiwan*
[2]*National Health Research Institutes, Stem Cell Reasearch Center, Miaoli County, 350 Taiwan*
[3]*Institute of Organismic and Cellular Biology, Academia Sinica, Taipei, 115 Taiwan*

The aim of this study was to illustrate the phenotypic modification of mitochondrion-rich (MR) cells and Na^+/K^+-ATPase (NKA) responses, including relative protein abundance, specific activity, and immunolocalization in gills of euryhaline tilapia exposed to deionized water (DW) for one week. The plasma osmolality was not significantly different between tilapia of the local fresh water (LFW) group and DW group. Remodeling of MR cells occurred in DW-exposed fish. After transfer to DW for one week, the relative percentage of subtype-I (wavy-convex) MR cells with apical size ranging from 3 to 9 μm increased and eventually became the dominant MR cell subtype. In DW tilapia gills, relative percentages of lamellar NKA immunoreactive (NKIR) cells among total NKIR cells increased to 29% and led to significant increases in the number of NKIR cells. In addition, the relative protein abundance and specific activity of NKA were significantly higher in gills of the DW-exposed fish. Our study concluded that tilapia require the development of subtype-I MR cells, the presence of lamellar NKIR cells, and enhancement of NKA protein abundance and activity in gills to deal with the challenge of an ion-deficient environment.

Key words: gill, mitochondrion-rich cell, Na^+/K^+-ATPase, *Oreochromis mossambicus*, tilapia, deionized water

INTRODUCTION

The epithelium serves a barrier function by controlling the movement of diverse ions for physiological homeostasis. Fish gills, amphibian skin, and mammalian renal collecting ducts are general models for addressing the mechanisms of transepithelial ion transport. Mitochondrion-rich (MR) cells are present in epithelium of these models and are responsible for ion transport. It is well accepted that different subtypes or subpopulations of MR cells are present in fishes, amphibians, and mammals, and that their major functions are associated with ion regulation (Larsen, 1991; Goss et al., 1992b; Brown and Breton 1996; Perry, 1997; Marshal, 2002; Evans et al., 2005; Hwang and Lee, 2007).

Aquatic environments that teleosts inhabit range from hypotonic to hypertonic media. Previous morphological, biochemical, and physiological studies revealed that MR cells secrete Cl^- actively in hypertonic environments, e.g., seawater (SW; Foskett and Scheffey 1982; Zadunaisky, 1984; Laurent and Perry, 1991; Hirose et al., 2003). Conversely, freshwater (FW) fishes have to actively absorb ions, e.g., Na^+, Cl^-, and Ca^{2+}, to counterbalance the continuous diffusive ion loss to hypotonic environments (Chang et al., 2001; Lin and Hwang, 2001; Chang et al., 2002, 2003; Perry et al., 2003; Chang and Hwang, 2004; Choe et al., 2007).

The relationship between subtypes and physiological functions of gill MR cells is complicated. By transmission electron microscopic (TEM) observation, Piasm et al. (1995) divided FW teleost gill MR cells into α and β types. Subsequently, three subtypes of MR cells with different apical surface morphologies have been recognized in tilapia (Lee et al., 1996; van der Heijden et al., 1997). These findings indicated that MR cell polymorphism is essential for teleosts to deal with ion uptake when the fish were acclimatized to hypotonic FW (Hwang and Lee, 2007). Development of different subtypes of MR cells in fish gills can be easily activated by manipulating the ionic composition of the aquatic environment (Chang et al., 2001; Lin and Hwang, 2001; Chang et al., 2002, 2003; Chang and Hwang, 2004), which illustrates the linkage between variable morphology and functions of MR cells. Together with the ion-flux data, these experiments demonstrated that one phenotype of MR cell corresponds to the regulation of one major ion (Chang et al., 2001, 2002, 2003; Chang and Hwang, 2004).

Mozambique tilapia tolerates salinities of up to 120‰

* Corresponding author. Phone: +886-4-2285-6141;
Fax : +886-4-2285-1797;
E-mail : thlee@dragon.nchu.edu.tw
† These authors contributed equally to this paper.
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(Stickney, 1986) and constitutes a good model animal for studying ionic and osmotic regulation in teleost fishes. Na^+/K^+-ATPase (NKA) is a universal membrane-bound enzyme that actively transports Na^+ out of and K^+ into animal cells. It is important not only for sustaining intracellular homeostasis but also for providing a driving force for many transporting systems in a variety of osmoregulatory epithelia, including fish gills (McCormick, 1995). Since FW teleosts might encounter special environments, such as soft water, in which the total ion composition is lower than the normal FW condition, the effects of ion-poor environments (e.g., deionized water; DW) on osmoregulation in teleosts are intriguing.

In this study, Mozambique tilapia was used to investigate the remodeling of cellular profiles, including densities and sizes of MR cell subtypes, after direct transfer from FW to DW for one week. Meanwhile, the effects of an ion-poor environment on NKA protein abundance and activity in gills of *O. mossambicus* were assayed. The distribution and number of NKA-immunoreactive (NKIR) cells were also determined, since MR cells have been demonstrated to be the major sites for localization of NKA in gill epithelia (Marshall, 2002; Wilson and Laurent, 2002; Evans et al., 2005).

MATERIALS AND METHODS

Animals and experimental environments

Tilapia (*Oreochromis mossambicus*) with body lengths of 3–5 cm were obtained from laboratory stocks. In this study, local fresh water (LFW) and deionized water (DW; Milli-RO60, Millipore, Billerica, MA, USA) were used as experimental media. Ionic compositions of LFW in mM were: Na^+ (2.60), K^+ (0.04), Ca^{2+} (0.58), Mg^{2+} (0.16) and Cl^- (0.18). DW, however, was devoid of detectable levels of those ions. Tilapia were kept in LFW at a photoperiod of 12L:12D for at least one month before the acclimatization experiments. Fish were fed daily with commercial pellets ad libitum, and water was continuously circulated through fabric-floss filters and changed every 2 days for maintenance of water quality.

Determination of plasma osmolality

Fish blood was collected from the caudal vein with heparinized 1-ml syringes and 27G needles. Collected blood was centrifuged at 1,000×*g* at 4°C for 10 min. Resultant plasma was stored at –20°C before analysis. A vapor pressure osmometer (5520 Vapro; Wescor, Logan, Utah, USA) was employed to determine the osmolality of plasma.

Ultrastructure and apical sizes of subtypes of mitochondrion-rich (MR) cells in gill filaments

After anesthetization with MS222 (100–200 mg/L), fish were killed and gills were excised. The first gill arch from each side was fixed at 4°C in fixative consisting of 5% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 12 hr. After rinsing with 0.1 M PB, specimens were postfixed with 1% (v/v) osmium tetroxide in 0.2 M PB for 1 hr. After rinsing with PB and dehydration in ethanol, specimens were critical-point dried using liquid CO_2 in a critical-point dryer (Hitachi HCP-2, Tokyo, Japan) and sputter coated for 3 min with a gold-palladium complex in a vacuum evaporator (Eiko 1B-2, Tokyo, Japan). Coated specimens were examined with a scanning electron microscope (SEM; Hitachi S-2500, Tokyo, Japan).

Based on previous studies (Hwang and Lee, 2007), subtypes of MR cells were identified by SEM observation, and the densities of different MR cell subtypes were determined. In brief, areas on the trailing edge of the filaments were chosen at random for counting at 1500X magnification. Two areas (4,000 μm^2) were counted on each of five gill filaments from each fish. Averages of 10 areas were obtained from five fish per group. The greatest linear diameters of MR cells, considered to indicate the apical surface sizes of MR cells (Franklin, 1990), were also determined.

Preparation of gill homogenates

Homogenizing procedures were performed as described by Lin et al. (2006). Immediately after fish were killed by spinal pithing, the gill arches of the fish were excised and blotted dry. The gills were immediately scraped off from the underlying cartilage with a scalpel. All procedures were performed on ice. Ten microliters of proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 ml aprotinin) were added to 1 ml of homogenization medium. Homogenization was performed in 2-ml tubes with a Brinkmann polytron homogenizer (PT1200E, Kinematica, Lucerne, Switzerland) at maximum speed for 25 strokes. The homogenates were centrifuged at 4°C and 13,000×*g* for 20 min, and the supernatants were stored at –80°C. Protein concentrations were determined with the reagents of a BCA Protein Assay Kit (Pierce, Hercules, CA, USA) using bovine serum albumin (Pierce) as a standard.

Antibodies

A monoclonal antibody (α5) raised against the α-subunit of avian NKA was used for immunoblots and immunohistochemitry. It was purchased from the Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA).

Immunohistochemical detection of gill Na^+/K^+-ATPase (NKA)

Immunohistochemical staining procedures for NKA were modified from Lin et al. (2003). The first left gill arch of tilapia was removed, fixed in 4% (w/v) paraformaldehyde for 24 h at 4°C, dehydrated in ethanol, and embedded in paraffin. Serial sections (7 μm) were cut parallel to the long axis of the filament and mounted on slides coated with poly-L-lysine. Sections were stained with the primary antibody (α5) to the catalytic subunit of NKA followed by a commercial kit (PicTure™, Zymed, South San Francisco, CA, USA). The immunostained sections were counterstained with hematoxylin. Negative control experiments in which PBS was used instead of the primary antibody were conducted (data not shown) to confirm the positive results. To quantify the distribution of NKIR cells, longitudinal sections of the gills, including lamellae and the cartilages of the filaments, were chosen and the numbers of immunoreactive cells in the filaments (F) and lamellae (L) were counted. Lamellar areas were defined as the parts projecting from filaments. The interlamellar regions, including the regions between lamellar bases and filaments, were considered to be filament areas. For each sample, 10 areas on the filaments, including symmetrical lamellae, were randomly selected.

Immunoblots

Immunoblotting procedures were carried out according to Tang and Lee (2007) with little modification. Proteins from samples were heated with sample buffer at 37°C for 30 min. The pre-stain protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). All samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels (25 μg protein/lane). The separated proteins were then transferred to PVDF membrane (Millipore) by electroblotting. After preincubation for 3 hrs in PBST buffer containing 5% (w/v) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 2 hrs at room temperature with the primary antibody (α5) diluted in 1% (w/v) BSA and 0.05% (w/v) sodium azide in PBST, then washed in PBST and incubated at room temperature for 1.5 hrs with the

secondary antibody. Blots were developed after incubation with a BCIP/NBT Kit (Zymed). Immunoblots were photographed and imported as TIF files into the commercial image-analysis software package MCID version 7.0, rev. 1.0 (Imaging Research Inc., Ontario, Canada). Results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

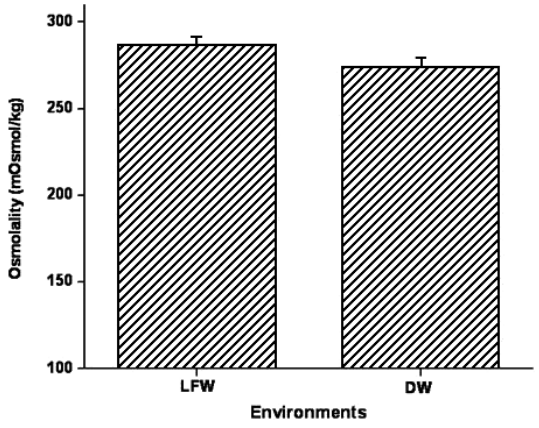

Fig. 1. Plasma osmolality in tilapia acclimatized to LFW or DW. No significant difference was found in plasma osmolality between LFW and DW. LFW, local fresh water; DW, deionized water.

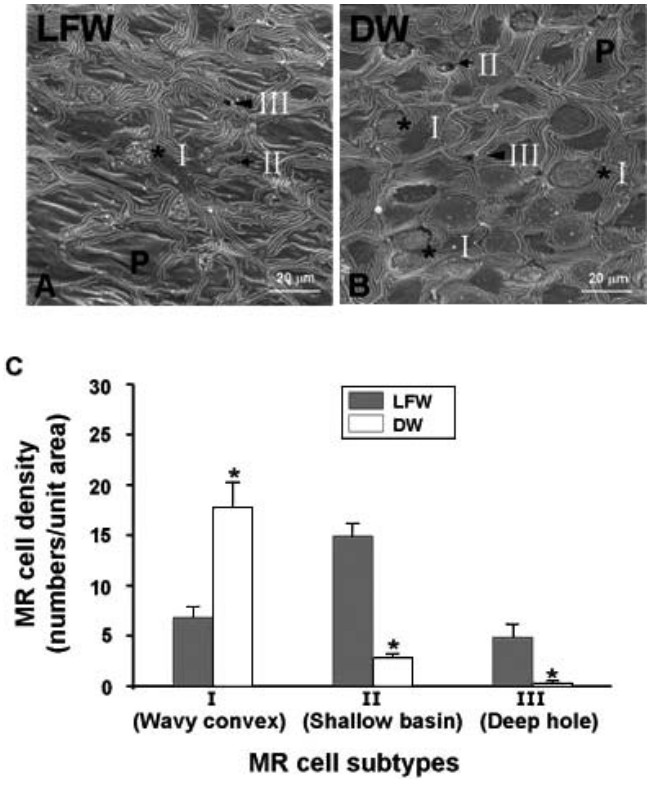

Fig. 2. Scanning electron micrographs of mitochondrion-rich (MR) cells in gill filaments of tilapia acclimatized to **(A)** LFW or **(B)** DW. Arrowheads, subtype-III MR cells; arrows, subtype-II MR cells; asterisks, subtype-I MR cells. Magnification 1500X. **(C)** Densities of different subtypes of MR cells in gill filaments of tilapia acclimatized to LFW or DW. Mean±SEM (N=4–6) is shown. The asterisks indicate significant differences of each subtype between different media. LFW, local fresh water; DW, deionized water.

Specific activity of NKA

Gill NKA 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 stopped by addition of 200 μl of ice-cold 30% (w/v) trichloroacetic acid. The inorganic phosphate concentration was determined by the method of Peterson (1978). The enzyme activity of NKA was defined as the difference between the inorganic phosphates liberated in the presence and in the absence of 5 mM ouabain in the reaction mixture.

Statistical Analyses

Statistical significance was determined by unpaired *t*-test ($P<0.05$) for group data analysis. Values were expressed as means±S.E.M.

RESULTS

Plasma osmolality

There was no significant difference in plasma osmolality between the local fresh water (LFW) and deionized water (DW) groups (Fig. 1).

Densities and apical sizes of different subtypes of mitochondrion-rich (MR) cells

The ultrastructures of gill MR cells of tilapia acclimatized to LFW or DW are shown in Fig. 2A and B. Density of

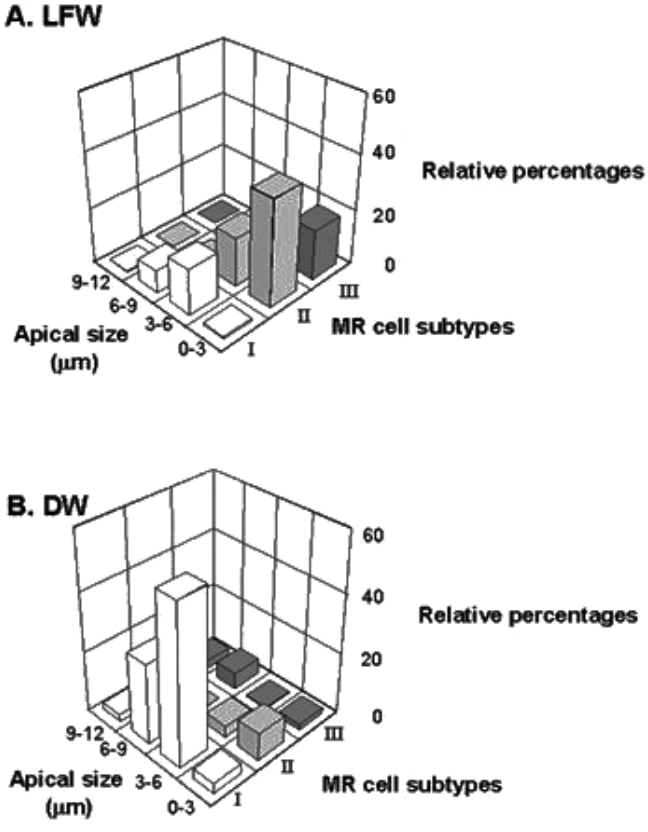

Fig. 3. Relative percentages and apical diameters (sizes) of different subtypes of MR cells in gill filaments of tilapia acclimatized to LFW and DW. The Y-axis of the figure is presented as percentages of subtypes of MR cells relative to total number of gill MR cells. LFW, local fresh water; DW, deionized water.

subtype-I (wavy-convex) MR cells in the DW group was significantly higher than that of the LFW group (Fig. 2C). In contrast, densities of subtype-II and -III MR cells in the DW group were less than in the LFW group (Fig. 2C).

Apical size, defined as the longest diameter of the apical opening of the MR cell, was used as an indicator of MR cell subtype. In the LFW group, the most abundant subtype population among the MR cells was subtype-II cells, with an apical size of less than 3 μm. Other populations of MR cells, including subtypes I and II with apical size of 3–6 μm and subtype III with an apical size of less than 3 μm, were also apparent (Fig. 3A). In DW tilapia gills, however, the most abundant subtype populations of MR cells were subtype-I cells with apical size of 3–6 or 6–9 μm (Fig. 3B).

Distribution and number of Na^+/K^+-ATPase immunoreactive (NKIR) cells

The distribution and number of NKIR cells in gills of LFW- and DW-acclimatized tilapia were visualized and counted using immunolocalization of the Na^+/K^+-ATPase (NKA) α-subunit on sections (Fig. 4). A representative micrograph of immunostaining shows that NKIR cells of LFW fish are distributed mainly in filaments (interlamellar regions) and basal regions of lamellae (Fig. 4A), while NKIR cells of DW tilapia are found in both filaments and lamellae (Fig. 4B). The number of NKIR cells in DW individuals was

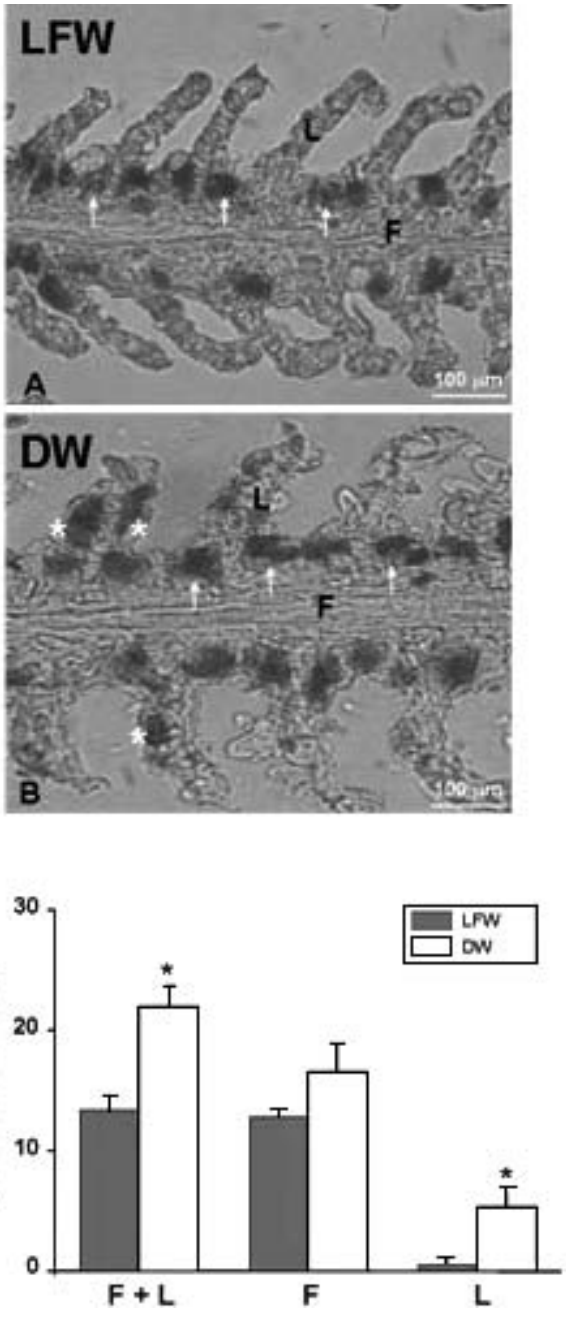

Fig. 4. Immunolocalization of Na^+/K^+-ATPase (NKA) in gills of tilapia acclimatized to **(A)** local fresh water (LFW) or **(B)** deionized water (DW). NKA immunoreactive (NKIR) cells in the filaments (arrows) and lamellae (asterisks) are indicated. Magnification × 300. **(C)** Density of NKIR cells in filaments and lamellae of tilapia transferred from local fresh water to deionized water. Mean±SEM (N=4–6) is shown. Asterisks indicate significant differences of all densities between different media. LFW, local fresh water; DW, deionized water; F+L, filaments and lamellae; F, filament; L, lamellae.

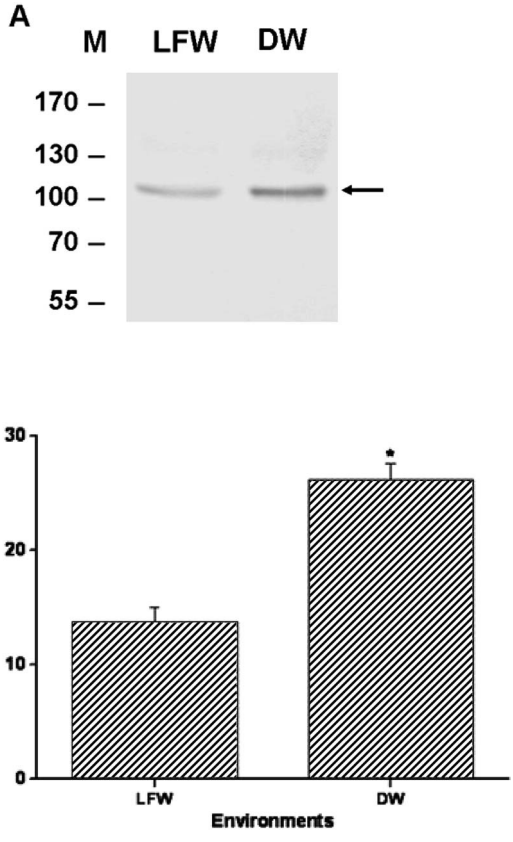

Fig. 5. (A) Representative immunoblot of tilapia gills probed with monoclonal antibody α5 to the NKA α-subunit. The immunoreactive bands have molecular masses centered at 105 kDa (arrow). The immunoreactive bands of DW-acclimatized fish are more intense than those of FW-acclimatized individuals. **(B)** Relative abundance of immunoreactive bands of NKA α-subunit in gills of different salinity groups (N=4). Expression of NKA α-subunit is 1.9 times as high in the DW group as in the FW group. The asterisk indicates a significant difference ($P<0.05$) by unpaired *t*-test. Values are means±S.E.M. M, marker; LFW, local fresh water; DW, deionized water.

significantly higher than that in the LFW group, due to the elevated number of NKIR cells on the lamellae (Fig. 4C).

Protein abundance and specific activity of NKA

Immunoblotting of homogenates of gill tissues from tilapia acclimatized to LFW or DW resulted in a single immunoreactive band of about 105 kDa (Fig. 5A). Quantification of immunoreactive bands between the two environmental groups revealed significant differences. Amounts of gill NKA protein were about 1.9 times as high in the DW-acclimatized fish as in the LFW group (Fig. 5B). Furthermore, the specific activity of gill NKA was significantly (3-fold) higher in the fish acclimatized to DW than in those acclimatized to FW (Fig. 6).

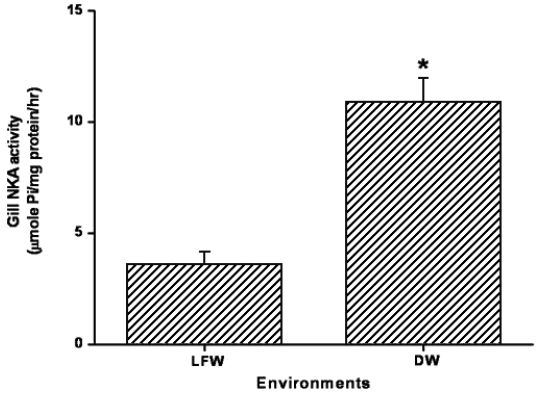

Fig. 6. Activity of branchial NKA in tilapia acclimatized to LFW or DW. Mean±SEM (N=4–6) is shown. The asterisk indicates a significant difference ($P<0.05$) by unpaired *t*-test. LFW, local fresh water; DW, deionized water.

DISCUSSION

Apical surfaces of gill mitochondrion-rich (MR) cells have been found to expand in salmonids acclimatized to ion-poor water or soft water (Laurent et al., 1994; Greco et al., 1996). In addition, the plasma osmolality of catfish (*Heteropneustes fossilis*) did not change for up to 14 days when DW was renewed only once a day; however, when DW was renewed four times a day for 25 days, a significant reduction in plasma osmolality was observed within 24 h (Parwez et al*.,* 1994). These studies indicate that modification in the morphology of MR cells is involved in physiological responses of teleosts to actively uptake ions from ambient diluted media to compensate for diffusive ion loss from plasma.

In this study, DW was utilized as ion-poor water to evaluate osmoregulatory responses of the Mozambique tilapia. Tilapia maintain constant osmolality in different environments (Lee et al., 2000, 2003), including DW (Fig. 1). Homeostasis of plasma osmolality could be achieved by an efficient capacity for active ion uptake from the external environment and enhanced ion reabsorption to compensate for ion loss. In the present study, the density of subtype-I MR cells increased when tilapia were acclimatized to DW (Fig. 2). Moreover, subtype-I MR cells with expanding apical surfaces became the predominant subpopulation of MR cells in gill filaments and lamellae of DW tilapia (Figs. 2C, 3B).

According to the ultrastructure of MR cell apical surfaces, Lee et al. (1996) first described three subtypes of MR cells in freshwater (FW) tilapia and named them, based on their external appearance, as the wavy convex (subtype I), shallow basin (subtype II), and deep hole (subtype III). In terms of physiological significance, FW MR cells with an expanding apical surface were supposed to result in changes in ion-uptake levels (Goss et al., 1992a). Moreover, MR cells with a large surface area were suggested to be in an active status (Lin and Hwang, 2004). Recent evidence obtained by manipulation of a single ambient ion level has illustrated the correlation between variable morphology and function in MR cells of tilapia (Chang et al., 2001, 2002, 2003; Chang and Hwang, 2004). Results of these studies demonstrated that the remodeling of MR cell subtypes led to changes in the capacity for ion uptake. The apical surface size of tilapia MR cell was demonstrated to be critical to the ion-uptake capacity. Different ranges of apical surface size, i.e., 0–4, 4–7, and >7 μm, of tilapia MR cells was proposed to be used as a reference index for MR cells that perform low-, middle-, and high-level ion-uptake, respectively (Chang et al., 2001, 2003). Taken together, this study suggests that euryhaline tilapia enhance the capacity for ion-uptake in ion-poor water by increasing the number of subtype-I MR cells with expanding apical surfaces.

Immunocytochemical studies on gill sections (reviewed by Wilson and Laurent, 2002) as well as biochemical studies on isolated MR cells (Kültz and Jürss, 1993; Wong and Chan, 1999) have demonstrated that these epithelial cells contained most of the Na^+/K^+-ATPase (NKA) activity as well as the protein abundance in fish gills. Dang et al. (2000) found that MR/NKIR cells were absent in gill lamellae of FW-acclimatized tilapia but present in lamellae of copper-exposed tilapia. Transfer to DW also led to the occurrence of lamellar NKIR cells in tilapia (Fig. 4B), similar to that in FW-acclimatized milkfish (*Chanos chanos*; Lin et al., 2003), Japanese Sea Bass (*Lateolabrax japonicus*; Hirai et al., 1999), and other FW-acclimatized euryhaline teleosts (Uchida et al., 1996; Sasai et al., 1998; Versamos et al., 2002). We attribute the significant elevation of total NKIR cell numbers in tilapia gills upon ion-poor shock to the appearance of lamellar NKIR cells (Fig. 4C). Lamellar MR/NKIR cells are thought to migrate from the germinal center, which is the area on the filamental epithelium which is immunoreactive for proliferating cell nuclear antigen (PCNA) (Dang et al., 1999). An increasing number of MR cells were found in rainbow trout (*Oncorhynchus mykiss*) acclimatized to ion-poor (soft) water (Greco et al*.,* 1996). Similarly, Sakuragui et al*.* (2003) reported that proliferation of MR cells was evident in DW-exposed erythrinid fish (*Hoplias malabaricus*). Lamellar MR/NKIR cells of tilapia that are in close contact with lamellar blood spaces might play an important role in active branchial ion absorption, as suggested in FW rainbow trout *Oncorhynchus mykiss* (Perry and Wood, 1985; Avella et al., 1987; Perry and Laurent, 1989).

Elevated MR/NKIR cell numbers in euryhaline teleosts are normally associated with an increase in NKA activity (Lin et al., 2006). NKA is a primary active transport pump that

provides the primitive driving force for ion transport. Since the first studies by Epstein et al. (1967) on killifish (*Fundulus heteroclitus*) and Kamiya and Utida (1968) on eels (*Anguilla japonica*), the significance of the role NKA plays in gill ion transport has been confirmed for other species (Marshall, 2002; Hwang and Lee, 2007). Previous studies have reported that different environmental salinities altered the protein amounts and specific activity of NKA in gills of tilapia (Lee et al., 2000, 2003). SW-acclimatized tilapia possessed about 2-fold higher protein abundance as well as specific activity of NKA in gills compared to FW-acclimatized fish (Lee et al., 2003). The protein abundance of gill NKA in DW-acclimatized fish was also increased compared to FW-acclimatized tilapia (Fig. 5). Meanwhile, significantly higher NKA activity in gills of DW tilapia (Fig. 6) could account for the enhanced ion-uptake capacity in DW-exposed fish observed in previous studies (McCormick 1995; Chang et al., 2001, 2003; Chang and Hwang, 2004). Taken together, a positive correlation between gill NKA protein abundance and specific activity was found in tilapia to change with the external environment (Lee et al., 2000; 2003).

To our knowledge, the present study is the first to integrate morphological modification of MR cells and the distribution, protein abundance, and specific activity of gill NKA in the osmoregulatory response of DW-exposed tilapia. It is reasonable to propose that homeostasis of plasma osmolality in DW-exposed tilapia can be maintained by utilization of enhanced gill NKA activity in the elevated number of MR cells with expanded apical surfaces to provide the greater driving force necessary for the efficient active uptake of ambient ions.

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