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Ambient Salinity Modulates the Expression of Sodium Pumps in Branchial Mitochondria-Rich Cells of Mozambique Tilapia, *Oreochromis mossambicus*

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ABSTRACT—Na,K-ATPase (sodium pumps) provide the primitive driving force for ion transport in branchial epithelial cells. Immunoblots of epithelial homogenates of both seawater (SW)- and freshwater (FW)-adapted tilapia gills as well as rat brain homogenate, a positive control, revealed one major band with a molecular weight of about 100 kDa. SW-adapted tilapia gills possessed larger (about 2-fold) amounts of sodium pumps compared with FW-adapted tilapia gills. ^{3}H-ouabain binding representing functional binding sites of Na,K-ATPase was also higher (about 3.5-fold) in gills of SW-adapted tilapia compared to that of FW-adapted fish. Moreover, specific activities of SW fish were higher (about 2-fold) than those of FW fish. Double labeling of Na,K-ATPase and Con-A, a fluorescent marker of MR cells, in tilapia gills followed by analysis with confocal microscopy showed that sodium pumps were localized mainly in MR cells, including the SW type and different FW types. Although more-active expression of Na,K-ATPase was demonstrated in gills of SW-adapted tilapia, no significant differences in densities of apical openings of MR cells were found between SW- and FW-adapted fish. These results indicate that, during salinity challenge, tilapia develop more "functional" Na,K-ATPase in SW-type MR cells to meet physiological demands.

Key words: salinity, Na,K-ATPase, gill, mitochondria-rich cells, teleost

INTRODUCTION

Gills are the most important extrarenal organ responsible for osmoregulation in fish. In the branchial epithelium, mitochondria-rich cells (MR cells, i.e., chloride cells) are the main site for exchange of ions which secrete ions in seawater (SW)-adapted fish and in freshwater (FW)-adapted fish, and absorb ions and maintain acid-base regulation (reviewed in Evans *et al*., 1999). MR cells are pleomorphic (see the review of Pisam and Rambourg, 1991), and MR cells with differing phenotypes are responsible for the transport of various ions (Tsai and Hwang, 1998; Lee *et al*., 2000; Chang *et al*., 2001). Variations in the morphology and number of MR cells thus reflect adaptive responses to particular environmental conditions.

Mozambique tilapia tolerate salinities of up to 120‰ (Stickney, 1986), and constitute a good model organism for studies on ionic and osmotic adaptations in teleost fishes. Branchial MR cells of tilapia change in density, size, and morphology (ultrastructure) in response to alterations in environmental salinities (Hwang, 1987; Kültz *et al*., 1995; Pisam *et al*., 1995; van der Heijden *et al*., 1997; Lee *et al*., 2000). In FW-adapted tilapia, scanning electron micrographs of gills revealed three types of MR cells with different features of the apical surfaces, i.e., wavy-convex (type I), shallow-basin (type II), and deep-hole (type III) (Lee *et al*., 1996; van der Heijden *et al*., 1997). Each type of MR cell was found to be dominant in media of particular ionic concentrations, and the relative abundances of the three cell types varied with the ionic composition of the water (Lee *et al*., 1996; Tsai and Hwang, 1998; Chang *et al*., 2001). Moreover, reversible changes in the morphology of MR cells occurred within a couple of hours following transfer to a new medium. On the other hand, in SW-adapted tilapia, only one type of MR cell with significant apical crypts similar to the type III (deep-hole) FW-MR cell was exhibited in the gill epithelium (van der Heijden *et al*., 1997; Lee *et al*., 2000). These deep-hole MR cells were also prominent in fish raised in water with higher Na^+/Cl^- concentrations (Lee *et al*., 1996), and they increased in number with environmental salinities (Lee *et al*., 2000). Type III MR cells were therefore correlated to SW-MR cells and are thought to be responsible

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for adaptation to high salinities. Using fluorescent-staining observations, comparison of branchial MR cell densities between SW- and FW-adapted tilapia revealed conflicting results: in SW, some reported that the density increased two- to three-fold (Kültz *et al.*, 1992; Uchida *et al.*, 2000), while others stated that the density decreased to two-thirds that found in FW (van der Heijden *et al.*, 1997).

Biochemical and morphological studies of teleostean gills have revealed a close relationship between MR cells and Na,K-ATPase (also known as the sodium pump) activity (reviewed in McCormick, 1995). The sodium pump is a widespread membrane-bound enzyme that provides a driving force for many transport systems in a variety of osmoregulatory epithelia including fish gills (McCormick, 1995). Na,K-ATPase is composed of a catalytic α-subunit with a molecular weight of about 100 kDa and a smaller glycosylated β-subunit with a molecular weight of approximately 55 kDa (Mercer, 1993). Immunocytochemical techniques have unequivocally demonstrated that Na,K-ATPase is located mainly in gill MR cells of SW- and FW-adapted tilapia (van der Heijden *et al.*, 1997; Lee *et al.*, 1998; Dang *et al.*, 2000; Uchida *et al.*, 2000).

Ouabain is a potent inhibitor of Na,K-ATPase which binds in a highly specific manner to the phosphorylated enzyme, and this binding is antagonized by potassium (Yoda and Yoda, 1988). [H^3]ouabain binding to gill homogenates and whole tissue has been used to measure the number of functional sodium pumps (McCormick, 1995). In mullet, the number of ouabain binding sites increases with increasing salinity (Hossler *et al.*, 1979). Autoradiographic methods using [H^3]ouabain have localized Na,K-ATPase to MR cells of the killifish (Karnaky *et al.*, 1976). Furthermore, a fluorescent derivative of ouabain, anthroylouabain, was demonstrated to localize functioning Na,K-ATPase in MR cells of tilapia transport epithelia (McCormick, 1990).

Na,K-ATPase activity is usually proportional to environmental salinities (McCormick, 1995). However, previous studies on salinity effects of branchial Na,K-ATPase activity of tilapia, like MR cell density, have produced dissimilar results. Some described increased activities with elevated external salinities (Dharmamba *et al.*, 1975; Dange, 1985; Hwang *et al.*, 1989; Kültz *et al.*, 1992; Morgan *et al.*, 1997; Uchida *et al.*, 2000), while others found no significant differences between SW- and FW-adapted tilapia (Verbost *et al.*, 1994; van der Heijden *et al.*, 1997; Nolan *et al.*, 1999).

Branchial sodium pump expression in the euryhaline Mozambique tilapia was obviously modulated by external salinities. Due to disparate results upon salinity challenge of the activity of branchial sodium pump and the number of epithelial MR cells in which the enzyme is concentrated, however, it is essential to verify these inconsistent consequences of previous studies. To validate salinity effects on the expression of the branchial Na,K-ATPase, several aspects including protein abundance, the numbers of functional sites, the specific activity, as well as localization in gill epithelium of FW- and SW-adapted tilapia were compared in the present study.

MATERIALS AND METHODS

Fish

Tilapia (*Oreochromis mossambicus*), 5.93±0.35 g body weight, were obtained from laboratory stocks. Fish were reared separately in aerated local tap water (FW: [Ca^{2+}], 0.20 mM; [Mg^{2+}], 0.10 mM; [Na^+], 0.20 mM; [K^+], 0.02 mM; [Cl^-], 0.18 mM), and seawater (SW: [Ca^{2+}], 11.90 mM; [Mg^{2+}], 57.20 mM; [Na^+], 419.90 mM; [K^+], 16.30 mM; [Cl^-], 520.84 mM) at 27–29°C with a daily 12-hr photoperiod for at least 4 weeks. The water was continuously circulated through fabric-floss filters and was partially refreshed every 3 days. Fish were fed a daily diet of commercial pellets.

Preparation of gill homogenates

Gill arches of fish from FW and SW were excised and blotted dry. The gill epithelia were immediately scraped off the underlying cartilage with a scalpel. All subsequent operations were carried out on ice. Gill scrapings (0.9–1.2 g wet wt) were suspended in 3 ml of homogenization solution according to Hwang *et al.* (1989) (100 mM imidazole-HCl buffer, pH 7.6; 5 mM Na_2EDTA; 200 mM sucrose; 0.1% sodium deoxychlolate). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a motorized Teflon pestle at 600 rpm for 20 strokes. The homogenate was then centrifuged at 6000 x*g* at 4°C for 10 min. Protein concentrations of the supernatant were determined with reagents of the Bio-Rad Protein Assay Kit using bovine serum albumin as a standard.

Antibodies

A mouse monoclonal antibody, α5, raised against the α-subunit of the avian sodium pump (Takeyasu *et al.*, 1988) and purchased from the Developmental Studies Hybridoma Bank was used in the present study. The secondary antibody for immunoblotting was alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce), while for immunofluorescent staining, it was FITC-conjugated goat anti-mouse IgG (Jackson).

Immunoblotting

The immunoblotting procedures were carried out as described by Lee *et al.* (2000) with little modification. Proteins within the homogenates were fractionated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels (100 μg of protein/lane), except that the homogenates were heated to 37°C for 5 min rather than to higher temperatures. Rat brain microsomes (Upstate Biotechnology) were used as a positive control for immunoblotting. The separated proteins were then transferred to PVDF membranes (Millipore) by electroblotting. After preincubation for 2 hr h in PBST buffer containing 1%-2% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 1 hr in primary antibody (α5) diluted in 5% BSA (1:1000 dilution), washed in PBST, and reacted for 30 min with secondary antibody (1:1000 dilution). Blots were developed after incubation with 0.015% nitroblue 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). Immunoblots were scanned and imported as JPG files into a commercial software package (Image-Pro Plus 1994). The finished images were interpreted numerically to show the relative intensities of the immunoreactive bands.

Ouabain binding

The assay of ouabain binding followed previous methods (Hwang *et al.*, 1999) with some modifications. Gill epithelia were prepared as described above except a different homogenization medium (25 mM Tris-HCl, 0.25 mM sucrose, 2 mM Na_2EDTA, protease inhibitors; pH 7.5) was used. Supernatant of gill epithelia was

added separately into the reaction medium (3 mM ATP, 6 mM $MgCl_2$, 100 mM NaCl and 50 mM Tris-HCl; pH 7.5) as a total binding reaction and non-specific binding medium (the reaction medium plus 5 mM Na_2 EDTA and 6.25 mM KCl) as a non-specific binding reaction. ^{3}H-labelled ouabain (10 pM/reaction, 40 Ci/mmol from Amersham) was added to the above reactions just before incubation. The total concentration of ouabain (cold plus hot) in the total binding reaction was 10 pM to 10 μM. For the non-specific binding reaction, 1×10^{-3} M ouabain was added to each concentration of ouabain in the total binding reaction. Assays were carried out in triplicate and incubated at 37°C for 1 hr. The reaction was stopped by chilling on ice, and the reacted product was collected on polyethylenimine-pretreated glass microfiber filters (GF/B, Whatman, USA) by vacuum filtration (sampling manifold 1225, Millipore) to separate bound and free ^{3}H-ouabain. The filter was mixed with Cocktail T (BDH), kept at room temperature overnight, and then counted in a β-counter (1211 Rackbeta, LKB). Protein concentrations of samples were determined with a protein assay kit (Bio-Rad) using bovine serum albumin as a standard. The specific binding (saturable) was obtained from total binding minus non-specific binding. The maximum binding of ouabain (B_{max}) was calculated by computer analysis of the saturation binding.

Activity of the sodium pump

Gill Na,K-ATPase activity was determined as described by Hwang *et al.* (1989). Aliquots of the suspension of gill homogenates prepared as described above were used for determination of protein and enzyme activities. Na,K-ATPase activity was assayed by adding the supernatant to the reaction mixture (500 mM imidazole-HCl buffer, pH 7.6, 625 mM NaCl, 750 mM KCl, 37.5 mM $MgCl_2$, 25 mM Na_2ATP). 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 37.5 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

Scanning electron microscopy (SEM)

Excised gill filaments were processed as described in Lee *et al.* (1996). In brief, tissues were fixed at 4°C in phosphate-buffered 4% paraformaldehyde plus 5% glutaraldehyde (pH 7.2) for 12 hr and then in 1% osmium textroxide (pH 7.2) for 1 hr at 4°C. Tissues were dehydrated in ascending concentrations of ethanol from 50% to absolute, then in 100% acetone, and were dried using a Hitachi HCP-2 critical-point drier. After sputter-coating with a gold-palladium complex for 3 min using an Eiko 1B-2 vacuum evaporator, specimens were examined with a Hitachi S-2500 scanning electron microscope. Densities of MR cells were also counted using SEM as described by Lee *et al.* (1996).

Transmission electron microscopy (TEM)

For TEM, gill filaments were fixed and dehydrated as described previously, then infiltrated and embedded in LR-white resin. Sections were cut with glass knives in an Ultracut E ultramicrotome (Reichert-Jung). Ultrathin gray sections were mounted on formvar-coated copper grids, double stained in uranyl acetate and lead citrate, and examined with a Hitachi H-7000 electron microscope at 75 kV.

Confocal laser scanning microscopy

Concanavalin A (ConA), a marker of apical crypts of MR cells (van der Heijden *et al.*, 1997) was combined with the monoclonal antibody, α5, for double-labeling of whole-mount preparations of gill filaments according to Lee *et al.* (2000). By using a Bio-Rad MRC600 confocal laser scanning microscope equipped with an argon laser (488 and 514 nm) for excitation and attaching the apparatus to a Nikon microscope, stained images of the Na,K-ATPase α-subunit were obtained with the use of an A1 (BP525-555 nm) filter set, and the ConA images were taken using an A2 (LP600 nm) filter set. With this setup, the emission wavelengths of antibody-FITC conjugate and ConA-Texas red conjugate were separated and transmitted to different multipliers. Pictures from each photomultiplier were subsequently merged in false color to simultaneously visualize the labels. For identifying different types of MR cells with differing apical openings, consecutive optical sections ('z-scans', 0.5 μm thick, 1 μm apart) were made in a plane parallel to the mucosal surface of the filament and to a depth of one cell layer. Merged z-scans clearly discriminated three types of MR cells according to the features of ConA-positive regions (i.e., with apical openings). Negative control experiments, in which normal goat serum was used instead of the primary antibody, were conducted (data not shown) to confirm the above positive results.

Statistics

Values were compared using Student's *t*-test, and $p<0.05$ was set as the significance level. Values are presented as the mean±S.E.M. unless stated otherwise.

RESULTS

Immunoblotting of the sodium pump

The immunoreactive bands of the Na,K-ATPase α-subunit were identified as having relative molecular masses of about 100 kDa in gill epithelia of both seawater (SW)- and freshwater (FW)-adapted tilapia. Rat brain microsomes were used as the positive control. These immunoreactive bands in SW fish were obviously darker and thicker than those of the FW fish (Fig. 1). Based on image analysis, the amounts of the sodium pump α-subunit in SW fish were about two-fold higher than those of FW fish (389.27±55 vs. 177.35±41 in arbitrary unit; n=4 of each group) (Fig. 2).

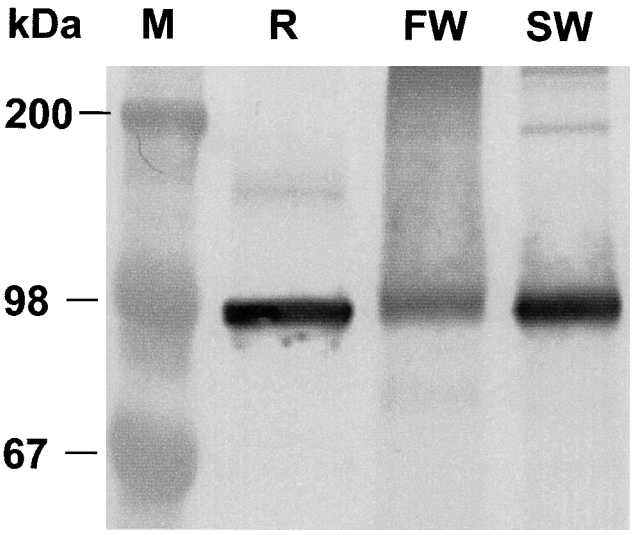

Fig. 1. Immunoblot of the Na,K-ATPase α-subunit from gill epithelium of tilapia adapted to fresh water (FW) or seawater (SW) revealing a single immunoreactive band corresponding to a molecular mass of about 100 kDa. R, rat brain microsomes as the positive control. M, marker. The immunoreactive band from SW fish was more intense than the band from FW fish.

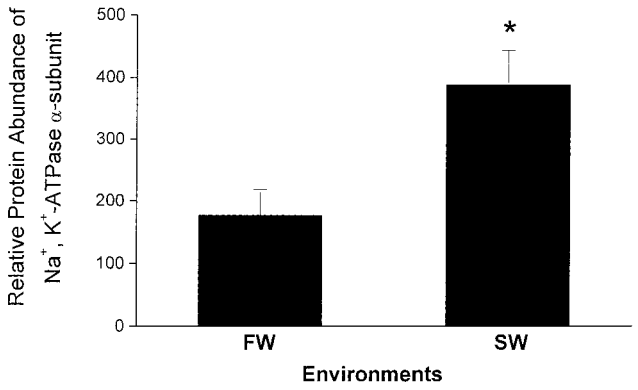

Fig. 2. Relative abundance of the Na,K-ATPase α-subunit protein expressed in gills of tilapia adapted to fresh water (FW) or seawater (SW). The amount of the sodium pump α-subunit in SW fish was about two-fold that of FW fish. An asterisk (*) indicates a significant difference between the FW and SW groups ($p<0.05$).

Ouabain binding and activity of the sodium pump

Binding of ^{3}H-ouabain in SW-adapted tilapia was significantly higher than that in FW-adapted fish (6.82±0.75 vs. 1.99±0.17 pmole·mg^{-1} protein; n=8 of each group) (Fig. 3a).

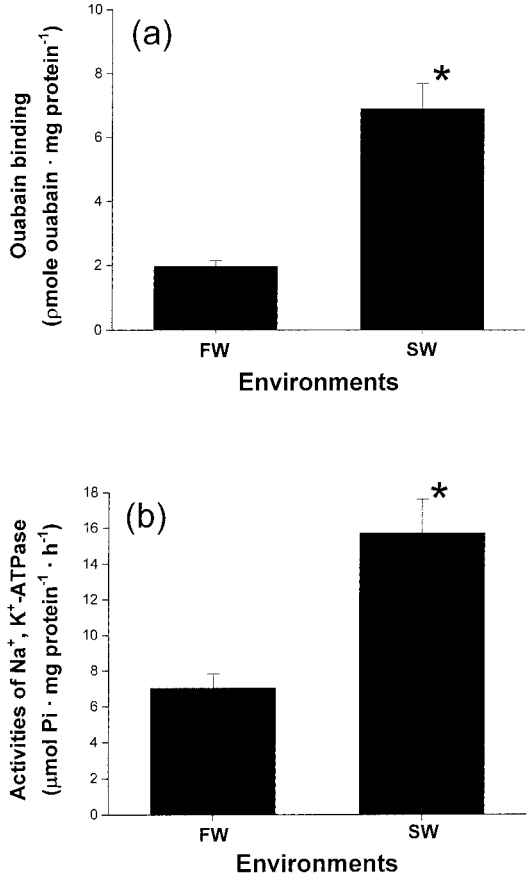

Fig. 3. Comparison of Na,K-ATPase activity and ouabain binding in gills of tilapia adapted to fresh water (FW) or seawater (SW). Both ouabain binding (a) and the activity of the sodium pump (b) were significantly higher (about two- to three-fold) in SW than in FW fish. An asterisk (*) indicates a significant difference ($p < 0.05$).

The data indicated that branchial Na,K-ATPase of SW tilapia contained more (about three- to four-fold) functional binding sites than did that of FW fish. Moreover, the specific

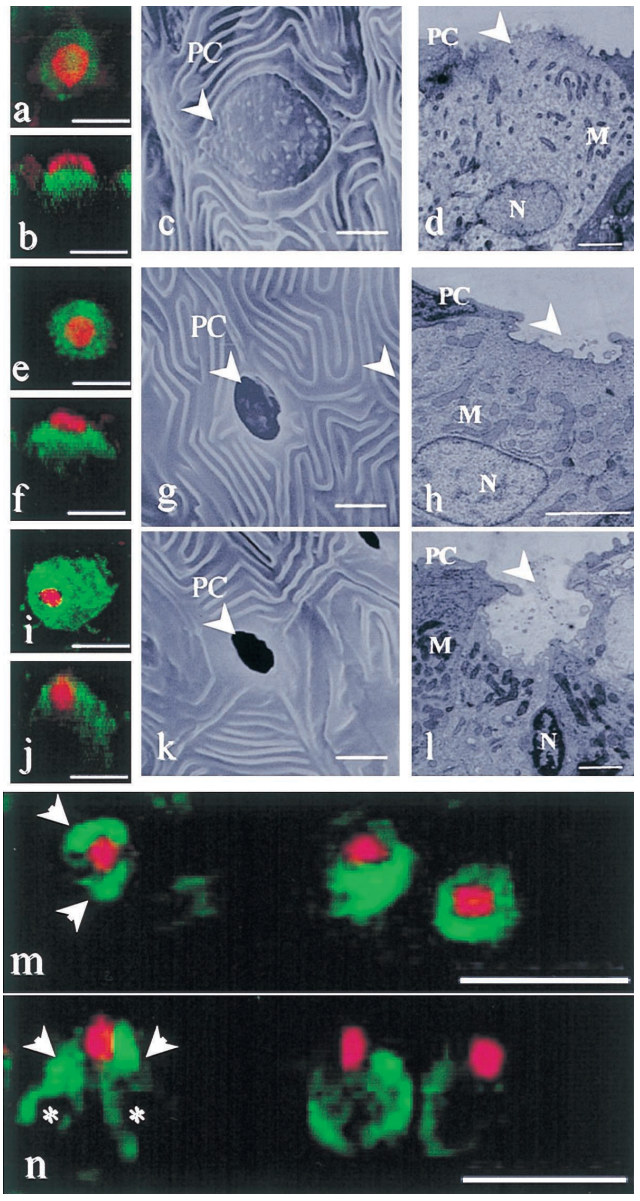

Fig. 4. SEM, TEM, and confocal micrographs of different types of mitochondrial-rich (MR) cells in tilapia gill epithelium. In freshwater (FW) fish, there were three types of MR cells according to the morphological features of apical openings: wavy convex of type I (a–d), shallow basin of type II (e–h), and deep hole of type III (i–l). Arrowheads in the SEM (c, g, k) and TEM (d, h, l) micrographs indicate the apical openings of MR cells. PC, pavement cells; N, nucleus; M, mitochondria. In the confocal micrographs, Texas-red-conjugated ConA clearly marked the apical openings of the three types of MR cells co-labeled with the sodium pump. 'Xy-scans' (a, e, i) and 'z-scans' (b, f, j) show features corresponding to the SEM and TEM observations, respectively. In seawater (SW) fish, all MR cells are identical (i.e., type III. m, n). The 'xy-scan' (m) and 'z-scan' (n) revealed that the apical crypt might be the opening of one single MR cell or a multicellular complex consisting of two adjacent MR cells as indicated by the arrowheads. An asterisk (*) indicates the nuclei of MR cells.

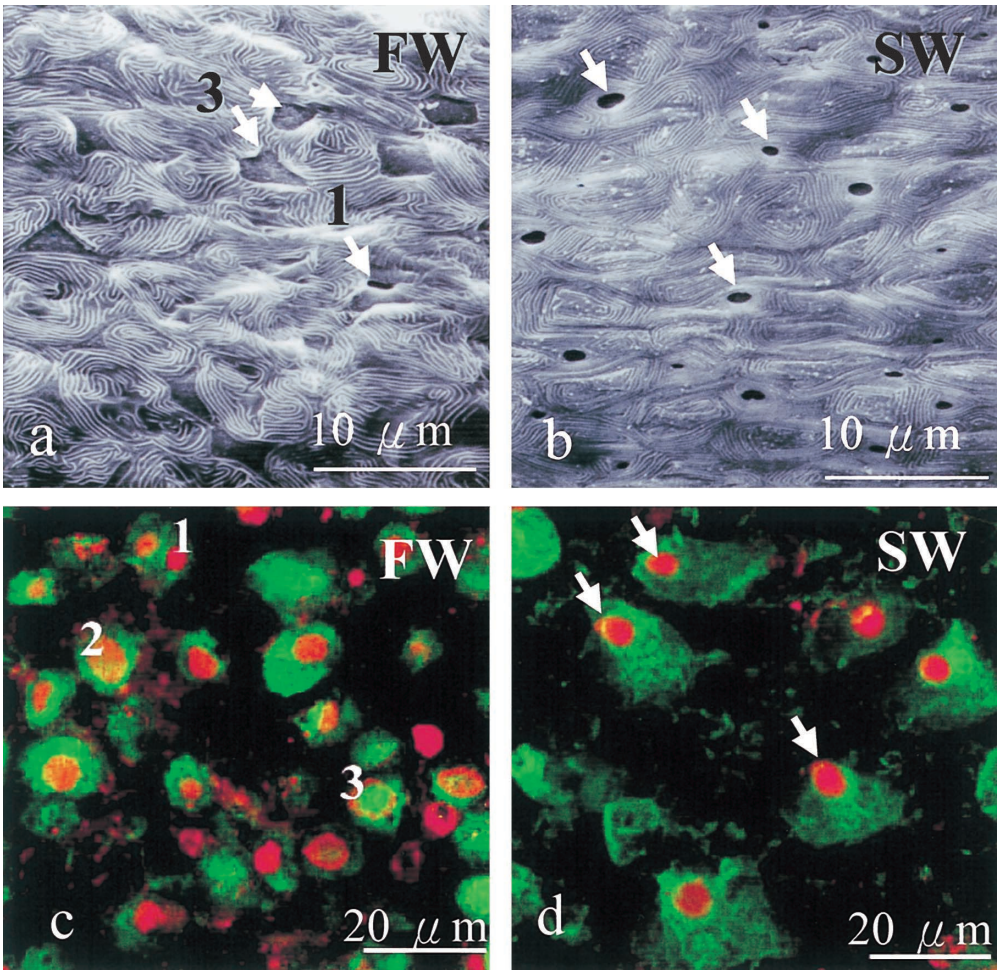

Fig. 5. SEM (a, b) and confocal (c, d) micrographs of gill epithelia revealing different types of MR cell apical openings indicated by 1, 2, or 3 in freshwater (FW)-adapted tilapia (a, c) and the apical openings (crypts) of mitochondria-rich (MR) cells in seawater (SW)-adapted tilapia (b, d) as indicated by the arrows.

activity of gill Na,K-ATPase in SW tilapia was significantly higher, about two-fold, than that in FW tilapia (15.72±1.88 vs. 7.02±0.80 μmol Pi · mg protein^{-1} · h^{-1}; n=8 of each group) (Fig. 3b).

Different types of gill MR cells

Lee *et al.* (1996) described three types of MR cells with various morphological features of apical surfaces: wavy-convex of type I, shallow-basin of type II, and deep- hole of type III. Confocal micrographs of co-labeling with ConA, a marker of MR cells, and an antibody to the Na,K-ATPase α-subunit demonstrated that these three types of MR cells exhibited abundant sodium pumps (Fig. 4a–d, type I; e–h, type II; i–l, type III) and thus were Na,K-ATPase immunoreactive (NKIR). In gills of SW-adapted tilapia, sometimes more than one NKIR cell (MR cells) was observed to share one apical crypt (Fig. 4 m and n, the apical crypt was indicated as staining positive for ConA) and formed a "multicellular complex" (Hwang, 1987; van der Heijden *et al.*, 1997).

Changes in phenotypes and densities of MR cells

SEM micrographs showed that in FW, the gill epithelium of tilapia exhibited different types of MR cell apical surfaces (types I–III; Fig. 5a), but in SW only one type of MR

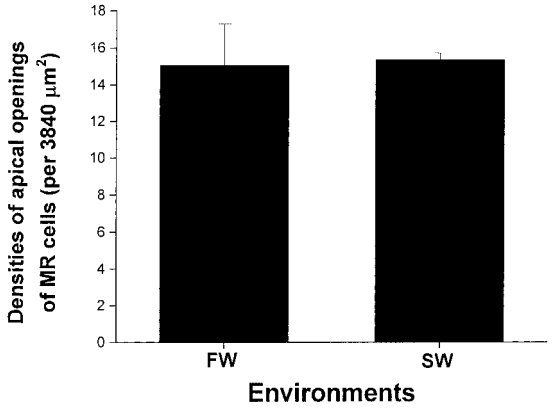

Fig. 6. Densities of apical openings of all types of branchial mitochondria-rich cells in tilapia. No significant difference was found between FW- and SW-adapted fish.

cell (type III) was found in gill epithelium (Fig. 5b). Corresponding staining of ConA and the sodium pump further revealed that although there were three types of FW MR cells and only one type of SW MR cells, those SW MR cells were larger in size than all types of FW MR cells (Fig. 5c and d). On the other hand, by SEM observation, no significant difference was found in densities of apical-surface MR cells in FW- and SW-adapted tilapia (Fig. 6; 15.05±2.25 vs. 15.38±0.34 per 3840 μm^2 of gill epithelium; n=5 of each group).

DISCUSSION

Present in all animal cells, Na,K-ATPase, an energy-dependent, ion-translocating enzyme, occurs in high concentrations in most transport epithelia; up to 10^8 sodium pumps might be present in a single MR cell (Karnaky, 1986). Na,K-ATPase plays a central role in the ion-transport function of MR cells through direct movement of sodium and potassium across the plasma membrane or indirect generation of ionic and electrical gradients. In general, Na,K-ATPase activity increases when euryhaline teleosts are transferred from FW to SW (McCormick, 1995). Gill ouabain binding and Na,K-ATPase activity are strongly correlated (McCormick, 1995). Gill Na,K-ATPase activity and ouabain binding increased two- to five-fold after adaptation of the eel to seawater (Kamiya, 1967; Sargent and Thomson, 1974). The present study demonstrates that in the euryhaline tilapia, *Oreochromis mossambicus*, raising environmental salinities resulted in increased abundance of the sodium pump protein, elevated levels of Na,K-ATPase activity, as well as a rising number of ouabain binding sites indicative of the number of sodium pump functional sites (Figs. 1–3). Although the results noted above contrast with some studies which describe no differences in sodium pump activities between SW- and FW-adapted tilapia (Verbost *et al*., 1994; van der Heijden *et al*., 1997; Nolan *et al*., 1999), similar changes in Na,K-ATPase activities in gills of tilapia with varying environmental salinities were reported by several other studies (Dange, 1985; Hwang *et al*.,1989; Kültz *et al*., 1992; Morgan *et al*., 1997; Uchida *et al*., 2000). Moreover, our previous experiments demonstrated that in tilapia, the abundance of sodium pump α-subunit mRNA was proportional to the external salinity (Hwang *et al*., 1998). Taken together, it is obvious that upon salinity challenge, the expression of branchial sodium pumps in tilapia increases from gene to functional protein to meet physiological demands and adapt to changes in the environment.

Due to their ionoregulatory roles in gill epithelium, functional MR cells should be in contact with both water (via the apical surface) and blood (via the basolateral membrane) (Zadunaisky, 1984). Moreover, the morphology of apical openings of MR cells changes in response to salinity variations as described above. Owing to these features, scanning electron microscope (SEM) has been used to identify or quantify functional MR cells including FW and SW types in numerous euryhaline species such as mullet (Hossler *et al*., 1979), killifish (Hossler *et al*., 1985), striped bass (King and Hossler, 1991), sea trout (Brown, 1992), tilapia (Kültz *et al*., 1995), and lampreys (Bartels *et al*., 1996). Generally on scanning electron micrographs, FW-type MR cells show flat or slightly invaginated surfaces like membrane patches with short cellular projections on them, while the SW type exhibits deeply invaginated surfaces with smaller orifices. Also using SEM, the total density of apical openings of MR cells in FW and SW tilapia in the present study revealed no significant differences (Fig. 6), although others have found an increase in MR cell number following seawater adaptation in this species by staining with a mitochondria fluorescent dye, DASPMI (Kültz *et al*., 1992, 1995). Since an abundance of mitochondria is found in mature (functional) MR cells with apical openings as well as in accessory, immature, or possibly in early degenerating MR cells without apical openings, it is likely that DASPMI labels most subtypes which would lead to an overestimation of the density of functional MR cells. On the other hand, van der Heijden *et al.* (1997) pointed out the possibility of underestimation of functional MR cells by SEM due to the presence of multicellular complexes. The ultrastructurally multicellular complex, however, still shares the same apical openings and could be thought as one functional unit at the cellular level. Hence, SEM analysis not only allows for the observation of morphological changes but also provides an estimation of the density of functional MR cells in tilapia gill filaments on the basis of the number of apical openings.

According to SEM observations, Lee *et al.* (1996) first described three types of MR cell apical surfaces in FW tilapia and named these types based on their external appearances: wavy convex (type I), shallow basin (type II), and deep hole (type III). Moreover, only one type of MR cell (deep hole) was found in SW fish, and this type of MR cells increased in density with elevated salinities (Lee *et al*., 2000). Similar results were presented by van der Heijden *et al.* (1997) who showed three types of apical pits (types I–III) in FW tilapia and only one type (type III) in SW fish. The present study using immunofluorescent staining further demonstrates that these types of MR cells, even the multicellular complex, are all Na,K-ATPase-immunoreactive (NKIR) cells whose apical openings have different morphologies (Fig. 4). The finding that the density of total functional MR cells (i.e., NKIR cells) was unchanged (Figs. 5 and 6), while the abundance of Na,K-ATPase α-subunit protein (Fig. 2) as well as the specific activity and the number of functional sites of Na,K-ATPase (Fig. 3) increased in seawater, suggests that the amount of Na,K-ATPase per cell had increased. This is consistent with the increased size (not the size of the apical surface) of MR cells in seawater (Fig. 5c and d) (Kültz *et al*., 1992; van der Heijden *et al*., 1997; Uchida *et al*., 2000), which is accompanied by expansion of the tubular system in those cells where the branchial Na,K-ATPase is located (Hwang, 1987; Lee *et al*., 1995; Dang *et al*., 2000; Uchida *et al*., 2000). Three-dimensional analysis

of corresponding fluorescent images of NKIR cells in tilapia gill filaments which represent the intensity of the immunoreaction for the sodium pump increased with elevated environmental salinities (Uchida *et al*., 2000) providing additional direct evidence that in SW-type MR cells, Na,K-ATPase is expressed more actively than in FW-type MR cells.

In summary, gill Na,K-ATPase expression in euryhaline tilapia was modulated by environmental salinity. Mature (functional) SW- and FW-type MR cells differed in both the morphology of the apical openings as well as the sodium pump contents per cell. Obviously, upon salinity challenge, functional MR cells changed into the SW type containing more Na,K-ATPase, thus driving a series of ion transporting systems to meet physiological requirements. Future studies will focus on the salinity effects on MR cell turnover.

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