Time-Course Changes in the Expression of Na, K-ATPase and the Morphometry of Mitochondrion-Rich Cells in Gills of Euryhaline Tilapia (*Oreochromis mossambicus*) During Freshwater Acclimation

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ABSTRACT   Changes in expression of Na, K-ATPase (NKA) and morphometry of mitochondrion-rich (MR) cells in gills of tilapia were investigated on a 96–hr time course following transfer from seawater (SW) to fresh water (FW). A transient decline in plasma osmolality and Na^+, Cl^- concentrations occurred from 3 hrs onward. Gills responded to FW transfer by decreasing NKA activity as early as 3 hrs from transfer. This response was followed by a significant decrease in the NKA isoform $\alpha1$–mRNA abundance, which was detected by real-time PCR at 6 hrs post transfer. Next, a decrease of $\alpha1$–protein amounts were observed from 6 hrs until 24 hrs post transfer. Additionally, during the time course of FW transfer, modifications in number and size of subtypes of gill MR cells were observed although no significant difference was found in densities of all subtypes of MR cells. These modifications were found as early as 3 hrs, evident at 6 hrs (exhibition of 3 subtypes of MR cells), and mostly completed by 24 hrs post transfer. Such rapid responses (in 3 hrs) as concurrent changes in branchial NKA expression and modifications of MR cell subtypes are thought to improve the osmoregulatory capacity of tilapia in acclimation from hypertonic SW to hypotonic FW. *J. Exp. Zool. 301A:85–96, 2004.*   © 2004 Wiley-Liss, Inc.

INTRODUCTION

Euryhaline teleosts inhabit environments ranging from fresh water (FW) to seawater (SW). Through effective mechanisms of osmoregulation, teleosts are able to retain an osmotic and ionic constancy in their internal milieus and survive in hypertonic SW or hypotonic FW. Gills are the most important extrarenal organs responsible for osmoregulation in fish. Epithelial mitochondria-rich (MR) cells are the main site for active transport of ions in gills. On the basis of studies of MR cell localization and dynamics in relation to changes in environmental salinity, it has been suggested that there are distinct MR cell subtypes and populations that are important in ion uptake and ion extrusion (Marshall, 2002; Wilson and Laurent, 2002). In the gill, immunocytochemical data provide evidence that Na, K-ATPase (NKA) is located mainly in epithelial MR cells (Wilson and Laurent, 2002). The number and size of MR cells as well as expression of branchial NKA in diadromous teleosts, i.e., salmonids (salmon), and anguillids (eel), normally increases concurrently with external salinity (Marshall, 2002).

The membrane-spanning enzyme NKA is responsible for the active transport of 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. NKA is a P-type ATPase consisting of an $(\alpha\beta)2$ protein complex. Four α (α 1–4) and three β (β 1–3) isoforms as well as a small γ subunit have been found in mammals and birds (Blanco and Mercer, '98). It has been suggested that tissue specific expressions of different isoforms are associated with various physiological functions. Among the three isoforms, α one that is predominantly expressed in the transporting epithelia in higher vertebrates functions primarily in a housekeeping capacity to maintain osmotic balance and cell volume regulation, whereas the other α -subunits fulfill more specialized requirements for cation transport necessary for differentiated cell-specific functions (Blanco and Mercer, '98). High levels of homology prevail among all the α -subunit sequences identified in animal species ranging from *Drosophila*, *Artemia* to vertebrates (Vasilets and Schwarz, '93), and including teleosts (Schönrock et al., '91; Culter et al., '95; Seidelin et al., 2001; Feng et al., 2002; Semple et al., 2002).

The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline cichlid that tolerates salinities of up to 120‰ (Stickney, '86). As in the salmon and the eel, tilapia branchial NKA expression, including the levels of mRNA and protein as well as its activity, is proportional to environmental salinities (Morgan et al., '97; Hwang et al., '98; Lee et al., 2000, 2003; Uchida et al., 2000). Gill NKA was observed mainly in epithelial MR cells of SW- and FW-adapted tilapia (Dang et al., 2000; Lee et al., 2000, 2003; Uchida et al., 2000). No significant difference was found in densities of MR cells in gills of SW- and FW-adapted fish (Lee et al., 2003). However, in FW-adapted tilapia, different subtypes of MR cells, i.e., wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III), were identified according to their features of the apical surfaces observed by scanning electron microscope (Lee et al., '96; van der Heijden et al., '97). Each subtype of FW-MR cell was found to be dominant in media of certain ionic concentrations, and the relative abundances of the three subtypes varied with the ionic composition of the water (Tsai and Hwang, '98; Lee et al., '96, 2000, 2003; van der Heijden et al., '97; Chang et al., 2001). Moreover, reversible changes in the morphology of MR cells were found within a few hours following transfer to a new environment (Lee et al., '96). On the other hand, in SW-adapted tilapia, only one type of MR cell with significant apical crypts

similar to the subtype III (deep-hole) FW-MR cell was exhibited in the gill epithelium (van der Heijden et al., '97; Lee et al., 2000, 2003). These deep-hole MR cells were also prominent in fish raised in water with higher Na^+ and Cl^- concentrations (Lee et al., '96), and increased in number with increasing environmental salinities (Lee et al., 2000). The subtype III FW-MR cells were therefore correlated to SW-MR cells and are thought to be responsible for adaptation upon salinity challenge.

Although the Mozambique tilapia is a euryhaline species, it dies during the initial critical phase of SW (35‰) acclimation but survives in SW with a pre-acclimation to 20‰ brackish water (BW) for 24 hours (Hwang, '87). The process of pre-acclimation allows tilapia to develop the osmoregulatory mechanisms including SW-MR cells and enhanced NKA activity to prevent serious dehydration in SW (Hwang, '87; Hwang et al., '89). In our preliminary study, however, SW-adapted tilapia was found to survive successfully in FW without pre-acclimation to BW. It is intriguing to verify the time-course alterations of ionoregulatory mechanisms in euryhaline tilapia from ion secretion in hypertonic SW to ion absorption in hypotonic FW. The present work using SW-adapted tilapia was thus performed to evaluate the time course leading to modifications in gill NKA activity after direct transfer to FW. Changes in the epithelial NKA isoform α1-mRNA levels and α1-protein variations were analyzed. Furthermore, concurrent modifications of densities and size frequencies of branchial MR cell subtypes were examined using the scanning electron microscope.

MATERIALS AND METHODS

Animals

The tilapia (*Oreochromis mossambicus*) obtained from laboratory stock were 10.2 ± 0.8 cm in total length. Seawater (SW) used in the present work was made of local tap water with proper amounts of synthetic sea salt added, (Instant Ocean, Aquarium Systems Co., Mentor, OH). Tilapia were reared in SW at 27–29 °C with a daily 12 hr photoperiod for at least one month before experiments. The water was continuously circulated through fabric-floss filters. Fish were fed a daily diet of commercial pellets ad libitum.

Freshwater adaptation

Preliminary tests of survival rate on FW acclimation were conducted first: (1) SW tilapia were directly transferred to FW, and (2) SW tilapia were exposed to 15 % saltwater for 6, 12, or 24 hrs, respectively, and then transferred to FW. Fish in the control group were treated identically to the FW-transfer animals but underwent a transfer from SW to SW. Tilapia survived in all tests. Hence, in this study, tilapia raised in SW ($[Na^+]$, 582.86 mM; $[K^+]$, 10.74 mM; $[Ca^{2+}]$, 15.75 mM; $[Mg^{2+}]$, 32.92 mM; $[Cl^-]$, 520.84 mM) were sampled after direct transfer to FW ($[Na^+]$, 2.6 mM; $[K^+]$, 0.04 mM; $[Ca^{2+}]$, 0.58 mM; $[Mg^{2+}]$, 0.16 mM; $[Cl^-]$, 0.18 mM) at 0, 3, 6, 12, 24, 48, and 96 hrs, respectively. Fish in the control group were moved from SW to SW and sampled with a time-course identical to the transfer group. Gills and blood were sampled for the following biochemical analysis and morphological observations. For sampling, the fish were anesthetized with ethylene glycol monophenyl ether (0.3ml/l; Merck) and then killed by a blow to the head.

Blood analysis

Fish blood was collected from the caudal veins using heparinized 1 ml syringes and 27 G needles and then injected into heparinized 0.5ml centrifuge tubes. After centrifugation at 1,000 g, 4°C for 10 min, the plasma was stored at $-20°C$ before analysis. Plasma osmolality was assessed with a WESCOR 5520 VAPRO Osmometer (Logan, Utah, USA). $[Na^+]$ was measured with a Hitachi Z–8000 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan). $[Cl^-]$ was evaluated by the Ferricyanide method (Franson, '85) using a Hitachi U–2001 spectrophotometer (Tokyo, Japan).

Oligonucleotide primers design

The Na, K-ATPase (NKA; GenBank Accession No. AAD11455) and GAPDH (glyceraldehydes 3–phosphate dehydrogenase) (unpublished observations) cDNA sequence was evaluated using the Primer Express software (Perkin–Elmer). Oligonucleotides were designed by the software. α1 isoform gene specific primer sequences were as follows (5' to 3'): TG3, GCCAACTGCCACACAGA CAC, and TCCCAATAAAACATTTCCACTG; GAP DH, CATCGAGGAGGCACTCATGA, and TCAAT GGTAAGCTGACAGGCAT (Hwang et al., '98).

Quantitation of NKA cDNA and real-time PCR

NKA α1–subunit mRNA was quantified by real-time PCR (ABI PRISM 5700 Sequence Detection System (SYBR green) real-time quantitative PCR). The principle and mRNA quantitation of real-time PCR is referred to Johnson et al. (2000). For each unknown sample the corresponding NKA and GADPH values were read using linear regression analyses from their respective standard curves (data not shown). A relative NKA expression value was then obtained by division of the NKA value by the GAPDH value.

NKA antibody

The mouse monoclonal antibody 6F raised against the α1–isoform of the avian NKA (Takeyasu et al., '88), was demonstrated to be α1 specific and to show broad species specificity (Arystarkhova and Sweadner, '96).

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), with the exception that homogenates were heated at 37°C for 15 min rather than at higher temperatures to minimize covalent linkage of NKA αβ dimmers (Pressley, '92). 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 hrs in PBST buffer containing 1%–2% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 1 h in primary antibody (6F) of diluted in 5% BSA (1:1000 dilution), washed in PBST, and reacted for 30 min with the secondary antibody (1:1000 dilution). Blots were developed after incubation 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). Immunoblots were scanned and imported as JPG files into a commercial software package (Image-Pro Plus, '94). The results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

Specific activity of NKA

Gill NKA activity was determined as described by Hwang et al. ('89). Aliquots of the suspension of gill homogenates, prepared as described above, were used for determination of protein and enzyme activities. NKA activity was assayed by adding the supernatant to the reaction mixture (100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, 5 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 measured according to Peterson's method ('78). The enzyme activity of NKA was defined as the difference between the inorganic phosphate liberated in the presence and absence of 3.75 mM ouabain in the reaction mixture (Hwang et al., '88). Each sample was assayed in triplicate.

Scanning electron microscopy (SEM)

Excised Gill filaments were processed as described by Lee et al. (2000). In brief, tissues were fixed at 4°C in a phosphate-buffered 4% paraformaldehyde plus 5% glutaraldehyde, at pH 7.2 for 12 h and then in 1% osmium textroxide, at pH 7.2, for 1 h at 4°C. Tissues were dehydrated in ascending concentrations of ethanol from 50% to absolute, in 100% acetone, and dried using a Hitachi HCP–2 critical-point drier. After sputter-coating with a gold-palladium complex for 3 min with an Eiko 1B–2 vacuum evaporator, the specimens were examined using a Hitachi S–2500 scanning electron microscope. MR cell densities were counted using SEM as described by Lee et al. (2000).

Abundances and sizes of subtypes of MR cells

Different subtypes of MR cells were identified under SEM observation according to size and morphology of the apical surfaces (Lee et al., '96). Areas on the trailing edge of the filaments were chosen at random for counting at 1250× magnification. Two areas (48×80 μm^2=3840 μm^2) were counted on each of five gill filaments from each fish. Averages of ten areas were obtained from four or five fish per group.

Statistical analysis

Values are expressed as means±SEM. Time course results were analyzed using a one way ANOVA followed by a posteriori comparisons (Tukey's multiple-comparisons test).

RESULTS

Plasma osmolality and ionic concentrations

No mortality was found in tilapia transferred directly from seawater (SW) to fresh water (FW), although significant changes in plasma osmolality and ionic concentrations of the fish were found in the transfer group (Fig. 1). Within 3 hrs post-transfer, a significant decrease in plasma osmolality occurred (Fig. 1A). After 3 hrs, the osmolality declined slowly with time and reached the same level of FW-acclimated tilapia at 48 hrs post-transfer (Fig. 1A). No change of the plasma osmolality was found in fish of the control group (Fig. 1A).

Fig. 1B reveals changes of plasma ion concentrations in tilapia in the control and transfer groups. Although the plasma [Na$^+$] decreased within 3 hrs after transfer, no significant change in [Na$^+$] was found at any time except for the lowest value at 48 hrs during the transfer to SW from FW (Fig. 1B). Within 3 hrs post-transfer, plasma [Cl$^-$] dropped significantly and reached a level similar to that of FW-acclimated fish (Fig. 1B).

Expression of Na, K-ATPase (NKA)

In tilapia transferred directly from SW to FW, (1) relative mRNA abundance of NKA α1-isoform decreased significantly at 6 hrs post-transfer (Table 1); (2) relative abundance of NKA α1–protein decreased gradually from 3 hrs post-transfer, was significant at 12 hrs, and became one-fifth of the amount in SW at 24 hrs post-transfer (Fig. 2A); (3) gill NKA activity dropped significantly within 3 hrs. After 3 hrs, NKA activity reached a stable level at 96 hrs (Fig. 2B). No significant change of mRNA, protein, or activity levels of gill NKA was shown in fish of the control group (Table 1, Figs. 2A and B).

Morphometry of mitochondrion-rich cells (MRCs)

Densities of the MRCs in gill epithelia of tilapia were constant during the time-course of four days post-transfer (Fig. 3). Three subtypes of MRCs were identified in gills of tilapia according to SEM observations of the apical surfaces of MRCs
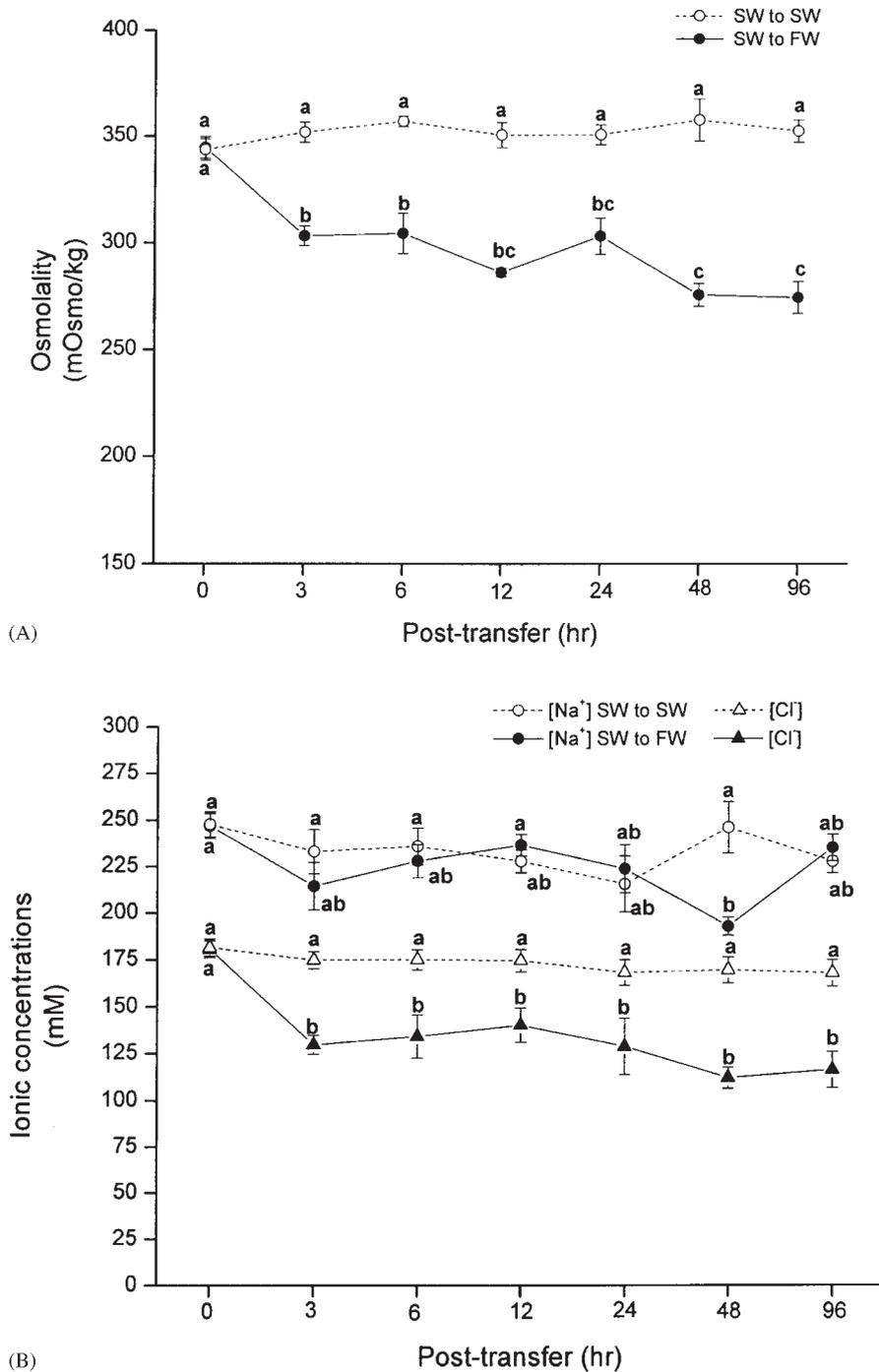
Fig. 1. Effects of direct transfer from seawater (SW) to fresh water (FW) on plasma osmolality (A) and ionic concentrations (B) of tilapia. A significant decrease in osmolality and [Cl⁻] occurred within 3 hrs in the transfer group. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means $\pm$ SEM (n=5–9).

(Lee et al., '96, 2000): subtype I (wavy convex), subtype II (shallow basin), and subtype III (deep hole). Modifications of MRC subtypes occurred in the transfer group within 48 hrs post-transfer (Figs. 4 and 5). Only subtype III MRCs were observed in SW tilapia (Figs. 4A and 5A). Within 3 hrs post-transfer from SW to FW, a few small-sized subtypes I and II MRCs (no more than 10% in frequency and 5μm in size) appeared (Figs. 4B and 5B). After 3 hrs in FW, a gradual rise in the
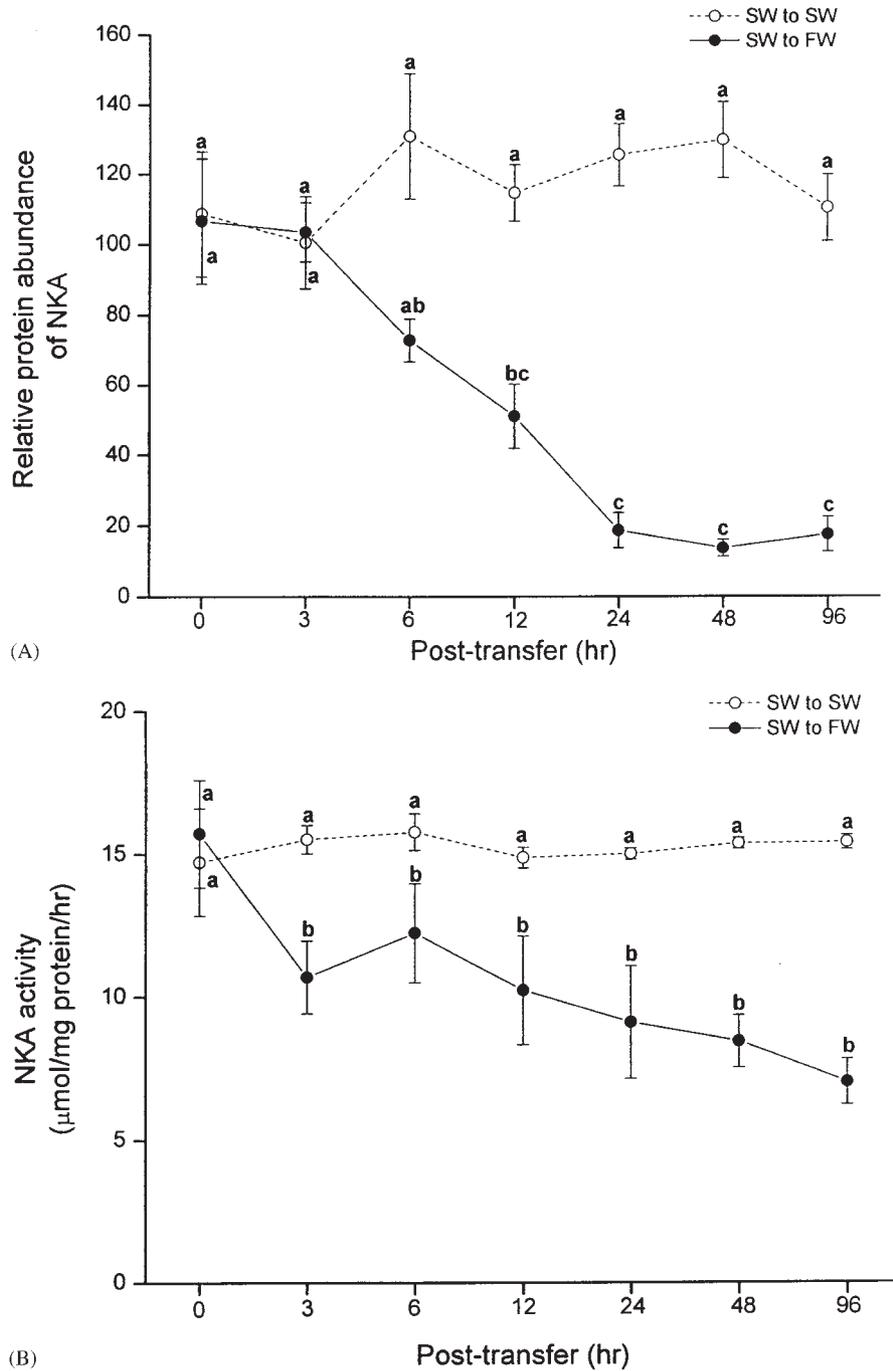
Fig. 2. Effects of direct transfer from SW to FW on protein abundance (A) and activity (B) of Na, K-ATPase (NKA) of tilapia gill. A significant decrease in NKA protein and activity occurred within 12 hrs and 3 hrs respectively, in the transfer group. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one way ANOVA. Values are means $\pm$ SEM (n=5–7).

number of subtypes I and II MRCs in the branchial epithelia, coincided with a drop in nearly half the number compared with subtype III MRCs (Figs. 4C, D and 5C, D). Meanwhile, the size of subtypes I and II MRCs increased with increasing density (Figs. 4C, D and 5C, D). At 24 hrs after transfer into FW, subtype I MRCs became dominant in number and larger in size compared with the other MRC subtypes (Figs. 4E, F and 5E, F).

TABLE 1. *Changes of relative mRNA abundance of Na, K-ATPase α-subunit (α1-isoform) of tilapia gill following transfer from sea water to fresh water. In the control group the fish were transferred from seawater to seawater[1]*

	Relative mRNA abundance (α1-isoform/GAPDH) of Na, K-ATPase
Control group	1.45 ± 0.21 (a)
Transfer group (post-transfer)	
3 hr	1.27 ± 0.40 (a)
6 hr	0.38 ± 0.04 (b)
12 hr	0.23 ± 0.06 (b)
24 hr	0.30 ± 0.09 (b)
48 hr	0.14 ± 0.08 (b)
96 hr	0.12 ± 0.06 (b)

[1]Dissimilar letters indicate a significant difference. Values are means ± SEM (n=4)
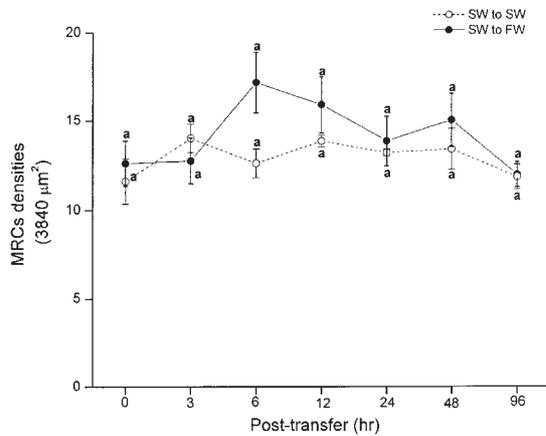

Fig. 3. Effects of direct transfer of tilapia from SW to FW on densities of apical openings of branchial mitochondria-rich cells (MRCs). No significant difference (p < 0.05) was found in either the control group (from SW to SW) or the transfer group using Tukey's multiple-comparison test following a one-way ANOVA. Values are mean ± SEM (n=4−5).

DISCUSSION

In the Mozambique tilapia, *Oreochromis mossambicus*, direct transfer from FW to SW does not induce rapid changes in branchial Na, K-ATPase (NKA) for the reason that the fish die of serious dehydration (Hwang, '87; Hwang et al., '89). However, the fish survive well in SW, with an initial moderate dehydration in 20‰ BW for 24 hrs, when gill NKA increases 3 hrs after transfer (Hwang, '87; Hwang et al., '89). Contrary to the previous experiments concerning hyperosmotic adaptation, in this study, no mortality was found in SW-adapted tilapia transferred directly to FW without any pre-acclimation.

In higher vertebrates, the α1 isoform of NKA is primarily expressed in the transporting epithelia (Blanco and Mercer, '98). The NKA α1 isoform has also been cloned with high levels of homology to teleosts including the white sucker (*Catostomus commersoni*: Schönrock et al., '91), European eel (*Anguilla anguilla*: Culter et al., '95), Atlantic salmon (*Salmo salar*: Seidelin et al., 2001), killifish (*Fundulus heteroclitus*: Semple et al., 2002), and Mozambique tilapia (*Oreochromis mossambicus*: Feng et al., 2002). Using different probes in Northern blots, the mRNA abundance of NKA α1- or α1-subunit have been found to increase during both smoltification of salmonids (D'Cotta et al., '96; 2000; Seidelin, et al., 2001) and SW acclimation of salmonids (Kisen et al., '94; Madsen et al., '95; D'Cotta et al., 2000; Seidelin, et al., 2000), anguillids (Cutler et al., '95), and cichlids (Hwang et al., '98; Feng et al., 2002). This study, to our knowledge, employed, for the first time, the more precise, real-time PCR method, to reveal time-course changes in abundance of NKA α1-mRNA in fish gills and to show a decline during the 6 hrs following transfer from SW to FW, after which the mRNA amounts remain constant (Table 1). Time-course studies on alteration of NKA α-mRNA abundance during SW acclimation of the salmonids revealed significant increases at 12 hrs (brown trout) and 24 hrs (Atlantic salmon) and declined thereafter to values close to those of FW fish at about 10 days post-transfer (D'Cotta et al., 2000; Seidelin et al., 2000). Early activation of regulatory mechanisms at the gene level seem to be crucial to euryhaline teleosts exposed to abrupt changes in environmental salinity.

In general Gill NKA is reported to be more active in SW-resident teleosts (see review of Marshall, 2002). Exposure of killifish (*Fundulus heteroclitus*) to 35‰ SW, induces a rise in gill NKA activity 3 hrs post-transfer (Mancera and McCormick, 2000), and exposure of tilapia (*O. mossambicus*) to 25‰ SW induces a rise in gill NKA activity 1 hr post-transfer (Weng et al., 2002). However, studies on time-course of salinity adaptation in salmon and trout reveal an apparent time lag between NKA α-subunit mRNA and the delay in the adaptive increase in gill NKA activity: normally the activity is not significantly increased until several days after transfer to SW, while the mRNA abundance is significantly elevated after only one day post-transfer (Mancera and McCormick, 2000; D'Cotta et al., 2000; Seidelin et al., 2000). This discrepancy suggests a two-stage modulation of NKA in the salmonids during SW
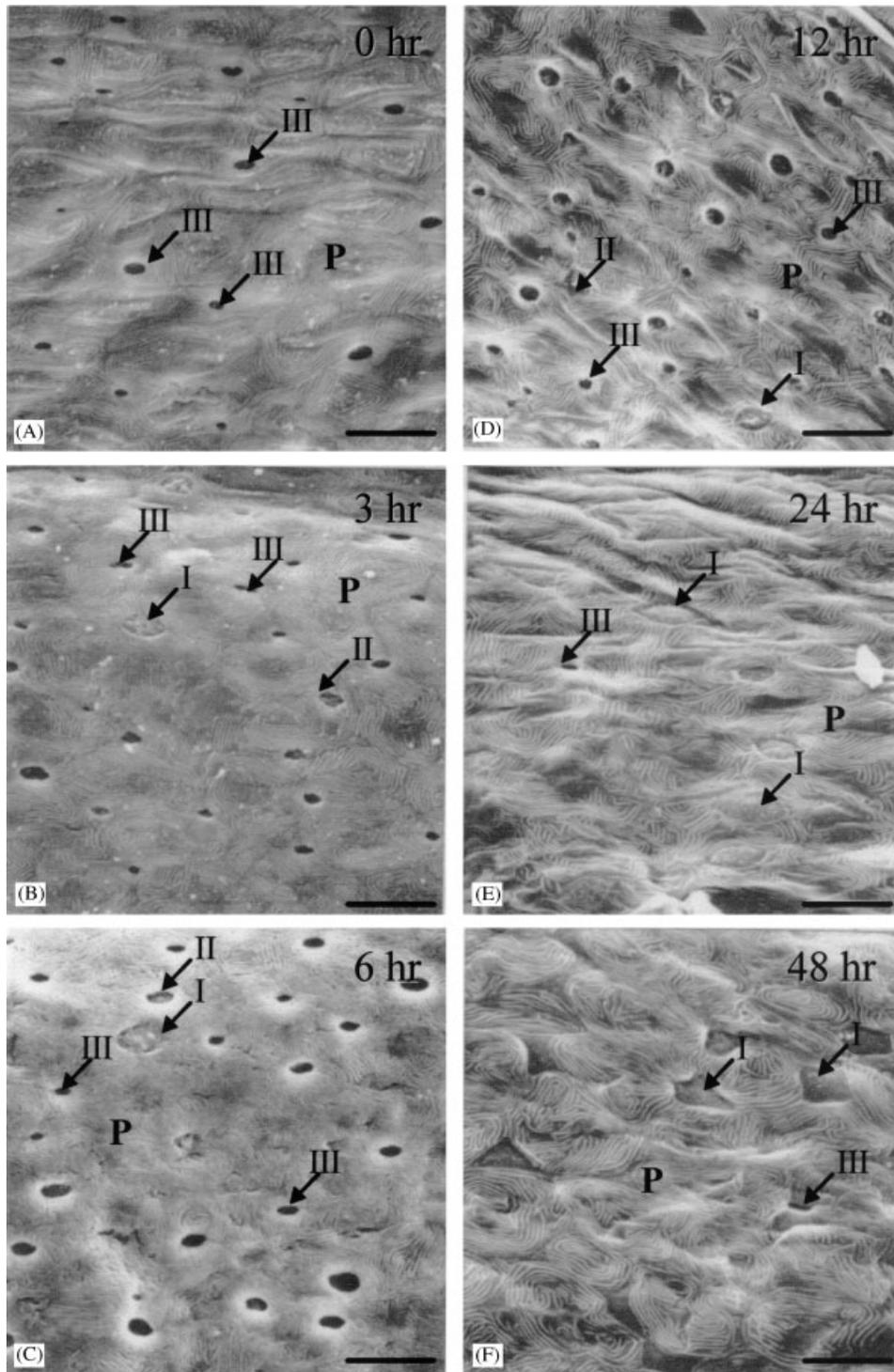
Fig. 4. SEM micrographs show the effects of direct transfer of tilapia from SW to FW on phenotypic changes of branchial MRCs. Gill filaments were examined at (A) 0, (B) 3, (C) 6, (D) 12, (E) 24, and (F) 48 hr after transfer. The apical openings of MRCs, including subtypes I, II, and III, are indicated by arrows. SW subtype III MRCs are expressed at 0 hr (A). FW subtypes I and II MRCs appeared in 3 hrs (B) and increased in density after 6 hrs (C, D, E, F). P, pavement cells. Scale bar: 10 μm.

acclimation: 1) the first modulation possibly acting on the α-gene expression seen at the transcriptional level and 2) the second regulation acting at a post-transcriptional stage, apparently affecting the α-translation, which eventually influences functional NKA (D'Cotta et al., 2000). Contrary
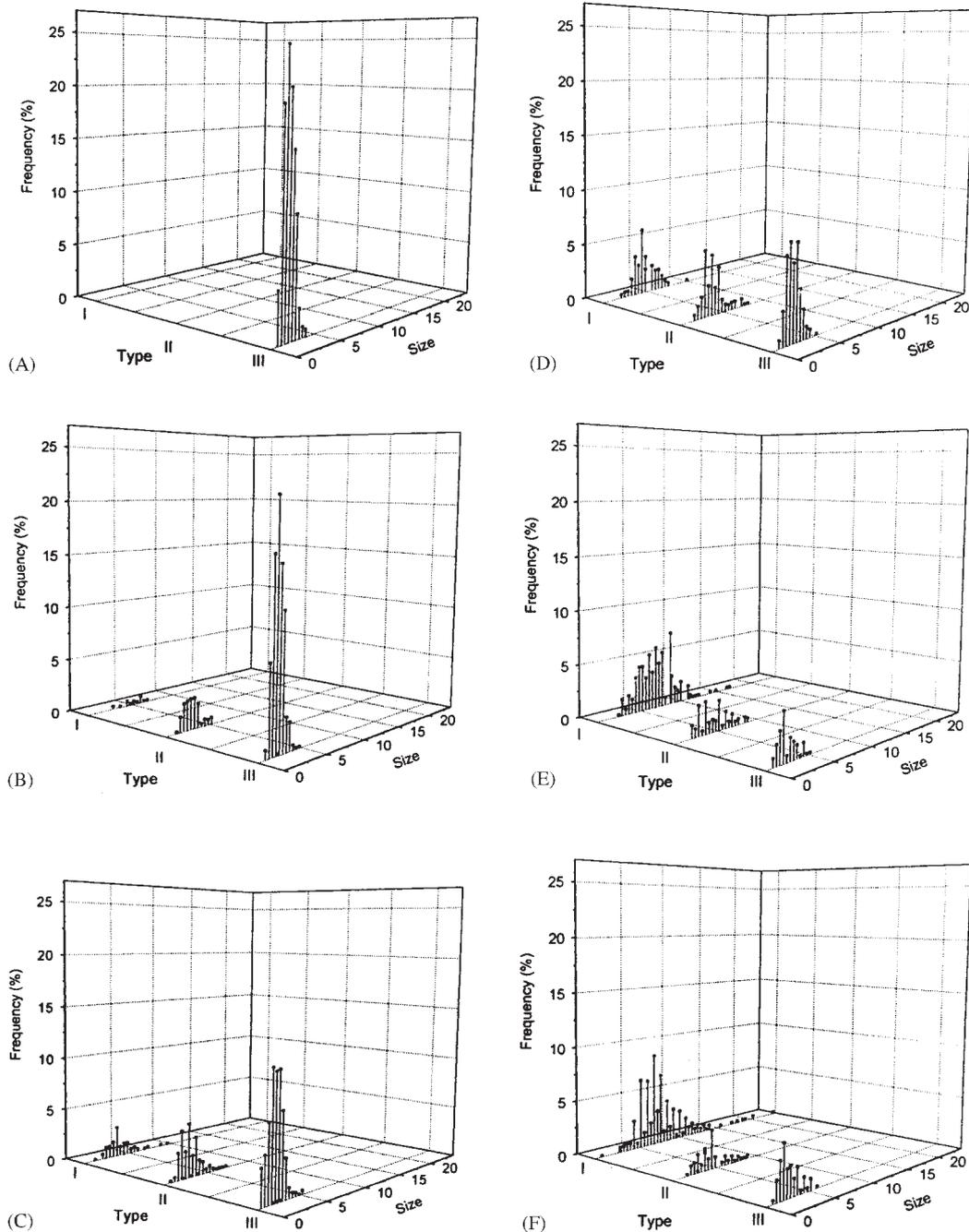
Fig. 5. Effects of direct transfer of tilapia from SW to FW on size (μm) and relative amounts of apical openings of various subtypes of MRCs (n=4−5). At 0 hr (SW), all the MRCs are subtype III and the size of apical membranes are less than 5 μm (A). At 3 hr post-transfer, MRCs of subtypes I and II appeared (B). At 6 hr, 12 hr, and 24 hr post-transfer (C, D, E), the frequency of subtype III MRCs decreased and the other two types increased. Also, the size of apical openings enlarged to more than 10 μm. At 48 hr post-transfer, subtype I became dominant (F).

to the time lag between enhanced expression of NKA gene and activity in salinity adaptation of salmon and trout, when the SW-adapted tilapia were transferred to FW, NKA activity decreased first to the level of FW tilapia as early as 3 hrs post-transfer (Fig. 2B), followed by a substantial drop of α1-mRNA abundance at 6 hrs (Table 1) and then a gradual decline of α1-protein amounts from 6 to 24 hrs post-transfer (Fig. 2A). The drop in α1–mRNA abundance could, however, involve a lower α1–gene expression and/or higher degradation rates.

Previous studies have reported the existence of more than one subtype of MR cells in gills of SW- and FW-adapted euryhaline teleosts, e.g., eel (*Anguilla anguilla*: Shirai and Utida, '70; Wong and Chan, '99); salmon (*Salmo salar*: Pisam et al., '88); trout (*Salmo trutta*: Brown, '92); killifish (*Fundulus heteroclitus*: Hossler et al., '85); and striped bass (*Morone saxatilis*: King and Hossler, '91). In FW-adapted tilapia, three subtypes (subtype I, II, and III) of branchial MR cells with different size and morphology of apical surfaces (i.e., wavy convex, shallow basin, and deep hole) were identified (Lee et al., '96; van der Heijden et al., '97), while in SW-adapted fish only one subtype of MR cell was expressed (Kültz et al., '95; van der Heijden et al., '97; Lee et al., 2000; Uchida et al., 2000). MR cells of SW- and FW-subtypes differed not only in the morphology of the apical openings but also in the NKA contents per cell (van der Heijden et al., '97; Uchida et al., 2000; Lee et al., 2003). A convincing relationship is apparent between the influx of ions and the relative densities of different subtypes of MR cells in tilapia (Tsai and Hwang, '98; Chang et al., 2001). These studies suggest that subtype I MR cells with wavy-convexed apical surfaces play a major role in maintaining constant $[Cl^-]$, while subtype II cells with shallow-basined apical surfaces are responsible for $[Ca^{2+}]$ uptake. Moreover, microscopic evidences of the chloride-secreting activity in SW-MR cells of tilapia have recently been provided (Kaneko and Shirishi, 2001). Hence, modification to the constitution and density of subtypes of MR cells would lead to changes in ion-transporting capacity in gills of euryhaline tilapia. Indeed, instead of only SW-subtype MR cells, three subtypes of FW-MR cells appeared within only 3 hrs after tilapia were transferred from SW to FW (Figs. 4 and 5). The relative abundance of subtypes I and II MR cells increases while subtype III cells decrease during the periods of acclimation (Fig. 5). Such rapid modification is reversible (Lee et al., '96) to complement the physiological demands of tilapia in varied environments.

After transfer to FW, the initial osmotic loss of ions and gain of water are evident from the pronounced decrease in plasma osmolality $[Cl^-]$, as well as the slight decline in $[Na^+]$, in tilapia after only 3 hrs (Fig. 1). The perturbations stabilized after 3 hrs, indicating an early modification of regulatory mechanisms, i.e., NKA expression. Seawater-adapted flounder (*Platichthys flesus*), long-jawed mudsuckers (*Gillichthys mirabilis*), chum salmon (*Oncorhynchus keta*), and sea

bream (*Sparus sarba*) are capable of tolerating direct exposure to hypotonic environments (Nonnotte and Truchot, '90; Kelly and Woo, '99; Yoshikawa et al., '93; Uchida et al., '97). In these teleosts, the "crisis" and "regulatory" phases also accompany abrupt transfer to hypoosmotic environments and express a rapid drop in plasma osmolality and $[Cl^-]$ in adapting to hypotonic environments. However, similar to plasma $[Na^+]$ of tilapia (Fig. 1B), sea bream maintain stability during acclimation to hypotonic environments (Kelly and Woo, '99). Through effective osmoregulatory mechanisms, i.e., rapid alteration in NKA expression, tilapia as well as the other euryhaline species are able to overcome changes in external salinity and reach a new steady state of blood ionic concentrations.

Thus, when exposed to FW, SW-adapted tilapia are capable of maintaining homeostasis within a short period of time as a result of their improved efficiency for ion-uptake capability, which derives from the rapid enhancement of FW-subtype MR cells. The present paper, together with analysis of the abundance of the $\alpha1$–mRNA, amount of $\alpha1$–protein, and the activity of NKA, reveals that, although the process is not lethal and the regulatory mechanisms start as early as 3 hrs post-transfer, 24 hrs are still necessary for full acclimation of the SW-adapted tilapia to FW.

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