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Short-term effects of hyposmotic shock on Na^+/K^+-ATPase expression in gills of the euryhaline milkfish, *Chanos chanos*

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Abstract

Changes in expression of gill Na^+/K^+-ATPase (NKA) on a short-term (96 h) time-course following hyposmotic shock (direct transfer to fresh water) of the euryhaline, marine milkfish were studied on gene, protein, and cell levels in this paper. Plasma osmolality and $[Na^+]$ responded with rapid declines in 3 h post-transfer yet, thereafter, remained constant. Plasma $[Cl^-]$ gradually fell to a significantly lower level at 6 h post-transfer. Gills responded to hyposmotic shock by a dual phase enhancement of NKA activity and protein abundance; (a) Before 24 h: NKA activity increased as early as 3 h and reached a maximum level from 6 to 12 h post-transfer coincided with the sustained lower levels of plasma osmolality, $[Na^+]$, and $[Cl^-]$ since 3 h post-transfer. This was followed by a gradual rise in α-subunit protein levels that peaked at 12 h post-transfer. Meanwhile, α-mRNA of NKA did no show significant change. (b) After 24 h: NKA activity as well as the amounts of α-subunit mRNA and protein increased significantly. Direct freshwater transfer induced a prompt and significant decrease of NKA immunoreactive (NKIR) cell abundance in filaments before 24 h, followed by a significant increase after 24 h due to their development in filaments and lamellae. Increased number of NKIR cells after 24 h of hyposmotic shock may occur in conjunction with rise of NKA activity as well as α-subunit mRNA and protein abundance. In conclusion, milkfish is able to avoid an excessive drop in plasma ions immediately upon hyposmotic shock and maintain plasma ions on a marginal lower level in fresh water. Notably, the initial increase in NKA activity (adjustive phase; 3–12 h) and delayed increase in NKA mRNA and protein abundance (regulatory phase; 48–96 h) indicate the importance of a higher level of the gill enzyme in milkfish upon hyposmotic shock.

Keywords: Milkfish; *Chanos chanos*; Euryhaline teleost; Gill; Na^+/K^+-ATPase; Time-course

1. Introduction

The ubiquitous membrane-spanning enzyme Na^+/K^+-ATPase (NKA) is responsible for the active transport of Na^+ out of and K^+ into animal cells. It is important for maintaining intracellular homeostasis as well as for providing a driving force for many transporting systems. The functional pump is composed of a catalytic α-subunit with a molecular weight of about 100 kDa, and an accompanying glycosylated β-subunit with a molecular weight of approximately 55 kDa (Scheiner-Bobis, 2002). High levels of homology prevail among all the α-subunit sequences identified in animal species ranging from *Drosophila*, *Artemia* to vertebrates (Vasilets and Schwarz, 1993), and including teleosts (Schönrock et al., 1991; Cutler et al., 1995; Seidelin et al., 2001; Feng et al., 2002; Semple et al., 2002). In gills of euryhaline teleosts, NKA energizes transport of NaCl across epithelia in both absorptive (fresh water; FW) and secretory (seawater; SW) modes (see reviews of Marshall, 2002; Perry et al., 2003; Hirose et al., 2003). In gill epithelia, immunocytochemical studies demonstrated that NKA is located mainly in mitochondrion-rich (MR) cells (Wilson and Laurent, 2002; Chen et al., 2004). Current models indicated that MR cells utilizing NKA as primary driving force have unique functions and transporter compositions dependent on the salinity of the

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surrounding environment (Marshall, 2002; Hirose et al., 2003).

Euryhaline teleosts are able to osmoregulate across a broad spectrum of salinities. Acclimation to changing environmental salinity requires pre-existing mechanisms to respond to altering conditions. A salinity challenge necessitates activation of gill NKA as well as MR cells, crucial for the fish acclimation (see review of Wilson and Laurent, 2002). According to previous studies, however, two major salinity-induced branchial NKA responses were found: (i) higher-NKA-in-hyperosmotic media and (ii) higher-NKA-in-hyposmotic media. The first group of euryhaline teleosts including salmon, eel, and tilapia, all responded to salinity challenge with higher NKA activities as well as MR cell densities, dominated conception of mechanisms used for salinity adaptation in fish gills (for reviews, see Marshall, 2002; Hirose et al., 2003). The other group of euryhaline teleosts responded to hyposmotic-media acclimation with higher NKA activities (reviewed by Marshall and Bryson, 1998; Kelly et al., 1999; Mancera et al., 2002; Lin et al., 2003) as well as NKIR (MR) cell densities (Lin et al., 2003). Effects of $[Na^+]$ or $[K^+]$ on gill NKA activity of FW- and SW-adapted tilapia, puffer (the first group), and milkfish (the second group) were compared and different affinities of NKA for sodium or potassium were found in these two groups of euryhaline teleosts (Lin and Lee, 2005). Moreover, in the higher-NKA-in-hyperosmotic media group, the salmonids showed a prolonged increase of NKA activity, usually in 3–7 days, after transfer to SW (see review of Marshall, 2002); while a more rapid activation (within a few hours) of gill NKA activity in killifish (Towle et al., 1977; Mancera and McCormick, 2000) and tilapia (Hwang et al., 1989; Weng et al., 2002) were reported during acclimation to hypertonic environments. Recently, gills of SW tilapia were found to respond to FW transfer rapidly by reducing NKA activity within 3 h, then NKA α1-subunit mRNA behaved similarly as did the amount of NKA α1-subunit protein within 6 h post-transfer (Lin et al., 2004a). On the other hand, upon hyposmotic shock, transient changes of responses and mechanisms in gills exploited by the higher-NKA-in-hyposmotic media euryhaline teleosts have been poorly studied: Caberoy and Quinitio (2000) reported changes of NKA activity and MR cell morphology in gills of grouper (*Epinephelus coioides*) larvae and juveniles following abrupt hyposmotic exposure; Tipsmark et al. (2004) reported the time-course alterations of some ion transporters including NKA in gills of the striped bass (*Morone saxatilis*) in 1 week after direct transfer from SW to FW. Time-course studies on different euryhaline species revealed that the source of changing NKA activity upon salinity challenge might be that of altered mRNA abundance (Scott et al., 2004; Seidelin et al., 2000; Singer et al., 2002), protein amounts (Tipsmark et al., 2002; Lee et al., 2000, 2003; Lin et al., 2003), or on both levels (D'Cotta et al., 2000; Lin et al., 2004a).

The milkfish (*Chanos chanos*) is a marine teleost widely distributed throughout the tropical and subtropical Indo-Pacific, yet it is euryhaline at all developmental stages (Bagrinao, 1994). Due to its euryhalinity, milkfish are commercially cultured in fresh, brackish, and oceanic waters (Chen, 1990). Lin et al. (2003) have compared the expression of gill NKA in milkfish adapted to environments of varying salinities. A marked enhancement of NKA activity as well as protein abundance, derived from a significant increase of lamellar NKA immunoreactive (NKIR) cell numbers, were found in FW-adapted milkfish rather than in BW- or SW-adapted individuals (Lin et al., 2003; Chen et al., 2004). Being a very well-suited subject for a study of marine fish hyperosmoregulation, it is intriguing to know more about the mechanisms making milkfish an efficient osmoregulator. The aim of this study was to evaluate the adaptive responses in gill epithelia of the marine milkfish upon hyposmotic shock. Special emphasis was given to the transient changes of NKA expression as well as the abundance and distribution of NKIR cells in gills.

2. Material and methods

2.1. Fish and experimental environments

Juvenile milkfish (*C. chanos* Forsskål, 1775) with 29.2± 9.3 g body mass were obtained from a fish farm in Chia-Yi, Taiwan. The fish used in the present study were kept in seawater (SW) at 27±1 °C with a daily 12 h photoperiod for at least two weeks. The fish were then divided into control and experimental groups. For the short-term hyposmotic shock experiment, milkfish of the experimental group were transferred directly to fresh water (FW; ionic concentrations in mmol L^{-1}: $[Na^+]$, 2.6; $[K^+]$, 0.04; $[Ca^{2+}]$, 0.58; $[Mg^{2+}]$, 0.16; $[Cl^-]$, 0.18), while fish of the control group were transferred to seawater (SW; ionic concentrations in mmol L^{-1}: $[Na^+]$, 582.86; $[K^+]$, 10.74; $[Ca^{2+}]$, 15.75; $[Mg^{2+}]$, 32.92; $[Cl^-]$, 520.84) simultaneously. Because fish were transferred by nets, the control group was used to monitor the effect of handling of fish alone. After transfer, the fish were sampled by netting at 3, 6, 12, 24, 48, and 96 h for the following analyses. Fish were fed a daily diet of commercial pellets ad libitum.

2.2. Plasma analyses

Milkfish blood was collected from the caudal vein with heparinized 1 mL syringes and 27 G needles. After centrifugation at 1000 ×*g*, 4 °C for 10 min, the plasma was stored at −20 °C. Plasma osmolality was assessed with the WESCOR 5520 VAPRO Osmometer (USA). $[Na^+]$ was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Japan). $[Cl^-]$ was determined by the Ferricyanide method (Franson, 1985) and photometric analysis was carried out using a Hitachi U-2001 spectrophotometer (Japan). Plasma osmolality and Na^+ and Cl^- concentrations of each fish were calculated as the mean of the replicate samples taken from that fish. Five fish were sampled for each salinity treatment.

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from the gill epithelium by using RNeasy Mini kit (Qiagen), following the manufacturer's instructions. RNA integrity was verified by 0.8% agarose gel electrophoresis. Extracted RNA samples were stored at −80 °C

after isolation. First-strand cDNA was synthesized by reverse transcribing 5 μg of the total RNA using a 1 μL Oligo(dT) [0.5 μg/μL] primer and a 1 μL PowerScript™ Reverse Transcriptase (Clontech), following the manufacturer's instructions.

2.4. Primers used for real-time PCR

The cDNA sequence of milkfish Na^+/K^+-ATPase (NKA; Lee et al., unpublished data) and β-actin (GenBank accession **no. DQ202397**) were aligned and compared with the sequences of other species from the database. NKA α-subunit gene-specific primer sequences used for real-time PCR were designed using the Primer3 software (Rozen and Skaletsky, 2000) as follows (5′ to 3′): forward—AGAGTTCCTCCTGGTCTTACAGA and reverse—GTGATTGTACAGTGGGCTGACT. β-actin primer sequences are as follows (5′ to 3′): forward—CCATTGAGCACGGTATTGTCA and reverse—GCAACACGCAGCTCGTTGTA.

2.5. Real-time PCR analysis

NKA α-subunit mRNA was quantified by a real-time PCR (Rotor-Gene™ 3000, Corbett Research, Sydney, Australia). For methods of quantifying mRNA by the real-time PCR, refer to Johnson et al. (2000). PCR reactions contained 5 μL of cDNA (100× dilution), 50 nM each of NKA α-subunit primer mixture or β-actin primer mixture, and 10 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR reactions were performed as follows: 1 cycle of 50 °C for 3 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 40 s. All samples were run in triplicate. Reactions for quantifying β-actin copy number were performed exactly as described above except for the use of different primers. NKA mRNA values were normalized against that of β-actin from the same DNA samples. For each unknown sample, the comparative Ct method with the following formula: $2\text{^}[(Ct_{NKA,n}-Ct_{GAPDH,n})-(Ct_{NKA,0}-Ct_{GAPDH,0})]$ was used to obtain the corresponding NKA and β-actin values, where Ct corresponds to the threshold cycle number.

2.6. NKA antibodies

A monoclonal antibody (α5) against the α-subunit of the avian NKA was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). This antibody has been demonstrated to react with NKA of different teleostean species (Witters et al., 1996; Schreiber and Specker, 1999; Dang et al., 2000; Wilson et al., 2000; Lee et al., 2003; Lin et al., 2004a,b) including milkfish (Lin et al., 2003; Chen et al., 2004) by immunoblotting or immunohistochemical studies.

2.7. Immunoblotting of NKA

The tissues scrapings were suspended in the mixture of homogenization medium (concentrations in mmol L^{-1}: imidazole-HCl: 100; Na_2EDTA: 5; sucrose: 200; 0.1% sodium deoxycholate, pH 7.6) and proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) (v/v: 100/1). Homogenization was performed with a motorized Teflon pestle at 600 rpm for 30 s. The homogenate was then centrifuged at 13,000 ×*g*, 4 °C for 20 min. The supernatants were used for determination of protein concentrations and immunoblotting. Aliquots containing 40 μg of gill homogenates and pre-stained molecular weight standards (Invitrogen, USA) were heated at 37 °C for 30 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from unstained gels to PVDF (PolyScreen, NEN, USA) using a tank transfer system (Bio-Rad, Mini Protean 3, USA). Blots were preincubated for 1 h in PBST (phosphate buffer saline with Tween 20) buffer (concentrations in mmol L^{-1}: NaCl:137; KCl: 3; Na_2HPO_4: 10; KH_2PO_4: 2; 0.2% (vol/vol) Tween 20, pH 7.4) containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding, then incubated at 4 °C with primary antibody (α5) diluted in PBST (1:5000) overnight. The blot was washed in PBST, followed by a 1-h incubation with AP-conjugated secondary antibody (Jackson, USA) diluted 2500× in PBST. Blots were visualized after incubation with a NBT/BCIP kit (Chemicon, UK). Immunoblots were scanned and images were imported as TIFF format into a commercial software package (Kodak Digital Science 1D, 1995) and the results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands. Gill homogenate from one milkfish was used as the internal control among different immunoblots (data not shown). The intensity of the immunoreactive band of the internal control was converted and adjusted to a numerical value of 100 in each immunoblot. The values of relative intensities for each time point came from the immunoblots of five individuals of milkfish.

2.8. NKA activity

Aliquots of the suspension of gill homogenates, prepared as described above, were used for determination of protein concentrations and enzyme activities. NKA activity was assayed by adding the supernatant to the reaction mixture (concentrations in mmol L^{-1}: imidazole–HCl buffer: 100, pH 7.6; NaCl: 125; KCl: 75, $MgCl_2$: 7.5; Na_2ATP: 5). Each sample was assayed in triplicate. The enzyme activity of NKA was defined as the difference between the inorganic phosphates liberated in the presence and absence of 3.75 mmol L^{-1} ouabain in the reaction mixture. 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 (1978).

2.9. Cryosection and immunohistochemical detection of NKA immunoreactive (NKIR) cells

Gills were excised and fixed in a mixture of methanol and DMSO (4:1 v/v) at −20 °C for 3 h. Before embedding with O.C.T. (optimal cutting temperature) compound (Tissue-Tek®,

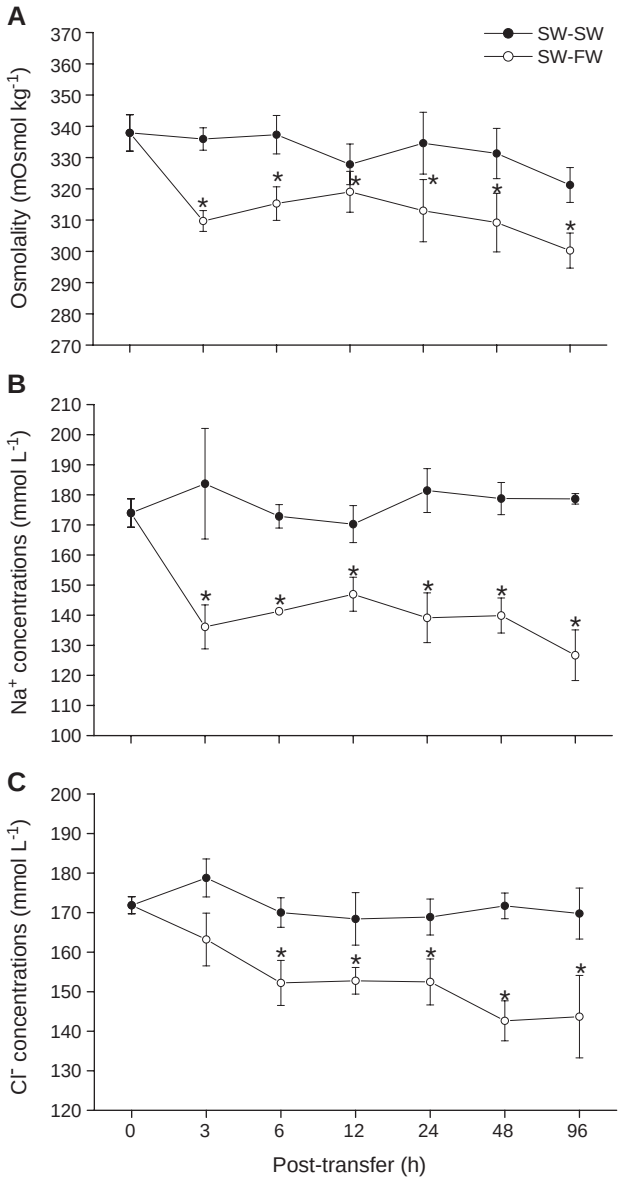

Fig. 1. Time-course changes of plasma osmolality, [Na^+], and [Cl^-] in milkfish transferred directly from SW to SW (control group) and from SW to FW (experimental group). The asterisks indicate significant differences ($p<0.05$) compared to the initial data. Values are means±SEM ($n=5$). Significant decrease in osmolality and [Na^+] occurred within 3 h, and in [Cl^-], within 6 h post-transfer.

Sakura, USA), fixed samples were washed with PBS, and the gill arch and one side of the filament were removed. Serial sections (7 μm) were cut parallel to the long axis of the filament, and mounted on slides coated with poly-L-lysine. To quantify the distribution of NKIR cells, cryosections were immunohistochemically stained with the monoclonal antibody (α5) to NKA α-subunit and then with a commercial kit (PicTure™, Zymed, USA) for visualization of the immunoreaction. The immunostained sections were then counterstained with Hematoxylin (Merck, Germany) and observed under a microscope (Olympus BX50) equipped with a differential interference contrast (DIC) device. Negative control experiments, in which mouse serum (Sigma, USA) was used instead of the primary antibody, were conducted (data not shown) to confirm the above positive results.

Most NKIR cells in gills of the milkfish were distributed in the interlamellar epithelium near the afferent side covering the cartilage of the filament (Chen et al., 2004). Hence longisections of the gills, including lamellae and the cartilages of the filaments, were chosen and the numbers of immunoreactive cells in the interlamellar regions of the filaments (F), the lamellae (L), and the basal regions of the lamellae (B) were counted. According to Lin et al. (2003), the basal region of the lamella was defined as the basal part of the lamella extending 10 μm up from the filament plus half the width of the filament itself. For each sample, 10 areas on the filaments including symmetrical lamellae were randomly selected. Lengths of lamellae and interlamellar regions were also measured to standardize cell counts to a fixed length (50 μm). Results are expressed as number of NKIR cells (1) per 50 μm of interlamellar region, (2) per 50 μm of lamella, and (3) per basal region of lamella. Four fish were sampled at each time point.

2.10. Statistical analysis

Values were expressed as means±SEM. The effect of FW-transfer at different time points was assessed by comparison with the 0 h value, using Dunnett's test at which salinity effects were detected by one-way ANOVA ($P<0.05$). Measured time-course variables of SW-transfer control group were assessed using the same method to elucidate the stress of handling fish upon transfer.

3. Results

3.1. Plasma analysis

A significant decline in plasma osmolality and [Na^+] were found in the experimental group in 3 h post-transfer (Fig. 1A

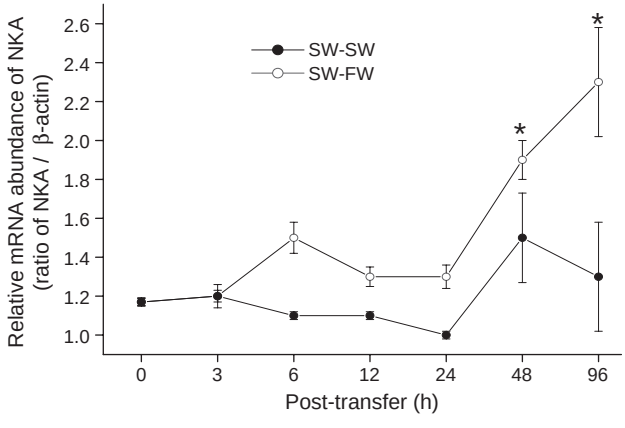

Fig. 2. Changes of relative mRNA abundance of Na^+/K^+-ATPase (NKA) α-subunit of milkfish gills following transfer from SW to SW (control group) and from SW to FW (experimental group). The asterisks indicate significant differences ($p<0.05$) compared to the initial data. Values are means±SEM ($n=5$). NKA α-subunit mRNA abundance increased significantly since 48 h post-transfer.

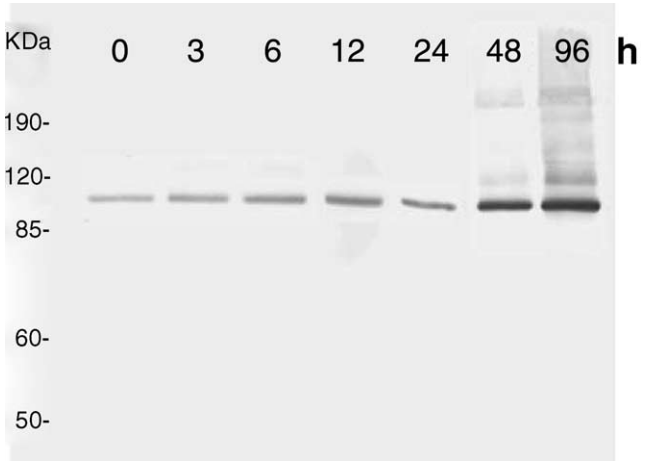

Fig. 3. Representative immunoblot of the Na^+/K^+-ATPase (NKA) α-subunit of milkfish gills sampled at time-course after transfer from SW to FW. A singe immunoreactive band was observed with α5 monoclonal antibody corresponding to a molecular mass of about 100 kDa.

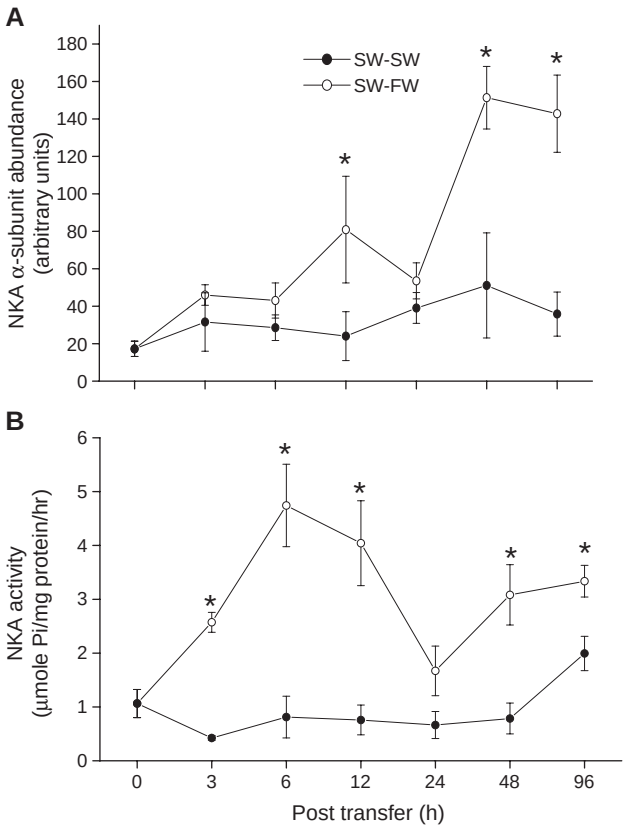

Fig. 4. Changes of relative protein amounts of Na^+/K^+-ATPase (NKA) α-subunit and NKA activity in gill epithelia of the milkfish transferred from SW to SW (control group) and from SW to FW (experimental group). The asterisks indicate significant differences ($p<0.05$) compared to the initial data. Values are means ± SEM ($n=5$). (A) Relative abundance of the NKA α-subunit protein increased gradually and peaked at 12 h, then decreased at 24 h, and enhanced significantly again at 48 and 96 h post-transfer. (B) Specific activity of NKA increased four to five folds at 6 and 12 h post-transfer, and dropped to the basal level at 24 h post-transfer, then elevated significantly again at 48 and 96 h.

and B). Compared to the initial data, $[Cl^-]$ decreased to a level significantly lower after 6 h (Fig. 1C). No change in plasma osmolality, $[Na^+]$ or $[Cl^-]$ was found in fish of the control group (Fig. 1A–C).

3.2. Gill Na+/K+-ATPase (NKA) expression

Hyposmotic shock induced a biphasic expression of NKA. During 24 h post-transfer, the abundance of NKA α-mRNA increased slightly, although not significantly, at 6 h (Fig. 2); the amounts of NKA α-protein increased gradually to achieve a significant maximum at 12 h, then declining slightly at 24 h (Figs. 3 and 4A), while the NKA activity began to rise by 3 h, reaching nearly a 5-fold increase at 6 and 12 h, and decreased thereafter to values very close to those of SW fish at 24 h (Fig. 4B). After 24 h post-transfer, the abundance of NKA α-mRNA increased significantly at 48 and 96 h (Fig. 2), paralleled by a near 7-fold increase of the amounts of NKA α-protein (Figs. 3 and 4A), and a 3-fold elevation of NKA activity (Fig. 4B). No significant difference was found in levels

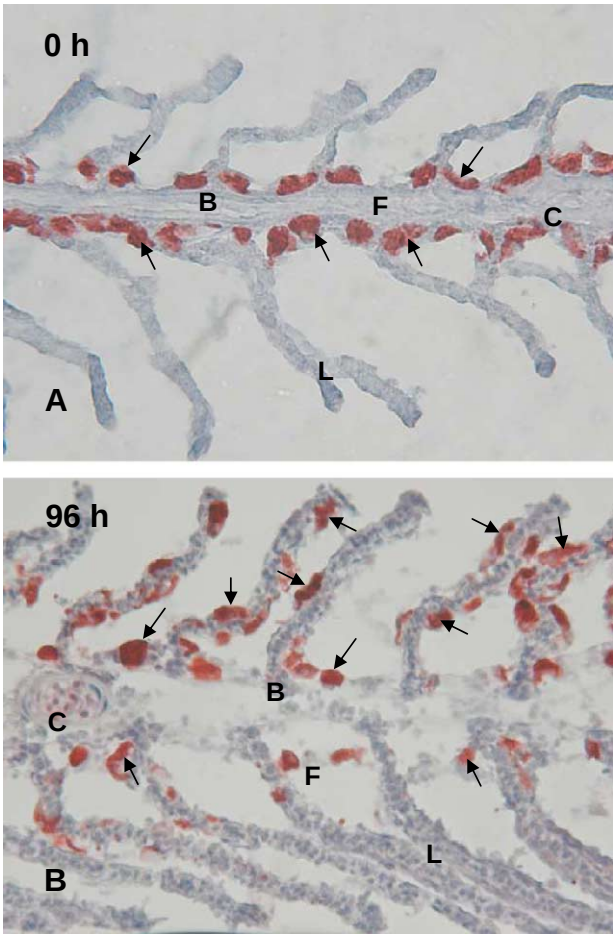

Fig. 5. Representative immunostaining of Na^+/K^+-ATPase (NKA) immunoreactive (NKIR) cells (arrows) in longitudinal sections of gill filaments from milkfish of 0 h (SW) group (A), and 96 h post-transfer to FW group (B). The objective lens was 40×. NKIR cells were found on interlamellar regions of the filament (F), basal regions of the lamellae (B), and/or lamellae (L; finger-like projections). More NKIR cells exhibited in the gill lamellae of milkfish of 96 h post-transfer group (B) than individuals of 0 h group (A). C, cartilage.

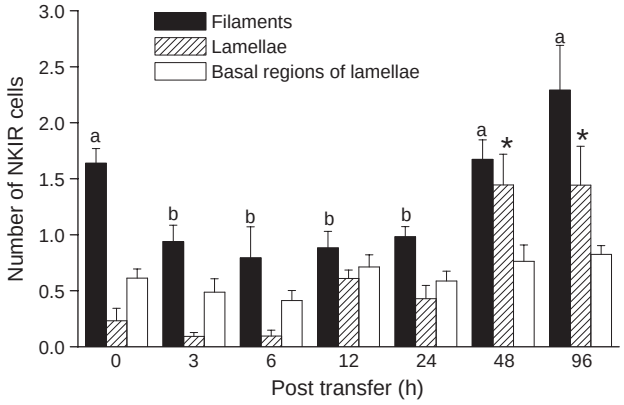

Fig. 6. Time-course changes of Na^+/K^+-ATPase immunoreactive (NKIR) cell numbers in gill epithelia of the milkfish transferred directly from SW to FW. For typical immunostaining patterns and NKIR cell numbers of SW and FW milkfish gills, see Chen et al. (2004) and Lin et al. (2003). Dissimilar letters and the asterisks indicate significant differences ($p<0.05$) of NKIR cell numbers in filaments (interlamellar regions) and lamellae, respectively, compared to the initial data. Values are means±SEM ($n=4$). From 3 to 24 h post-transfer the numbers in filaments declined and rose again at 48 and 96 h. In the lamellae, NKIR cell numbers increased significantly at 48 and 96 h post-transfer. Consistent numbers of NKIR cells were found in the basal regions of lamellae.

of NKA α-mRNA, α-protein, and NKA activity during the time-course of the control-group fish (Figs. 2 and 4).

3.3. Distribution and morphometry of NKA immunoreactive (NKIR) cells

Time-course changes in the distribution and number of gill NKIR cells after transfer to FW were visualized and counted using immunolocalization of the NKA α-subunit on cryosections (Figs. 5 and 6). The representative micrograph of immunostaining showed that at 0 h (SW) and 96 h post-transfer NKA was restricted to relatively large cells (presumably MR cells) distributed mostly in filaments (interlamellar regions), basal regions of lamellae, or lamellae of milkfish gills (Fig. 5). Before 24 h post-transfer, NKIR cell abundance (in the interlamellar regions of the filaments) experienced a slight, although significant, decrease; while in lamellae and the basal regions of lamellae, NKIR cells numbers remained constant at a low level (Fig. 6). However, since 48 h post-transfer, (i) filamental NKIR cell abundance increased above that of fish from 3 to 24 h; (ii) lamellar NKIR cell numbers increased more than 3-fold; and (iii) in the basal regions of lamellae, the numbers among various groups were similar (Fig. 6). The sum of the mean values revealed a pronounced elevation of NKIR cell numbers at 48 and 96 h post-transfer.

4. Discussion

After transfer to fresh water (FW), the initial diffusive loss of ions and gain of water are evident from the decrease in plasma osmolality, $[Na^+]$ and $[Cl^-]$ in milkfish in 6 h (Fig. 1). Ferraris et al. (1988) reported a similar observation in milkfish transferred from seawater (SW) to FW: the plasma osmolality and chloride concentrations decreased significantly during one day post-transfer, and restored gradually until 14 days. The present study revealed that decrease in plasma osmolality in milkfish stabilized in 3 h after hyposmotic shock (Fig. 1A). Sustained lower levels of plasma ions to a FW level after a rapid drop following FW transfer indicated an early and efficient modification of regulatory mechanisms of milkfish. The other euryhaline, marine species, i.e., black sea bream (*Mylio macrocephalus*, Kelly et al., 1999), flounder (*Platichthys flesus*, Nonnotte and Truchot, 1990; *Paralichthys orbignyanus*, Sampaio and Bianchini, 2002), long-jawed mudsuckers (*Gillichthys mirabilis*, Yoshikawa et al., 1993), European sea bass (*Dicentrarchus labrax*, Jensen et al., 1998), silver sea bream (*Sparus sarba*, Kelly and Woo, 1999), grouper (*E. coioides*, Caberoy and Quinitio, 2000), and puffer (*Tetraodon nigroviridis*, Lin et al., 2004b), are capable of tolerating direct exposure to hypotonic environments. The European sea bass, black sea bream, and flounder, however, experienced a substantial and lasting decrease in blood osmolality when transferred to FW.

Most euryhaline teleosts exhibit adaptive changes in gill NKA activity following salinity changes. NKA is composed of an $(\alpha\beta)_2$ protein complex with four α (α1–4) and three β (β1–3) isoforms as well as a small γ subunit (Scheiner-Bobis, 2002). Responsible for catalytic and transport work of NKA, α-subunit isoforms revealed distinct differences in their affinities to Na^+ and K^+ in rat brain and kidney (Urayama and Nakao, 1979), and adipocytes (Lytton et al., 1985). Different isoforms of NKA α-subunit (i.e., α1 and α3) were also found in gills of euryhaline teleosts (eel, Cutler et al., 1995; salmon/trout, D'Cotta et al., 1996; Seidelin et al., 2001; Richards et al., 2003; killifish, Semple et al., 2002; tilapia, Hwang et al., 1998; Feng et al., 2002). SW-adapted tilapia revealed different levels of increase of α1 and α3 mRNA as well as protein in the gills from that of FW-adapted fish (Lee et al., 1998; Feng et al., 2002). In rainbow trout gills, the changes in distribution of four NKA α-isoforms (α1a, α1b, α1c, and α3) were accompanied by an elevation in gill NKA activity by 10 days after transfer to 80% SW (Richards et al., 2003). Taken together, expression of various levels of NKA isoforms may lead to different Na^+ or K^+ affinities and fulfill some of the requirements for altered enzyme behavior in gills of SW- or FW-acclimated euryhaline teleosts. The exhibition and switching of NKA α-isoforms in gills of milkfish following salinity transfer will be the subject of further investigation.

Previous papers described two major salinity-induced gill NKA responses: the "higher-NKA-in-hyperosmotic media" response, i.e., NKA expression increase to their greatest extent after transfer to hyperosmotic environments, has been observed as a general pattern of tilapia, eel, and salmon (see reviews of Marshall, 2002; Hirose et al., 2003). These species also have been reported to possess higher levels of the V-type H^+-ATPase in FW (Lin and Randall, 1998; Hiroi et al., 1998; Seidelin et al., 2001). This supports the implication that Na^+ uptake through epithelial Na^+ channels in FW is driven by the electrical gradient produced by apical H^+-ATPase (Perry et al., 2000; Reid et al., 2003). In euryhaline teleosts of this group, e.g., tilapia and puffer, no change was found in the affinity of gill NKA for Na^+

in FW- or SW-adapted individuals (Lin and Lee, 2005). Contrary to the higher-NKA-in-hyperosmotic media response, however, in milkfish and some other euryhaline species, e.g., killifish and striped bass, exhibit an alternative NKA response—"higher-NKA-in-hyposmotic media" response: the NKA expression were upregulated to a greater extent after FW transfer than after SW transfer (Lin et al., 2003; Scott et al., 2004; Tipsmark et al., 2004). The transcription or activity of H^+-ATPase was unaffected by salinity transfer in killifish and striped bass, respectively (Scott et al., 2004; Tipsmark et al., 2004). Meanwhile, several pieces of evidence suggested that Na^+, H^+-exchanger instead of H^+-ATPase may be important for Na^+ absorption in gills of FW killifish (Scott et al., 2004). In milkfish, the affinity of NKA for Na^+ is higher in FW-adapted fish than in SW-adapted fish (Lin and Lee, 2005). The discrepancy in NKA responses upon salinity challenge between these two groups may therefore relate to the suggestion that apical Na^+, H^+-exchanger, rather than sodium channels coupled to H^+-ATPase, is responsible for Na^+ uptake in the higher-NKA-in-hyposmotic media group. Thus, without the electrochemical gradient created by active apical extrusion of protons by H^+-ATPase, higher NKA activity may be required in the gills of milkfish (the higher-NKA-in-hyposmotic media group) to maintain a favorable Na^+ gradient across the apical membrane.

During FW acclimation, a biphasic strategy is exploited by the marine milkfish (Figs. 2–4). Before 24 h post-transfer, termed the adjustive phase, the abundance of NKA α-mRNA increased slightly, although not significantly, at 6 h, then a significant but slight increase of NKA α-protein abundance appeared at 12 h (Figs. 2, 3 and 4A), preceded by elevated NKA activity, with the peak occurring from 6 to 12 h post-transfer (Fig. 4B). The fact that NKA activity increases prior to mRNA and protein abundance of the catalytic subunit suggests that modulation of the hydrolytic rate of the enzyme by phosphorylation via protein kinase A and cAMP or recruitment of latent enzymes is taking place as in the gill epithelium of a few other teleosts, e.g., Atlantic cod (Crombie et al., 1996), European eel (Marsigliante et al., 1997), and striped bass (Tipsmark and Madsen, 2001; Tipsmark et al., 2004). At 24 h post-transfer, both the values of NKA α-protein abundance and activity differed minimally from the pre-transfer level (Fig. 4A and B). After 24 h post-transfer, termed the regulatory phase, the abundance of NKA α-mRNA as well as α-protein elevated markedly (Figs. 2 and 4A), paralleled by an increase of NKA activity (Fig. 4B). In this phase, the increases in gill NKA activity were attributed to accompaniment of increased abundance of NKA α-subunit mRNA and protein. Similar mechanisms were found in salmonids (Kisen et al., 1994; Madsen et al., 1995; D'Cotta et al., 2000; Seidelin et al., 2000; Singer et al., 2002), European eel (Cutler et al., 1995), European sea bass (Jensen et al., 1998), and tilapia (Hwang et al., 1998; Feng et al., 2002). Our results demonstrate that higher expression of gill NKA found in hyposmotic shock milkfish derives from a two-phase modulation. Upon hyperosmotic shock, similar two-phase increases of gill NKA expression were also reported in the other euryhaline teleosts, e.g., killifish (Mancera and McCormick, 2000; Scott et al., 2004) and tilapia (Lee et al., unpublished data). Meanwhile, time-course studies revealed that rapid decrease of NKA expression (from mRNA to activity) was found in gills of tilapia upon hyposmotic shock (Lin et al., 2004a), opposite to the trend of NKA expression in milkfish gills.

Immunocytochemical studies on gill sections (reviewed by Wilson and Laurent, 2002) as well as biochemical studies on isolated mitochondrion-rich (MR) cells (Kültz and Jürss, 1993; Wong and Chan, 1999) have clearly demonstrated that these epithelial cells contain most NKA activity and protein in gills. Hence, NKA immunoreactive (NKIR) cells represent MR cells. Elevated MR/NKIR cell numbers in euryhaline teleosts of the higher-NKA-in-hypertonic media group are normally associated with an increase in NKA activity. Concomitant elevation of NKA activity, protein abundance, as well as NKIR cell numbers, was also found in FW-adapted milkfish (Lin et al., 2003). Similarly, studies using either scanning electron microscopic or immunocytochemical observations indicated that, in the other euryhaline, marine teleosts, the number of MR/NKIR cells in gill epithelia increased when acclimated to hypotonic milieu (e.g., *M. macrocephalus*, Kelly et al., 1999; *S. sarba*, Kelly and Woo, 1999) or FW (e.g., *Lateolabrax japonicus*, Hirai et al., 1999; *Dicentrachus labrax*, Versamos et al., 2002). The data (Fig. 6) in the present study showed that, upon hyposmotic shock, significant elevation of NKIR cell numbers occurred only in the regulatory phase, i.e., 48 and 96 h post-transfer. An increase of NKA protein or activity may be due to the elevation of MR/NKIR cell numbers (Versamos et al., 2002), or an increase in the amount of NKA in each MR/NKIR cell in response to salinity challenge (Uchida et al., 2000; Lee et al., 2003). Hence, upon hyposmotic shock of milkfish, significant increase of NKA activity found in the adjustive period (i.e., before 24 h post-transfer; Fig. 4B) could be attributed to the utilization of latent enzyme in MR/NKIR cells. Thereafter, in the regulatory period (i.e., after 24 h post-transfer), significant elevation of NKA activity (Fig. 4B) coincident with NKA mRNA and protein abundance (Figs. 2 and 4A) might be derived from the elevated numbers of NKIR cells (Fig. 6).

In FW- and SW-adapted euryhaline teleosts, MR/NKIR cells are generally found to be abundant in the gill filament epithelia (Wilson and Laurent, 2002). MR/NKIR cells also appear on the lamellar epithelia in stenohaline FW teleosts (Lee et al., 1996) or FW-adapted euryhaline species (Wilson and Laurent, 2002), including the milkfish (Fig. 5; Lin et al., 2003; Chen et al., 2004). MR/NKIR cells are effective at eliminating ions in hypertonic SW as well as absorbing ions in hyposmotic FW (Chang et al., 2002; Marshall, 2002; Perry et al., 2003). In the euryhaline Japanese sea bass (*L. japonicus*), European sea bass (*D. labrax*), as well as the milkfish, a significant elevation of lamellar NKIR cell numbers was observed in FW-adapted individuals (Hirai et al., 1999; Versamos et al., 2002; Lin et al., 2003). Moreover, in milkfish, significant increases in the number of lamellar NKIR cells occurred in the regulatory phase of FW acclimation, i.e., 48 and 96 h post-transfer (Fig. 6). On the other hand, reduced lamellar MR/NKIR cells have been reported in SW-adapted diadromous species, i.e., the anguillids (Sasai et al., 1998) and salmonids (Brown, 1992; Uchida et al.,

1996; Seidelin et al., 2000). The occurrence of lamellar MR/NKIR cells has thus been suggested as the mechanism to meet the physiological demand of ion-uptake in some FW-adapted teleosts (Uchida et al., 1996; Sasai et al., 1998; Hirai et al., 1999; Seidelin et al., 2000; Versamos et al., 2002). It is important to stress, however, that the putative involvement of lamellar MR/NKIR cells in ion absorption in the FW-adapted teleosts does not exclude the possibility that a subpopulation (subtype) of filament MR/NKIR cells may also be involved in ion uptake in euryhaline teleosts (Laurent and Perry, 1990; Marshall et al., 2002; Lee et al., 2003; Lin and Sung, 2003; Wu et al., 2003).

Taken together, upon hyposmotic shock, the milkfish is able to avoid an excessive drop in plasma ions indicating that hyper-osmoregulatory mechanisms are readily activated including a two-phase modulation of NKA: (a) the adjustive phase, acts directly on NKA possibly by post-translational mechanisms; and (b) the regulatory phase, affects α-transcription and α-translation localized to the newly developed MR/NKIR cells. The nature of these rapid alterations appears to be an important response of milkfish to hyposmotic shock and may reflect greater tolerance to salinity change. Future work will be focused on the Na^+ uptake mechanisms in gills of the euryhaline, marine milkfish.

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