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ORIGINAL PAPER

Elevated Na^+/K^+-ATPase responses and its potential role in triggering ion reabsorption in kidneys for homeostasis of marine euryhaline milkfish (*Chanos chanos*) when acclimated to hypotonic fresh water

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Abstract The milkfish (*Chanos chanos*) is an economic species in Southeast Asia. In Taiwan, the milkfish are commercially cultured in environments of various salinities. Na^+/K^+-ATPase (NKA) is a key enzyme for fish iono- and osmoregulation. When compared with gills, NKA and its potential role were less examined by different approaches in the other osmoregulatory organs (e.g., kidney) of euryhaline teleosts. The objective of this study was to investigate the correlation between osmoregulatory plasticity and renal NKA in this euryhaline species. Muscle water contents (MWC), plasma, and urine osmolality, kidney histology, as well as distribution, expression (mRNA and protein), and specific activity of renal NKA were examined in juvenile milkfish acclimated to fresh water (FW), seawater (SW 35‰), and hypersaline water (HSW 60‰) for at least two weeks before experiments. MWC showed no significant difference among all groups. Plasma osmolality was maintained within the range of physiological homeostasis in milkfish acclimated to different salinities, while, urine osmolality of FW-acclimated fish was evidently lower than SW- and HSW-acclimated individuals. The renal tubules were identified by staining with periodic acid Schiff's reagent and hematoxylin. Moreover, immunohistochemical staining showed that NKA was distributed in the epithelial cells of proximal tubules, distal tubules, and collecting tubules, but not in glomeruli, of milkfish exposed to different ambient salinities. The highest abundance of relative NKA α subunit mRNA was found in FW-acclimated milkfish rather than SW- and HSW-acclimated individuals. Furthermore, relative protein amounts of renal NKA α and β subunits as well as NKA-specific activity were also found to be higher in the FW group than SW and the HSW groups. This study integrated diverse levels (i.e., histological distribution, gene, protein, and specific activity) of renal NKA expression and illustrated the potential role of NKA in triggering ion reabsorption in kidneys of the marine euryhaline milkfish when acclimated to a hypotonic FW environment.

Keywords Euryhaline teleost · Milkfish · Osmoregulation · Kidney · Na^+/K^+-ATPase

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Introduction

Maintaining a stable internal environment is important for vertebrate animals to survive in a variety of habitats. In response to changes of environmental conditions, the ion-transporting epithelia play the roles of modulating ion fluxes. The principle of successful adaptation from hypertonic to hypotonic environments is to switch from active hypoosmoregulation (ion excretion) to active hyperosmoregulation (ion uptake) to counter the passive diffusion fluxes imposed by concentration gradients (Wilson et al. 2004).

The osmoregulation in teleosts is mediated by a group of structures including the gills, kidneys and intestines

(Marshall and Grosell 2006). The kidney plays an important role in osmoregulation of both freshwater (FW) and seawater (SW) teleosts, although its function is entirely different under these two environments (Miyazaki et al. 2002). In FW teleosts, the hypotonic environments result in water loading and ions loss through their permeable body surface, and accordingly, the primary kidney function is to excrete excess water and reabsorb filtered solutes. In contrast, SW teleosts gain excess ions, lose water to their surrounding hypertonic environment, and consequently ingest SW to prevent dehydration (Katoh et al. 2006). In marine teleosts, the osmolality of urine is similar to that of the body fluid, while the predominant electrolytes of urine are Mg^{2+}, SO_4^{2-}, and Ca^{2+}, rather than Na^+ and Cl^- (Marshall and Grosell 2006). Moreover, various adaptive changes were reported in fish kidneys during salinity acclimation, such as changes in morphology, excretion and absorption of ions, glomerular filtration rate, urine production, and metabolism (Beyenbach 1995; Miyazaki et al. 2002; Renfro 1995; Sangiao-Alvarellos et al. 2003; Wong and Woo 2006).

Hickman and Trump (1969) reported an extensive review on the functional morphology of fish nephrons. The nephron of teleosts is composed of a well-developed glomerulus (although some teleosts are aglomerular), a ciliated neck segment, a proximal tubule, an intermediate segment, a distal tubule, and a collecting tubule. However, the kidney of teleosts lacks the loop of Henle and exhibits neither zonation, such as the cortex and medulla, nor a countercurrent system of tubular elements (Hickman and Trump 1969). Therefore, teleosts are not able to excrete hypertonic urine (Miyazaki et al. 2002).

Na^+/K^+-ATPase (NKA) is a universal membrane bound enzyme which actively transports Na^+ out of and K^+ into animal cells (Post and Jolly 1957). NKA is composed of a catalytic-α subunit with a molecular weight of approximately 100 kDa and a smaller glycosylated β subunit with a molecular weight of approximately 55 kDa (Mercer 1993). However, the expression of both α and β subunits is required for NKA activity. The β subunit regulates assembly of $\alpha\beta$ heterodimers to modulate the number of NKA transported to the plasma membrane (McDonough et al. 1990). Because NKA is a primary active transport protein, it is important for sustaining intracellular homeostasis as well as providing a driving force for many transporting systems in a variety of osmoregulatory epithelia, including fish gills and kidneys (Marshall and Grosell 2006; McCormick 1995). Most studies on renal NKA expression of the teleosts, however, examined the specific activity only (Arjona et al. 2007; Gallis and Bourdichon 1976; Herrera et al. 2009; Kelly et al. 1999; Krayushkina et al. 2006; Lasserre 1971; Romao et al. 2001; Venturini et al. 1992). So far, very few papers investigated NKA expression in fish kidneys from diverse aspects, e.g., mRNA and protein abundance (Deane and Woo 2004; Lin et al. 2004).

The milkfish (*Chanos chanos*) is a marine inhabitant and an efficient osmoregulator (Ferraris et al. 1988). It is widely distributed throughout the tropical and subtropical Indo-Pacific (Bagrinao 1994). Farming of the milkfish is an important aquaculture industry in Indonesia, Philippines, and Taiwan. This species occurs naturally and is commercially cultured in fresh, brackish, and oceanic waters as well as in hypersaline lagoons (Crear 1980). It demonstrates extremely euryhaline behaviors throughout its life history although it does not appear to require a FW environment for any part of its life cycle (Bagrinao 1994). Because the size and cost of juvenile milkfish are smaller and cheaper than adult fish, the juvenile milkfish are well suited to rear in laboratory for studies on osmoregulation. NKA expression (i.e., distribution, mRNA, protein, and activity) in the kidneys of euryhaline teleosts, however, is less reported when compared with that in the gills. The present study, therefore, was to assess osmolality of plasma and urine as well as renal NKA expression in euryhaline milkfish acclimated to FW, SW (35‰), and hypersaline water (HSW 60‰) to illustrate the relationship between renal NKA and their osmoregulatory plasticity.

Materials and methods

Fish and experimental environments

Juvenile milkfish (*Chanos chanos*) with 26.7 ± 0.9 g body weight and 13.6 ± 0.2 cm total length were obtained from a fish farm in Taiwan. Seawater (SW 35‰) and hypersaline water (HSW 60‰) were prepared from local tap water with appropriate amounts of synthetic sea salt (Aquarium Systems, Mentor, OH, USA). Milkfish were reared in fresh water (FW) ($[Na^+]$ 2.6 mM; $[K^+]$ 0.04 mM; $[Ca^{2+}]$ 0.58 mM; $[Mg^{2+}]$ 0.16 mM; $[Cl^-]$ 0.2 mM; pH = 7.6 ± 0.1), SW, or HSW, respectively, at 27 ± 1°C with a daily 12 h photoperiod for at least 2 weeks before experiments. Feeding was terminated 48 h prior to the following experiments. The water was continuously circulated through fabric-floss filters. Fish were fed daily with commercial pellets.

Analysis of plasma and urine osmolalities and muscle water contents (MWC)

Fish blood was collected from the heart with heparinized 1 ml syringes and 29-G needles and centrifuged at 1,000*g* at 4°C for 10 min ($n = 8$). The procedures of urine collection were carried out according to Nebel et al. (2005).

The milkfish were captured with a hand-net and transferred to a black 10-l plastic container filled with FW, SW, or HSW, and covered by a black plastic sheet. The containers were kept still in a silent room for 2 h. The anesthetic (MS-222; Sigma, St. Louis, MO, USA) was then gently introduced into the water under the plastic cover. The urinary pore of each anaesthetized fish were quickly rinsed with distilled water and carefully dried with kitchen towel. Urine was collected following its emission induced by probing the urinary pore and gently inserting the micropipette into the pore ($n = 5$). Plasma and urine osmolalities were measured immediately by the Wescor 5520 vapro osmometer (Logan, UT, USA). MWC procedures were carried out according to Tang et al. (2009). MWC was measured gravimetrically after drying at 100°C for 48 h ($n = 8$).

Tissue collection

Fish were anaesthetized by immersion in MS-222 (Sigma). Following anesthesia, fish were sampled immediately after killing by decapitation. The organs except kidney in the abdominal cavities were removed. For paraffin sections, kidneys located beneath the spinal cord in the body cavity were pre-fixed onsite by dropping Bouin's fluid (Sigma) for 1 h before sampling. After pre-fixation, the whole kidney tissue was harder and easier to be excised. For RNA extraction and immunoblotting analysis, the kidneys were directly excised and immersed in liquid nitrogen immediately for subsequent homogenization.

Total RNA extraction and reverse transcription PCR

Total RNA was extracted from the kidney by RNeasy® Mini kit (Qiagen, Valencia, CA, USA) and contaminating DNA was digested with RNase-free DNase Set (Qiagen) according to manufacturer's protocol. After isolation, the RNA integrity was checked by 0.8% agarose gel electrophoresis to determine the presence of 28S, 18S, and 5S ribosomal RNA bands. The concentration of extracted RNA was measured by NanoDrop 2000 (Thermo, Wilmington, DE, USA). First-strand cDNA was synthesized by reverse transcribing 5 μg of the total RNA using 1 μl PowerScript™ Reverse Transcriptase (Clontech, Franklin Lakes, NJ, USA) and 1 μg oligo dT primer (GeneMark, Taichung, Taiwan) according to the manufacturer's protocol.

Real-time PCR

Na^+/K^+-ATPase α1-subunit mRNA (accession number: DQ512799) was quantified by the real-time PCR (Rotor-Gene™ 3000 real-time multiplexing system, Corbett Research, Sydney, Australia). Primers for both NKA α1-subunit and β-actin genes (accession number: DQ202397) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm) with the following sequences (5′–3′): NKA α1-subunit, AGAGTTCCTCCTGGTC TTACAGA and GTGATTGTACAGTGGGCTGACT; β-actin, CCATTGAGCACGGTATTGTCA and GCAACA CGCAGCTCGTTGTA. The PCR products were sequenced to check the specificity of the primers. Furthermore, real-time PCR reactions contained 5 μl of cDNA (100× dilution), 50 nM of NKA α1-subunit primers, and 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All real-time PCR reactions were performed as follows: 50°C for 3 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 40 s. All samples were run in duplicate. Reactions for quantifying β-actin copy number were performed exactly as described above except for the primers. Non-template control reactions were conducted with sterile deionized water instead of cDNA template to determinate the level of background. A SW sample was used as the internal control for standardization of each PCR reaction. For each unknown sample, the comparative Ct method with the following formula: $2^{\wedge}$ $[(Ct_{NKA,n} - Ct_{\beta\text{-actin},n}) - (Ct_{NKA,c} - Ct_{\beta\text{-actin},c})]$ (Livak and Schmittgen 2001) was used to obtain the corresponding NKA values where Ct corresponded to the threshold cycle number.

Antibodies

The primary antibodies used in the present study were the mouse monoclonal antibodies, α5 (Takeyasu et al. 1988) and 24 (Fambrough and Bayne 1983), which were against NKA α and β subunits, respectively (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The secondary antibody for immunoblotting of NKA α and β subunits were alkaline phosphatase (AP)-conjugated IgG (Jackson ImmunoResearch, West Grove, PA, USA) and horseradish phosphatase (HRP)-conjugated (Pierce, Rockford, IL, USA) goat anti-mouse IgG, respectively.

Histological examination

Pre-fixed kidneys were fixed in Bouin's fluid for periodic acid Schiff's (PAS) reagent (PAS; Merck, Darmstadt, Germany) staining for 24 h at 25°C. Subsequently, the tissues were dehydrated through a graded ethanol series and embedded in paraffin. Then, the serial sections of 5 μm were mounted onto gelatin-coated glass slides. The sections were stained in PAS counterstained with Gill's hematoxylin, then mounted with cover slips. Sections were observed with an optic microscope (Olympus BX50,

Tokyo, Japan) and micrographs were taken with a digital camera (Nikon COOLPIX5000, Tokyo, Japan).

Immunohistochemical staining

According to Lin et al. (2004), the sections of kidneys prepared as described above were immunohistochemically stained with the monoclonal primary antibody (α5) followed by staining with a commercial kit, PicTure™ (Zymed, San Francisco, CA, USA) for visualization. Negative control experiments, in which PBS (phosphate buffer saline) was used to replace the primary antibody, were conducted to confirm the positive results (data not shown).

Immunoblotting

The immunoblotting procedures were carried out as described by Tang et al. (2009) with not much modification. The homogenization was performed in a 2-ml tube with the homogenizer (PT1200E, KinematicaAG, Lucerne, Switzerland) at maximal speed for 20–30 s. After homogenization, the homogenate was then centrifuged at 13,000*g* for 20 min at 4°C. Protein concentrations were identified by BCA protein assay reagents (Pierce, Rockford, IL, USA) using bovine serum albumin (Pierce) as a standard. Aliquots containing 50 μg protein of renal homogenates were heated at 37°C for 30 min and fractionated by electrophoresis on SDS-containing 7.5% or 10% polyacrylamide gels for NKA α and β subunit, respectively, with the pre-stained protein marker (Fermentas, Hanover, MD, USA). Separated proteins were transferred from unstained gels to PVDF membrane (PerkinElmer, Waltham, MA, USA). Blots were preincubated in PBST (phosphate buffer saline with Tween 20) buffer containing 5% (wt/vol.) nonfat dried milk at room temperature for 2 h, then incubated overnight at 4°C with primary antibodies (α5 and 24) diluted in 1% BSA and 0.05% sodium azide in PBST (1:5,000 and 1:200), washed in PBST, followed by 1.5 h incubation with HRP-conjugated (1:20,000) secondary antibodies diluted in 1% BSA and 0.05% sodium azide in PBST. Blots were developed after incubation with an ECL kit for HRP-conjugated system (Pierce, Rockford, IL, USA). To confirm the loading, amounts in each lane were the same and the gels were stained by Coomassie Blue for quantification. Coomassie Blue-stained gels and developed immunoblots were photographed and imported as TIFF files. The lanes of the gels stained with Coomassie Blue and immunoreactive bands were analyzed using a software package (MCID software; Imaging Research, ON, Canada). The results were converted to numerical values to compare the relative protein abundance. Gill homogenates from one individual were used as the internal control among different immunoblots (data not shown) for calibration of relative abundance.

Specific activity of NKA

A method using 96-well microplate to measure the inorganic phosphate concentrations for determination of NKA activity was developed. Aliquots of the suspension of kidney homogenates, prepared as described above, were used for the determination of protein concentration and NKA enzyme activities. The reaction medium (final concentration: 100 mM imidazole–HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$) was prepared according to Hwang et al. (1988). Then 10 μl supernatant, 50 μl 5 mM ouabain (specific inhibitor of NKA) or deionized water, and 100 μl 10 mM Na_2ATP was added to 340 μl of the reaction medium. The enzyme activity was defined as the difference between the inorganic phosphates liberated in the presence and absence of ouabain in the reaction mixture. The reaction mixture was incubated at 28°C for 20 min followed by immediate ice-bath for 10 min to stop the reaction (Cheng et al. 1999). Because van der Heijden et al. (1997) and Sardella et al. (2008) have demonstrated that the specific NKA activity of fish gills measured at environmental temperature correlates with the level of in vivo activity, while measured at 35°C provided an estimate of total amount of functioning NKA exhibiting in the studied organ (total NKA capacity), the reaction in this study was run at 28°C. The concentrations of inorganic phosphate were measured according to Doulgerakia et al. (2002) with not much modification. The color reagent was consisted of 1% Tween-20 and 1% ammonium molybdate in 0.9 M H_2SO_4. The reaction mixtures and color reagent were 1:1 (v/v) mixed and incubated for 3 min at 28°C before samples were determined by a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Each sample was determined in triplicate. Some protocols determined the concentrations of inorganic phosphate by measuring the color of molybdenum blue which was the reducing product of phosphomolybdate. The instability of color formation and reagents, however, were variables in those protocols (Fiske and Subbarow 1925; Taussky and Shorr 1953). The formation of the unreduced phosphomolybdate in the present study is directly proportional to the amounts of inorganic phosphate.

Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) (Tukey's pairwise method). Values were expressed as mean ± SEM.

Results

Plasma osmolality and MWC

There was no significant difference in MWC among milkfish exposed to fresh water (FW), seawater (SW 35‰) and hypersaline water (HSW 60‰) (Fig. 1a). Plasma osmolality of milkfish acclimated to environments of various salinities was maintained in a range which was appropriate for physiological homeostasis, although it was slightly, but significantly higher in the SW and HSW groups (Fig. 1b). Urine osmolality of FW-acclimated fish, on the other hand, was evidently lower than SW- and HSW-acclimated individuals (Fig. 1c).

Histology of kidney

In the kidney of euryhaline milkfish, the nephron could be distinguished into different segments using PAS staining and the nuclei were counterstained with hematoxylin to observe the standard morphological features of milkfish kidneys (Fig. 2). The distinct segments of milkfish kidney included the proximal tubules (P), distal tubules (D) and collecting tubules (C). The PAS staining method was used to label the proximal tubules as this segment has a brush border coated with glycocalyx (Katoh et al. 2008). The proximal tubules were identified by the well-developed PAS-positive microvilli in the lumen. The distal tubules in milkfish could be identified as their lumen was PAS negative and the nuclei were round and located in the central portion of the cells. The lumen of the collecting tubules was also PAS negative. However, the collecting tubules could be recognized by the feature of pseudostratified columnar epithelium. Similar renal histology was observed in milkfish acclimated to environments of different salinities, the glomerulus, proximal, distal, and collecting tubules were presented in all groups (Fig. 2a–c).

Renal Na^+/K^+-ATPase distribution and expression

Na^+/K^+-ATPase was immunohistochemically stained using monoclonal antibody α5 in kidneys of different salinity-acclimated (FW, SW, and HSW) milkfish. The immunoreaction revealed that NKA was distributed in proximal, distal, as well as collecting tubules, but not in the glomeruli among all salinity groups (Fig. 3). NKA immunoreaction was detected in the basolateral membrane of epithelial cells of all observed renal tubules. Furthermore, the real-time polymerase chain reaction (PCR) analysis showed that the relative mRNA levels of renal NKA α subunit in FW-acclimated milkfish were significantly higher than SW- and HSW-acclimated groups (Fig. 4). In addition, immunoblotting of NKA α and β subunits in kidney tissues of milkfish of different salinity groups (FW, SW, and HSW) resulted in single immunoreactive bands at about 105 kDa (α subunits; Fig. 5a) and 58 kDa (β subunits; Fig. 6a), respectively. Quantification of immunoreactive bands of each group showed that the relative protein abundance of the FW-acclimated individuals was significantly higher than SW- and HSW-acclimated individuals in both α (Fig. 5b) and β- (Fig. 6b) subunits. The pattern of renal NKA-specific activity was similar to that of mRNA and protein abundance. The specific activity of renal NKA of fish

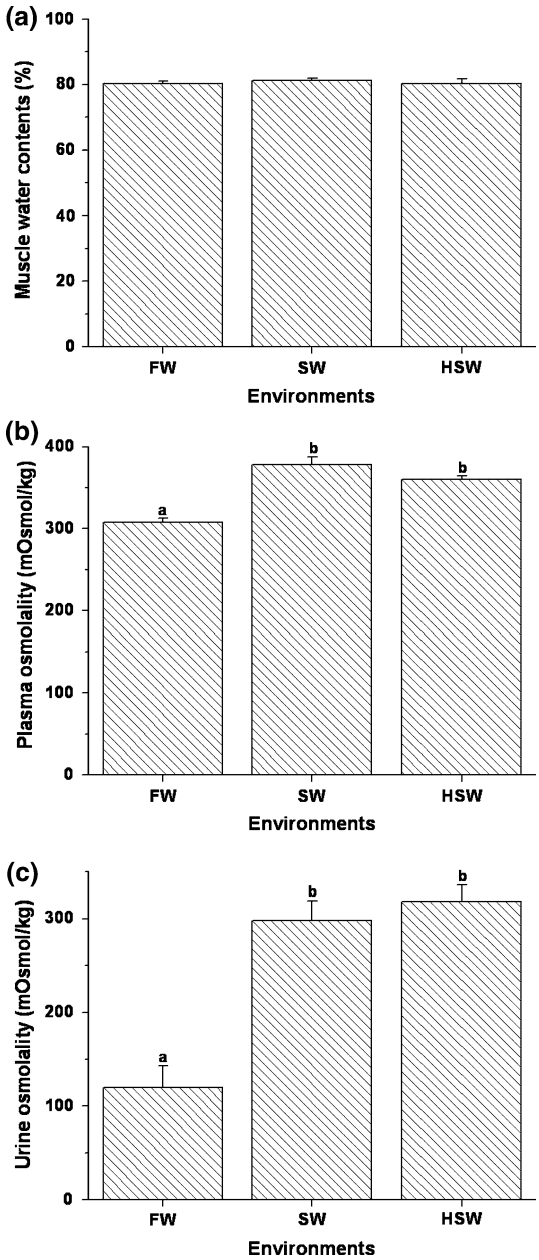

Fig. 1 Muscle water contents (MWC) ($n = 8$) and plasma ($n = 8$) and urine ($n = 5$) osmolality in milkfish (*Chanos chanos*) acclimated to different environments of salinities. The *asterisk* indicates a significant difference ($P < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values were mean ± SEM. *FW* fresh water, *SW* seawater (35‰), *HSW* hypersaline water (60‰)

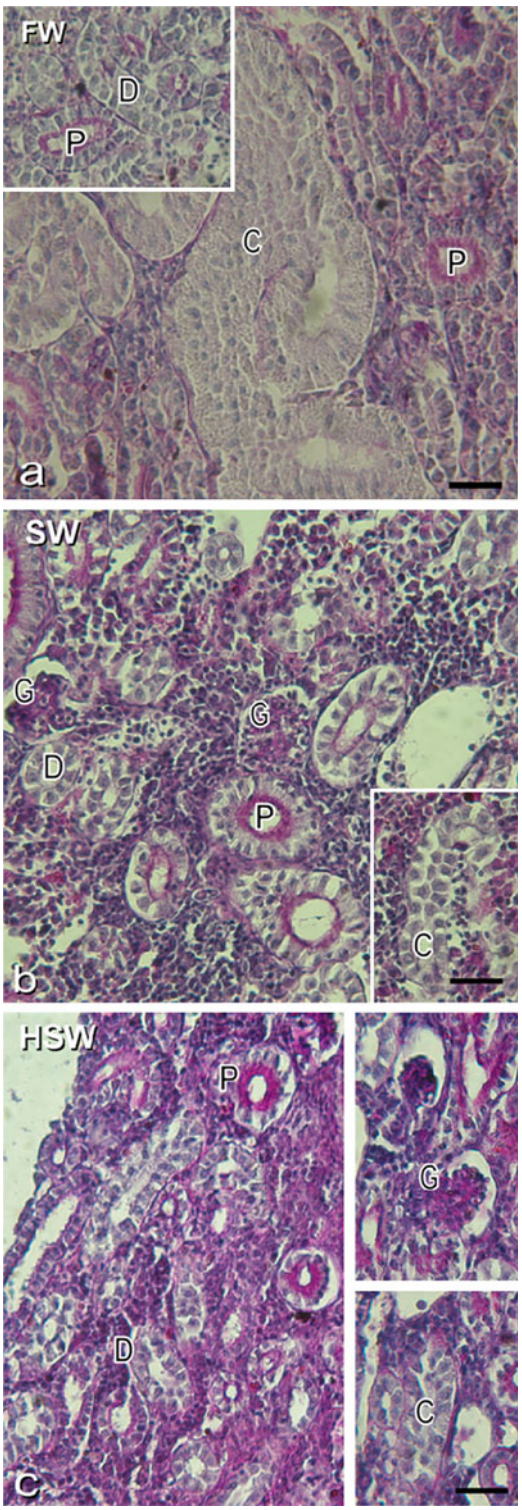

Fig. 2 Paraffin sections were stained with PAS reagent and hematoxylin to examine the general morphology of milkfish kidney. PAS-stained sections showed the structural features of the kidney in FW (**a**), SW (**b**), and HSW (**c**) milkfish. The significant *pink circles* in the cross sections of renal tubules indicated the apical brush border structures of epithelial cells constituting the renal tubules. *G* glomerus, *P* proximal tubule, *D* distal tubule, *C* collecting tubule. *Scale bar* 20 μm

acclimated to FW was significantly higher than that of fish acclimated to SW and HSW (Fig. 7). There was no significant difference in renal NKA mRNA abundance, protein amounts, as well as specific activity between SW and HSW-acclimated fish (Figs. 4, 5, 6, 7).

Discussion

Around 5% of teleosts species are euryhaline, having the capacity to withstand large changes in environmental salinity, a trait that is found among teleost lineages and has apparently evolved many times (Evans 1984). Milkfish (*Chanos chanos*) is an economic species commonly cultured in various salinity environments, including fresh water (FW), brackish water (BW), and seawater (SW) in Taiwan (Chen 1990). The culture conditions with a wide range of salinities show that the milkfish is an excellent euryhaline osmoregulator. The euryhaline osmoregulators usually maintain plasma osmolality in a narrow physiological range. In addition, MWC acts as a marker of the osmoregulatory capacity and as a measure of the degree of euryhalinity in fish (Freire et al. 2008). MWC was not dramatically changed and plasma osmolality was maintained in an isotonic range in milkfish acclimated to FW, SW (35‰) and HSW (60‰) (Fig. 1a, b). These two physiological parameters also remained in the homeostatic range in the other euryhaline teleosts acclimated to a wide range of salinities, such as gilthead sea bream, *Sparus aurata* (Sangiao-Alvarellos et al. 2003), spotted green pufferfish, *Tetraodon nigroviridis* (Lin et al. 2004; Tang et al. 2009), tilapia, *Oreochromis mossambicue* (Tipsmark et al. 2008a), two medaka species, *Oryzias latipes* and *O. dancena* (Kang et al. 2008), southern flounder *Paralichthys lethostigma* (Tipsmark et al. 2008b), and sailfin molly, *Poecilia latipinna* (Yang et al. 2009). The urine osmolality was found to be isotonic in SW- and HSW-acclimated milkfish (Fig. 1c). In FW-acclimated milkfish, however, urine osmolality was hypotonic (Fig. 1c), similar to FW-acclimated juvenile sea bass, *Dicentrarchus labrax* (Nebel et al. 2005). This result indicated that the ion-reabsorption mechanism might be triggered when milkfish were exposed to hypotonic FW environment.

The regulation of plasma osmolality in teleosts is achieved by integrating ion and water transport of the osmoregulatory organs, including gills, kidneys, and intestines (Marshall and Grosell 2006). When compared with the gills, however, the role of osmoregulation in kidneys has been less studied. The kidney structure of teleosts was classified into five types (Ogawa 1961). The kidney of milkfish was similar to most marine telesots and showed the morphological characteristics of the type III

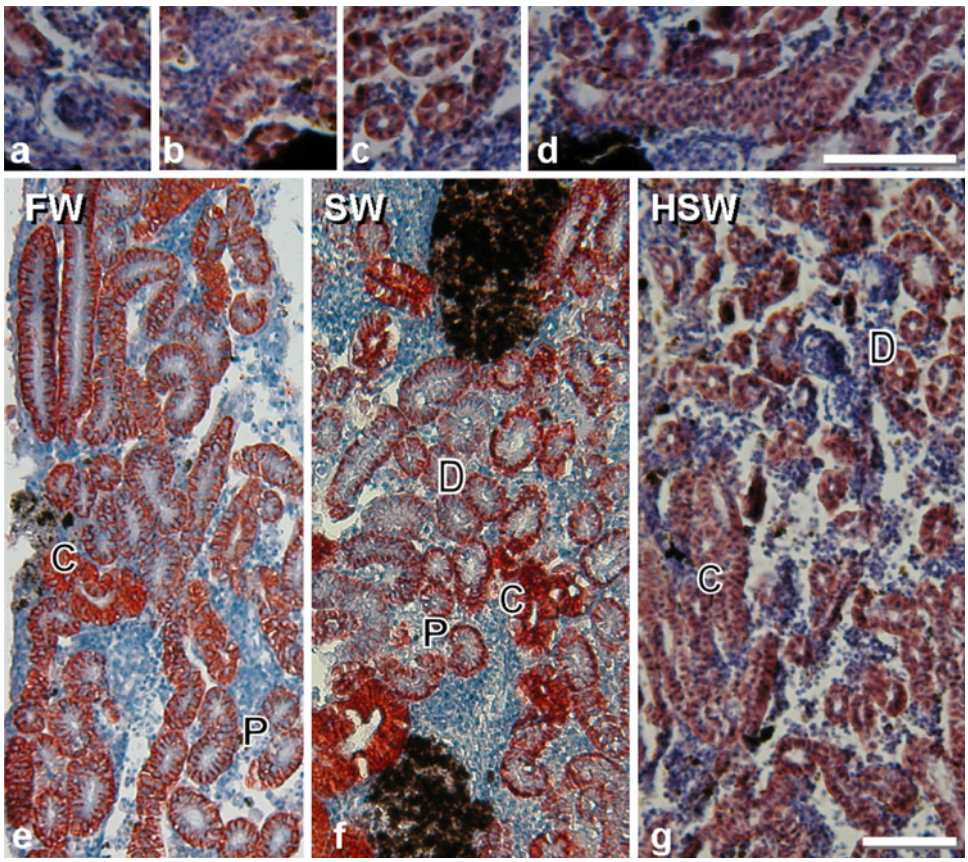

Fig. 3 Immunohistochemical localization of Na^+/K^+-ATPase (NKA) recognized by α5 antibody in milkfish kidneys acclimated to FW (**e**), SW (**f**), and HSW (**g**). In all groups of milkfish, positive immunostaining was found in the basolateral membrane of epithelial cells of proximal (**b**), distal (**c**), and collecting (**d**) tubules, but not in the glomerulus (**a**). The distribution pattern of renal NKA was similar among all groups (**e**, **f**, and **g**). *Scale bars* 50 μm. *P* proximal tubule, *D* distal tubule, *C* collecting tubule, *G* glomerus

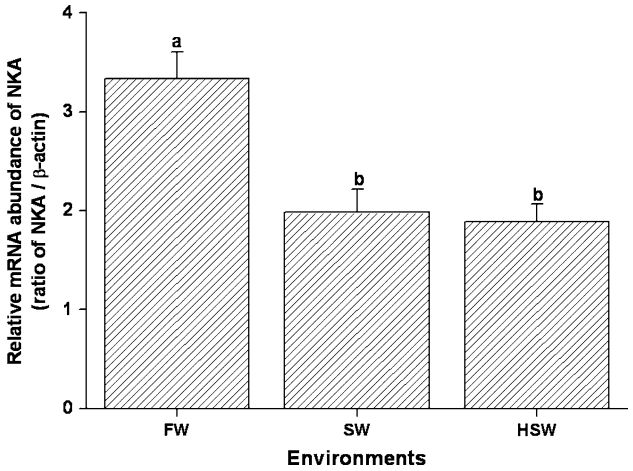

Fig. 4 Relative mRNA abundance of Na^+/K^+-ATPase (NKA) α1 isoform in kidneys of milkfish acclimated to environments of different salinities ($n \geq 8$). The *asterisk* indicated significant differences by one-way ANOVA, Tukey's pairwise-comparison test ($P < 0.05$). The mRNA level was significantly higher in the FW group than that in the SW and HSW groups. Values were mean ± SEM. *FW* fresh water, *SW* seawater (35‰), *HSW* hypersaline water (60‰)

kidney with a two-branched anterior portion and a fused posterior portion (Ogawa 1961). Moreover, the milkfish kidney exhibited the typical components of FW or euryhaline teleosts (Wendelaar bonga 1973; Endo and Kimura 1984; Lin et al. 2004, Katoh et al. 2008) with the glomerulus, proximal convoluted tubule, distal convoluted tubule, and collecting tubule in the nephron (Fig. 2).

Furthermore, the osmoregulatory organs are rich in NKA which provides the driving force for secondary transport system (McCormick 1995; Marshall and Grosell 2006). The significance of the osmoregulatory role of branchial NKA in teleosts has been investigated by different approaches through years in many species (see reviews of Marshall 2002; Hwang and Lee 2007). When compared with the results in the gills, previous studies on renal NKA of euryhaline teleosts mainly focused on NKA activity (Arjona et al. 2007; Gallis and Bourdichon 1976; Herrera et al. 2009; Kelly et al. 1999; Krayushkina et al. 2006; Lasserre 1971; Romao et al. 2001; Venturini et al. 1992). However, the present study integrated different approaches including immunohistochemistry, real-time PCR, immunoblotting, and specific activity assay to illustrate the potential roles of renal NKA in osmoregulation. Immunohistochemical staining showed that NKA was detected in the basolateral membrane of proximal, distal, and collecting tubules, but not found in glomeruli of milkfish kidneys of various groups (Fig. 3). Similar results were also reported in the other euryhaline teleosts (Lin et al. 2004; Ura et al. 1996). Localization of different ion

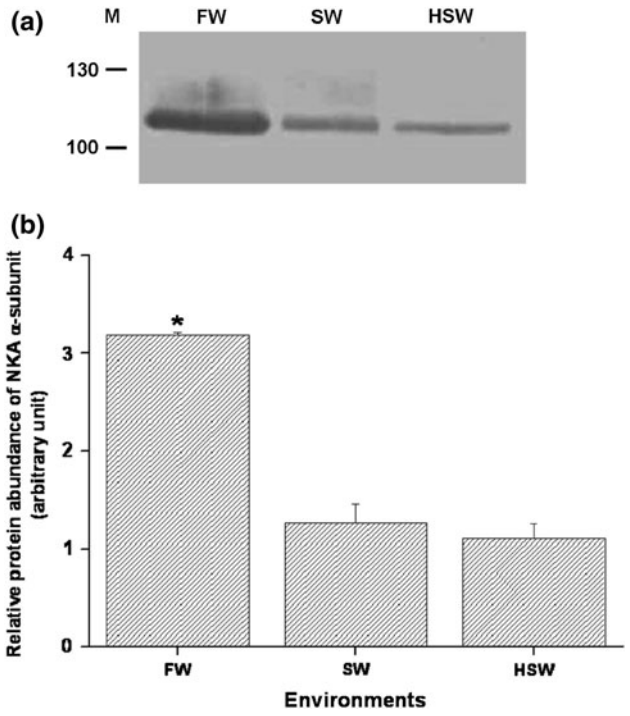

Fig. 5 Immunoblots of kidneys of milkfish acclimated to various salinities probed with a monoclonal antibody α5 to Na^+/K^+-ATPase (NKA) α subunit. **a** The representative immunoblot showed a single immunoreactive band in each environment with a molecular mass centered at 105 kDa. The immunoreactive bands of FW-acclimated fish were more intensive than SW and HSW-acclimated individuals. **b** Relative protein abundance of NKA α subunit in kidneys of milkfish in different environments ($n = 5$). NKA α subunit protein amounts were significantly higher in the FW group than the SW and HSW groups. The a*sterisk* indicated a significant difference by one-way ANOVA, Tukey's pairwise-comparison test ($P < 0.05$). Values were mean ± SEM. *FW* fresh water, *SW* seawater (35‰), *HSW* hypersaline water (60‰)

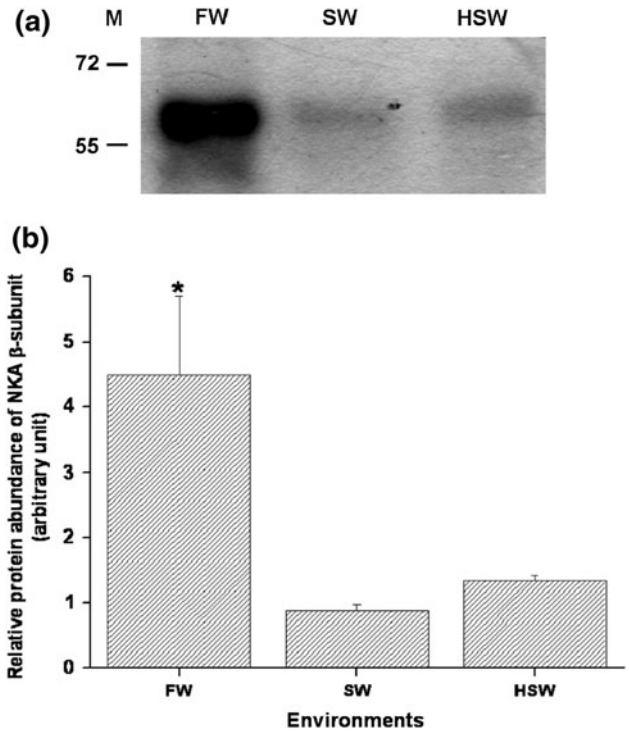

Fig. 6 Immunoblots of kidneys of milkfish acclimated to diverse salinities probed with a monoclonal antibody 24 to Na^+/K^+-ATPase (NKA) β subunit. **a** The representative immunoblot showed a single immunoreactive band in each environment with a molecular mass centered at 58 kDa. The immunoreactive bands of FW-acclimated fish were more intensive than SW and HSW-acclimated individuals. **b** Relative protein abundance of renal NKA β subunit in milkfish acclimated to different salinities ($n = 5$). NKA β subunit protein amounts were significantly higher in the FW group than the SW and HSW groups. The *asterisk* indicated a significant difference by one-way ANOVA, Tukey's pairwise-comparison test ($P < 0.05$). Values were mean ± SEM. *M* marker, *FW* fresh water, *SW* seawater (35‰), *HSW* hypersaline water (60‰)

transporter proteins (e.g., $Na^+/K^+/Cl^-$ cotransporter, NKCC; kidney specific chloride channel, ClC-K) will be examined in future studies to further identify the functions of different segments of renal tubules.

In higher vertebrates, the α1 isoform of NKA is primarily expressed in the transporting epithelia (Blanco and Mercer 1998). In teleosts, NKA α1 isoform has been cloned with high levels of homology including the white sucker, *Catostomus commersoni* (Schonrock et al. 1991), European eel, *Anguilla anguilla* (Cutler et al. 1995), Atlantic salmon, *Salmo salar* (Seidelin et al. 2001), killifish, *Fundulus heteroclitus* (Semple et al. 2002), and Mozambique tilapia, *O. mossambicus* (Feng et al. 2002). The full length of NKA α1 isoform in milkfish was also cloned (accession number: DQ512799). In this study, the expression of renal NKA α1 mRNA was significantly increased in FW milkfish as compared to SW and HSW fishes (Fig. 4) and the pattern was similar to protein abundance of NKA α subunit (Fig. 5). The responses of renal NKA α subunit, therefore, were regulated from transcriptional level to increase the protein amount when the milkfish were exposed to FW. Moreover, elevated protein amounts of renal NKA were also found in FW-acclimated pufferfish (Lin et al. 2004) and FW-successfully adapted sea bass (*D. labrax*). On the other hand, renal NKA contents were similar between SW-successfully- and FW-unsuccessfully-adapted sea bass (Nebel et al. 2005). These results obviously indicated the importance of renal NKA protein expressed higher in FW-acclimated euryhaline teleosts.

NKA has been demonstrated to be a heterodimer enzyme (α and β). Because NKA α subunit is a catalytic subunit, the protein amounts of α subunit were detected in several studies (Hiroi and McCormick 2007; Lin et al. 2003, 2004; Tang and Lee 2007; Tang et al. 2008; Tipsmark et al. 2002, 2004, 2008a, b). Nevertheless, the expression of both α and β subunits is required for NKA enzyme activity and inhibition of glycosylation of

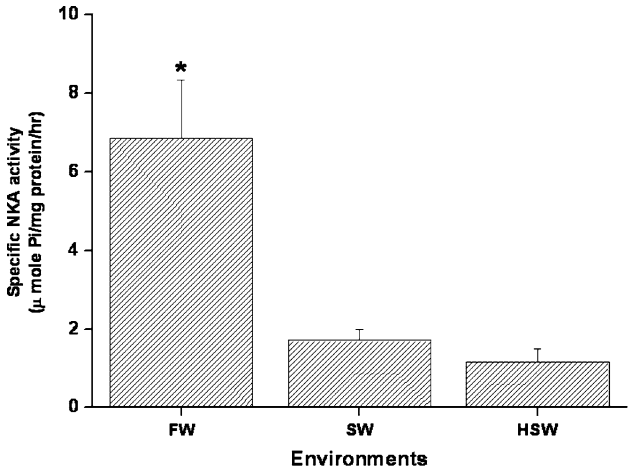

Fig. 7 Na^+/K^+-ATPase (NKA) activity of kidney in milkfish acclimated to environments of different salinities; $n = 5$ for all groups. FW-acclimated group was significantly higher than the other groups. The *asterisk* indicated a significant difference by one-way ANOVA, Tukey's pairwise-comparison test ($P < 0.05$). Values were mean ± SEM. *FW* fresh water, *SW* seawater (35‰), *HSW* hypersaline water (60‰)

β subunit can result in decrease in the cellular protein synthesis of both α and β subunits (McDonough et al. 1990). There are few studies, however, that detected both α and β subunits expression in gills (Deane and Woo 2004; Nilsen et al. 2007; Tse et al. 2006, 2007) or kidneys (Deane and Woo 2004). The present study examined protein abundance of NKA α subunit as well as β subunit simultaneously in milkfish kidneys (Figs. 5, 6). The 55-kDa immunoreactive band of β subunit (Fig. 6a) indicated that the glycosylated form (McDonough et al. 1990) was detected in kidneys of milkfish. The protein abundance of the NKA β subunit in kidney of FW-acclimated milkfish was higher than the other groups (Fig. 6b). The expression of both α and β subunits was greater in the kidney of FW-acclimated milkfish and this paralleled the pattern of specific activity of renal NKA (Fig. 7).

To minimize NaCl loss, FW-acclimated teleosts reabsorb approximately 95% of the NaCl that enters the glomerular filtrate and consequently they produce diluted urine (Fig. 1c; Perry et al. 2003). Although active ion reabsorption is often coupled with the osmotic water transport to the blood, the rate of water transport depends on hormonal stimulation, expression of aquaporins and rate of water delivery by the nephrons. Usually, the water permeability of the collecting ducts is very low in FW teleosts (Nishimura et al. 1983; Nishimura and Fan 2003). Moreover, whether urine was concentrated or diluted was decided due to the activity of the NaCl reabsorbing mechanism (Miyazaki et al. 2002). The responses of renal NKA (i.e., mRNA, protein, and activity) of FW-acclimated milkfish were greater than those of milkfish acclimated to SW or HSW. Meanwhile, no significant difference was found between renal NKA responses of SW- and HSW-acclimated milkfish. These results reflected the fact that fish could not produce urine hypertonic to blood due to the lack of Henle's loop in their kidney structures (Marshall and Grosell 2006). Renal NKA activity was also found to be higher in FW individuals than in SW or BW groups in several other euryhaline species, including killifish, *F. heteroclitus* (Epstein et al. 1969), thicklip gray mullet, *Crenimugil labrosus* (Lasserre 1971; Gallis and Bourdichon 1976), European sea bass, *D. labrax* (Lasserre 1971; Nebel et al. 2005; Venturini et al. 1992), thinlip mullet, *Liza ramada* (Gallis and Bourdichon 1976), striped bass, *Morone saxatilis* (Madsen et al. 1994), black seabream, *Mylio Macrocephalus* (Kelly et al. 1999), spotted green pufferfish, *T. nigroviridisand* (Lin et al. 2004), and three sturgeon species, *Acipenser* ruthenus, *A. gueldenstaedtii*, and *A. stellatus* (Krayushkina et al. 2006). In addition, kidney NKA activity was significantly increased when FW rainbow trout (*Oncorhynchus mykiss*) (Sloman et al. 2001) and goldfish (*Carassius auratus*) (Chasiotis et al. 2008) were exposed to ion-deficient water. Distinct from the kidney, the patterns of NKA activity in gills of euryhaline teleosts can be distinguished into two groups: (1) higher NKA activity in hyperosmotic media and (2) higher NKA activity in hypoosmotic media (reviewed by Hwang and Lee 2007). In addition, branchial NKA activities in milkfish acclimated to FW (Lin et al. 2003, 2006) as well as HSW (unpublished data) were higher than those of milkfish acclimated to their natural habitat, SW. Taken together, elevated renal NKA responses in most studied euryhaline teleosts indicated that, when acclimated to FW, NKA in teleost kidneys played a crucial role for ion reabsorption.

Euryhaline milkfish naturally inhabit or commercially cultured in environments of various salinities and they are capable of maintaining homeostasis. This study investigated different levels (i.e., histology, gene, protein, activity) of NKA expressed in kidneys of milkfish to illustrate the relationship between the osmoregulatoy plasticity and the role of renal NKA. To integrate our results with other euryhaline species, when acclimated to FW, the renal NKA showed higher responses and the urine was diluted and hypotonic. Hence, renal NKA of euryhaline fish might provide more driving force for the epithelial transporting system to efficiently trigger the mechanisms of ion reabsorption in the kidneys when they were acclimated to FW or ion-poor environments. Future studies will focus on the regulatory mechanisms (e.g., FXYD protein) of renal NKA in euryhaline teleosts.

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