

# Relative Changes in the Abundance of Branchial $\text{Na}^+/\text{K}^+$ -ATPase $\alpha$ -Isoform-Like Proteins in Marine Euryhaline Milkfish (*Chanos chanos*) Acclimated to Environments of Different Salinities

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**ABSTRACT** Previous studies revealed that upon salinity challenge, milkfish (*Chanos chanos*), the euryhaline teleost, exhibited adaptive changes in branchial  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) activity with different  $\text{Na}^+$  and  $\text{K}^+$  affinities. Since alteration of activity and ion-affinity may be influenced by changes in different isoforms of NKA  $\alpha$ -subunit (i.e., the catalytic subunit), it is, thus, intriguing to compare the patterns of protein abundance of three major NKA  $\alpha$ -isoform-like proteins (i.e.,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) in the gills of euryhaline milkfish following salinity challenge. The protein abundance of three NKA  $\alpha$ -isoform-like proteins in gills of milkfish reared in seawater (SW), fresh water (FW), as well as hypersaline water (HSW, 60‰) were analyzed by immunoblotting. In the acclimation experiments, the SW group revealed significantly higher levels of NKA  $\alpha 1$ - and  $\alpha 3$ -like proteins than the FW or HSW group. Time-course experiments on milkfish that were transferred from SW to HSW revealed the abundance of branchial NKA  $\alpha 1$ -like and  $\alpha 3$ -like proteins decreased significantly after 96 and 12 hr, respectively, and no significant difference was found in NKA  $\alpha 2$ -like protein. Furthermore, when fish were transferred from SW to FW, the amounts of NKA  $\alpha 1$ - and  $\alpha 3$ -like proteins was significantly decreased after 96 hr. Taken together, acute and chronic changes in the abundance of branchial NKA  $\alpha 1$ - and  $\alpha 3$ -like proteins may fulfill the requirements of altering NKA activity with different  $\text{Na}^+$  or  $\text{K}^+$  affinity for euryhaline milkfish acclimated to environments of various salinities. *J. Exp. Zool.* 311A:522–530, 2009. © 2009 Wiley-Liss, Inc.

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Maintaining a stable internal environment is important for vertebrates to survive in various habitats. Compared to terrestrial animals, fish must manage more challenging osmotic and ionic gradients from aquatic environments with diverse salinities, ion compositions, and pH values (Hwang and Lee, 2007). Salinity adaptation in euryhaline teleosts is a complex process involving a set of physiological responses to environments with differing ionoregulatory requirements. Although ionoregulation in fish is mediated by a group of structures including the intestine and kidney, the gill is the major organ for the balance of ion movement between gains and losses (Hirose

et al., 2003; Evans et al., 2005). To maintain osmolality and ionic balance, marine teleosts drink seawater (SW) to compensate for passive water loss and actively secrete salt via the gills as well as kidneys. On the contrary, freshwater (FW) teleosts do not drink (or drink very little) water,

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produce diluted urine via the kidneys to balance the passive water gain, and actively absorb salts through the gills from the environment (Marshall and Grosell, 2006). Effective ionoregulatory mechanisms thus enable teleosts to retain an osmotic and ionic constancy in their internal milieu and survive in hypertonic or hypotonic environments.

$\text{Na}^+/\text{K}^+$ -ATPase (NKA) is a membrane-spanning protein that couples the exchange of two extracellular  $\text{K}^+$  ions for three intracellular  $\text{Na}^+$  ions to the hydrolysis of one molecule of ATP (Post and Jolly, '57). It is crucial not only for sustaining intracellular homeostasis, but also for providing a driving force for many transporting systems including fish gills (Hwang and Lee, 2007). The  $\alpha$ -subunit of NKA containing the binding sites for ATP,  $\text{Na}^+$ , and  $\text{K}^+$  is considered to be the catalytic subunit. The  $\beta$ -subunit is a type II glycosylated polypeptide that is thought to assist in the folding and placement of the  $\alpha$ -subunit into the cell membrane (Blanco and Mercer, '98). A third, nonessential  $\gamma$ -subunit has been identified in mammals (Reeves et al., '80) and is thought to play roles in modulating  $\text{Na}^+$ ,  $\text{K}^+$ , and ATP binding affinities to the NKA  $\alpha\beta$  complex (Therien et al., '99). Immunocytochemical studies demonstrated that NKA was localized mainly in epithelial mitochondrion-rich cells of teleostean gills. The current model depicted that mitochondrion-rich cells in gill epithelia of teleosts used NKA as the primary driving force for operation of the different ion transporters depending on the environmental salinity (Hwang and Lee, 2007; Evans, 2008).

NKA exhibits four  $\alpha$  ( $\alpha 1$ -4) and three  $\beta$  ( $\beta 1$ -3) isoforms. Sequence variations in the amino terminus and 11 amino acids in the large intracellular loop between transmembrane domains 4 and 5 distinguished four isoforms of the NKA  $\alpha$ -subunit (Takeyasu et al., '90; Pressley, '92). These  $\alpha$ -subunit isoforms, which showed tissue-specific and developmentally dependent patterns of expression in birds and mammals, have been suggested to be presented in all vertebrate classes, including teleosts (Takeyasu et al., '90; Pressley, '92). Comparing the sequences of one orthologous isoform from many different species yields an amino acid identity of 90% or greater (Blanco and Mercer, '98). In teleosts, full length sequence of NKA  $\alpha$ -isoforms have been cloned in the white sucker ( $\alpha 1$ ; *Catostomus commersoni*; Schönrock et al., '91), the European eel ( $\alpha$ ; *Anguilla anguilla*; Cutler et al., '95), tilapia ( $\alpha 1$  and  $\alpha 3$ ; *Oreochromis mossambicus*; Feng et al., 2002), killifish ( $\alpha 1$  and

$\alpha 2$ ; *Fundulus heteroclitus*; Semple et al., 2002), and rainbow trout ( $\alpha 1a$ ,  $\alpha 1b$ ,  $\alpha 1c$ ,  $\alpha 2$ , and  $\alpha 3$ ; *Oncorhynchus mykiss*; Richards et al., 2003). In addition, nine distinct NKA  $\alpha$ -subunit genes have been identified in zebrafish (*Danio rerio*) genomic databases. Among them, four ( $\alpha 1a.1$ ,  $\alpha 1a.2$ ,  $\alpha 1a.4$ , and  $\alpha 1a.5$ ) of the seven  $\alpha 1$  genes were found to be expressed in the mucous cells of skin (Blasiolo et al., 2002; Canfield et al., 2002). In addition, partial sequences of  $\alpha$ -isoforms were reported in the spiny dogfish ( $\alpha 3$ ; *Squalus acanthias*; Hansen, '99) and the Antarctic nototheniid ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ; *Trematomus bernachii*; Guynn et al., 2002).

Milkfish (*Chanos chanos*) is a marine teleost widely distributed throughout the tropical and subtropical Indo-Pacific. The euryhalinity of milkfish was demonstrated throughout its life history, although it did not appear to require the fresh water (FW) for any part of its life cycle (Bagrinao, '94). Milkfish are found naturally or cultured commercially in FW, SW, and hypersaline water (HSW) (Crear, '80). These characteristics make milkfish a good experimental species for studies on osmoregulation. Previous studies revealed that milkfish, like other euryhaline teleosts, exhibited adaptive changes in branchial NKA responses (i.e., mRNA, protein, activity, immunoreactive cell number) upon salinity challenge (Lin et al., 2003, 2006). Moreover, Pagliarani et al. ('91) demonstrated that NKA proteins isolated from FW- and SW-acclimated fish gills had different biochemical properties and suggested that different NKA isoforms might correlate with ion regulation in different environmental salinities. Since  $\alpha$ -subunit is the catalytic subunit of NKA, alteration of NKA  $\alpha$ -isoforms protein expression was supposed to be a crucial mechanism for euryhaline teleosts to adapt to environments of various salinities. Feng et al. (2002) provided evidence of enhanced transcription of branchial NKA  $\alpha 1$  and  $\alpha 3$  genes in Mozambique tilapia following transfer from FW to SW. Recent studies focused on mRNA expression patterns of two NKA  $\alpha$ -isoforms ( $\alpha 1a$  and  $\alpha 1b$ ) revealed their different responses to salinity changes in salmonid fishes (Richards et al., 2003; Shrimpton et al., 2005; Bystriansky et al., 2006, 2007a,b; Nilsen et al., 2007). However, Morrison et al. (2006) reported that changes in the protein, but not mRNA levels of NKA  $\alpha$ -subunit isoforms (i.e.,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ), occurred in the gills of warm acclimated Antarctic teleost (*Trematomus bernachii*). This motivates this study to investigate the protein expression patterns of gill NKA  $\alpha$ -subunit isoforms in euryhaline milkfish following salinity-

transfer, to illustrate the potential role of NKA  $\alpha$ -isoforms in this marine euryhaline teleost upon salinity challenge.

## MATERIALS AND METHODS

### *Experimental animals*

Juvenile milkfish (*C. chanos*) with  $13.6 \pm 0.2$  cm total length and  $22.1 \pm 5.9$  g body weight were obtained from a local fish farm. Values of total length and body weight were expressed as the mean  $\pm$  SEM. SW (35‰) or HSW (60‰) were prepared from local tap water (i.e., FW) added with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH). The water was continuously circulated through fabric-floss filters. Milkfish were kept in SW at  $28 \pm 1^\circ\text{C}$  with a daily 12 hr photoperiod for at least two weeks before experiments. Fish were fed a daily diet of commercial fodder.

### *Acclimation experiments*

Milkfish kept in the laboratory for at least two weeks were transferred into experimental tanks with HSW, SW, and FW, respectively, and the culture conditions were identical to those described above. Milkfish were held in experimental tanks for two weeks prior to sampling.

### *Time-course environments*

For time-course experiments, milkfish of the experimental group were transferred directly from stock SW to FW or HSW, while fish in the control group were transferred to SW simultaneously. Because fish were transferred by nets, the control group was used to monitor the effect of handling fish alone. After transfer, the fish were caught by netting at 12, 24, 48, 96, and 168 hr and sampled for immunoblotting.

### *Preparation of gill homogenates*

Gills were excised and blotted dry. After removing the gill arches, gills were immediately cut into small pieces. All subsequent procedures were performed on ice. Gill scrapings were suspended in the mixture of homogenization solution (100 mM imidazole-HCl buffer, pH 7.6; 5 mM  $\text{Na}_2\text{EDTA}$ ; 200 mM sucrose; and 0.1% sodium deoxycholate) and proteinase inhibitors (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidin dissolved in 5 mL aprotinin) (v/v: 100/1). Homogenization was performed with a homogenizer (Polytron PT 1200E KINEMATICA, Lucerne, Switzerland) at a

maximum speed for 25 strokes. The homogenates were then centrifuged at 13,000g for 20 min at  $4^\circ\text{C}$ . The supernatants were used to determine protein concentrations and for immunoblotting. Protein concentrations were identified by BCA protein assay reagents (PIERCE, Rockford, IL) using bovine serum albumin (Sigma, St. Louis, MO) as a standard. All gill lysates were stored at  $-80^\circ\text{C}$  before immunoblotting.

### *Antibodies*

NKA  $\alpha 1$ -isoform antibody, mouse monoclonal antibody (a6F) raised against the  $\alpha 1$ -isoform of the avian NKA was purchased from the Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA). The dilution rate is 1:5,000 for immunoblotting. NKA  $\alpha 2$ -isoform antibody was the commercial goat polyclonal antibody (sc-16049), raised against a peptide mapping within an internal region of the NKA  $\alpha 2$ -subunit of human origin (Santa Cruz Biotechnology, Santa Cruz, CA). The dilution rate is 1:500 for immunoblotting. NKA  $\alpha 3$ -isoform antibody was the commercial goat polyclonal antibody (sc-16052), raised against a peptide mapping within an internal region of NKA  $\alpha 3$  of human origin (Santa Cruz Biotechnology). The dilution rate is 1:100 for immunoblotting. Alkaline phosphatase-conjugated goat anti-mouse or rabbit anti-goat antibodies (Jackson Immuno Research, West Grove, PA) were used as secondary antibodies for immunoblotting.

### *Immunoblotting analysis*

Immunoblotting procedures were performed according to Tang et al. (2008) with little modification. Seventy-five microgram of protein from gill homogenates was heated with sample buffer at  $37^\circ\text{C}$  for 30 min and fractionated by electrophoresis on sodium dodecyl sulfate-containing 7.5% polyacrylamide gels. The prestained protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD). Separated proteins were then electro-transferred to PVDF membranes (Millipore, Bedford, MA). Blots were preincubated in PBST (phosphate buffer saline with Tween 20) buffer ( $137 \text{ mmol L}^{-1}$  NaCl;  $3 \text{ mmol L}^{-1}$  KCl;  $10 \text{ mmol L}^{-1}$   $\text{Na}_2\text{HPO}_4$ ;  $2 \text{ mmol L}^{-1}$   $\text{KH}_2\text{PO}_4$ ; 0.2% Tween 20; pH 7.4) containing 5% w/v nonfat dried milk to minimize nonspecific binding, then incubated at  $4^\circ\text{C}$  overnight, with the primary antibodies diluted in PBST containing 1% BSA and 0.05% sodium azide. The blots were washed in PBST and incubated at room temperature for 1 hr with

secondary antibodies. Blots were developed after incubation with BCIP/NBT kit (Zymed, South San Francisco, CA). Immunoblots were scanned and images were imported as TIF files into the commercial image analysis software package (MCID software; Imaging Research, Ontario, Canada). Results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

### Statistical analysis

Values of relative intensities of immunoreactive bands were expressed as the mean  $\pm$  SEM and compared using the one-way analysis of variance (ANOVA) followed by Tukey's pair-wise method for acclimation experiments or Dunnett's test for time-course experiments.

## RESULTS

### Long-term acclimation of milkfish to environments with different salinities

The antibodies corresponding to different NKA  $\alpha$ -isoforms ( $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 3) were used to determine the protein expression patterns in milkfish gills. An approximately 105 kDa NKA  $\alpha$ 1-like immunoreactive protein was detected in gill homogenates preparations from milkfish acclimated to different salinities (Fig. 1A). The intensity of immunoreactive bands in the SW-acclimated group was about 4-fold higher than the FW-acclimated fish or HSW-acclimated fish (Fig. 1B). Immunoblots of NKA  $\alpha$ 2-like protein resulted in a single immunoreactive band of about 114 kDa (Fig. 2A). The immunoreactive bands were similar among all groups. Relative protein abundance in the immunoreactive bands revealed the protein abundance of NKA  $\alpha$ 2-like protein in the gills were unaffected by environmental salinity (Fig. 2B). Immunoblots of NKA  $\alpha$ 3-like protein also resulted in a single immunoreactive band of about 105 kDa (Fig. 3A). Similar to the pattern of  $\alpha$ 1-like protein, milkfish exposed to SW environment exhibited a significant increase (about 1.5-folds) in NKA  $\alpha$ 3-like protein expression compared with individuals exposed to FW and HSW (Fig. 3B).

### Abrupt exposure of milkfish from SW to HSW

Like the experiment of long-term acclimation, a 105 kDa NKA  $\alpha$ 1-like immunoreactive protein was detected in the gill of milkfish (Fig. 4A). Compared to the 0 hr group, the levels of NKA  $\alpha$ 1-like protein

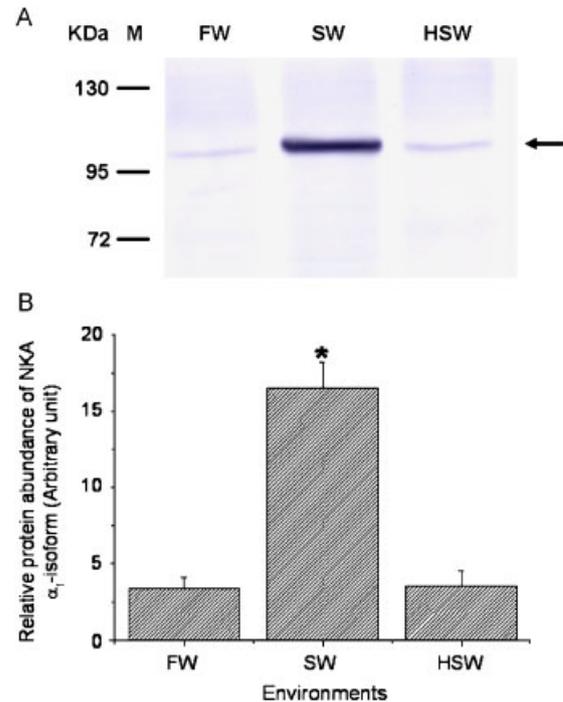


Fig. 1. The protein expression of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ 1-like protein in the gills of milkfish acclimated to environments of different salinities for two weeks. (A) Representative immunoblot probed with a monoclonal antibody. The indicated immunoreactive band is approximately at 105 kDa (arrow). The immunoreactive bands of SW-acclimated fish were more intensive than FW- or HSW-acclimated individuals. (B) The relative protein abundance of immunoreactive bands of the SW group was 4-fold higher than those of FW or HSW groups. Values were mean  $\pm$  SEM ( $n = 6$ ). The asterisk indicated a significant difference ( $P < 0.05$ ) using Tukey's pair-wise comparison test following a one-way ANOVA. M, marker; FW, fresh water; SW, seawater; HSW, hypersaline water.

in gills declined gradually within 48 hr post-transfer and were decreased 4-fold at 96 hr and sustained to 168 hr after salinity transfer (Fig. 4B). The representative immunoblot of NKA  $\alpha$ 2-like protein resulted in a single immunoreactive band of about 114 kDa (Fig. 5A). Compared to the 0 hr group, the abundance of branchial NKA  $\alpha$ 2-like protein was not significantly different following salinity transfer (Fig. 5B). Moreover, NKA  $\alpha$ 3-like immunoreactive proteins were detected in gill protein preparations from milkfish. The NKA  $\alpha$ 3-like protein was approximately 105 kDa (Fig. 6A). Compared to the 0 hr group, protein levels of gill NKA  $\alpha$ 3-like protein decreased significantly since 12 hr post-transfer (Fig. 6B). No significant difference was found in the levels of three NKA  $\alpha$ -isoform-like proteins during the time-course in the control group fish (Figs. 4B, 5B, and 6B).

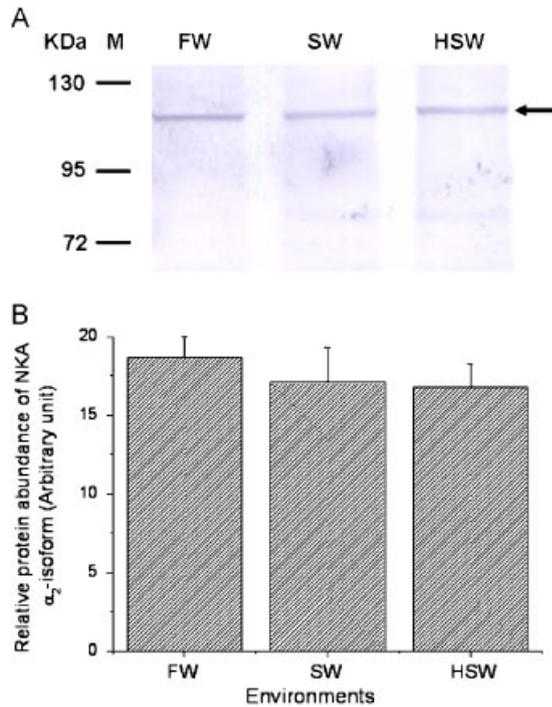


Fig. 2. The protein expression of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha_2$ -like protein in the gills of milkfish acclimated to environments of different salinities for two weeks. (A) Representative immunoblot probed with a polyclonal antibody. The indicated immunoreactive band is approximately at 114 kDa (arrow). The immunoreactive bands of all groups were similar. (B) The relative protein abundance of immunoreactive bands showed no significantly different. Values were mean  $\pm$  SEM ( $n = 6$ ). Different letters indicated significant differences ( $P < 0.05$ ) using Tukey's pair-wise comparison test following a one-way ANOVA. M, marker; FW, fresh water; SW, seawater; HSW, hypersaline water.

### ***Abrupt exposure of milkfish from SW to FW***

The representative immunoblot of NKA  $\alpha_1$ -like protein resulted in a single immunoreactive band of about 105 kDa (Fig. 4A). The amounts of NKA  $\alpha_1$ -like protein decreased at 12 hr post-transfer, increased gradually to a peak at 48 hr post-transfer, and then were decreased 3-fold at 96 and 168 hr after salinity transfer (Fig. 4B). The representative immunoblot of NKA  $\alpha_2$ -like protein resulted in a single immunoreactive band of about 114 kDa (Fig. 5A). Similar to transfer from SW to HSW experiment, the abundance of branchial NKA  $\alpha_2$ -like protein was not significantly different following transfer from SW to FW (Fig. 5B). The representative immunoblot of NKA  $\alpha_3$ -isoform resulted in a single immunoreactive band of about 105 kDa (Fig. 6A). During the first 48 hr post-transfer, NKA  $\alpha_3$ -like protein abundance decreased gradually and the levels of NKA  $\alpha_3$ -like

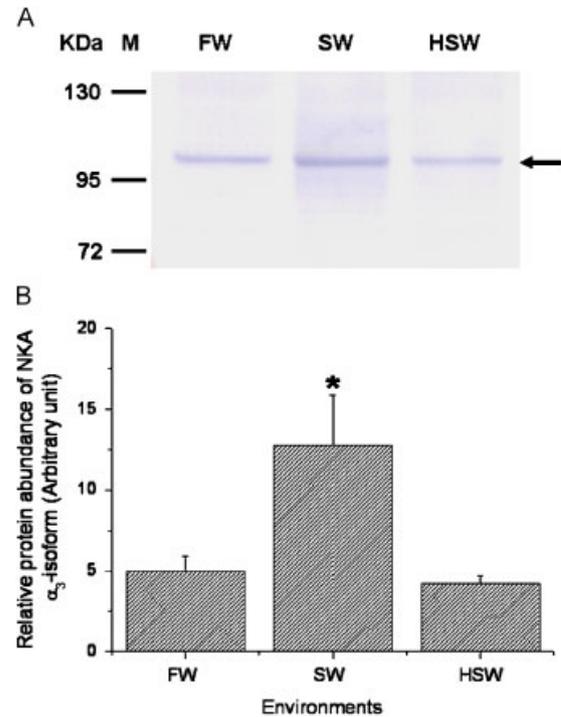


Fig. 3. The protein expression of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha_3$ -like protein in the gills of milkfish acclimated to environments of different salinities for two weeks. (A) Representative immunoblot probed with a polyclonal antibody. The indicated immunoreactive band is approximately at 105 kDa (arrow). The immunoreactive bands of SW-acclimated fish were more intensive than those of FW- or HSW-acclimated individuals. (B) The relative protein abundance of immunoreactive bands of the SW group was 1.5-fold higher than those of FW or HSW groups. Values were mean  $\pm$  SEM ( $n = 6$ ). The asterisk indicated a significant difference ( $P < 0.05$ ) using Tukey's pair-wise comparison test following a one-way ANOVA. M, marker; FW, fresh water; SW, seawater; HSW, hypersaline water.

protein significantly decreased at 96 and 168 hr following salinity transfer (Fig. 6B). In fish of the control group, no significant difference was found in the amounts of three NKA  $\alpha$ -isoform-like-proteins in the time-course experiment (Figs. 4B, 5B, and 6B).

## **DISCUSSION**

The osmoregulatory systems used by euryhaline teleosts to adapt to FW and SW differ not only in the direction of ion and water movements but also in their molecular components. Euryhaline fish adapts to FW and SW by switching these systems efficiently (Kato et al., 2005). In the gills of euryhaline teleosts, NKA activity changing with environmental salinity is thought to provide the driving force for transporting ions across epithelia

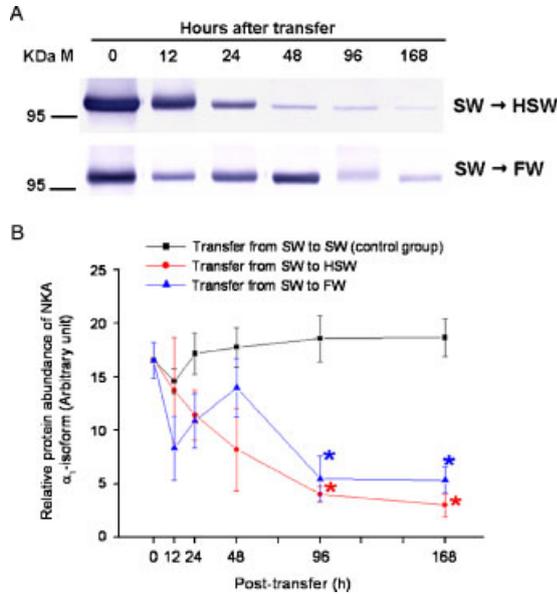


Fig. 4. The summary of  $\text{Na}^+/\text{K}^+$ -ATPase (NKA)  $\alpha_1$ -like protein in the gills of milkfish sampled at a series of different times after transfer from SW to HSW or FW. (A) Representative immunoblots of NKA  $\alpha_1$ -like protein probed with a monoclonal antibody revealed single immunoreactive bands at approximately 105 kDa. (B) The relative protein abundance following transfer from SW to HSW or FW indicated that NKA  $\alpha_1$ -like protein levels declined gradually during the first 48 hr post-transfer and decreased 4-fold at 96 and 168 hr after transfer. When fish were transferred from SW to FW, NKA  $\alpha_1$ -like protein levels decreased during the first 12 hr post-transfer, then increased gradually until 48 hr post-transfer, and finally decreased 3-fold at 96 and 168 hr post-transfer. Values were mean  $\pm$  SEM ( $n = 6$ ). The asterisks indicated significant differences ( $P < 0.05$ ) compared with the 0 hr using Dunnett's test following a one-way ANOVA. M, marker; SW, seawater; HSW, hypersaline water.

in both absorptive (in FW) and secretory (in SW) modes (Marshall, 2002; Hwang and Lee, 2007). The branchial NKA expression (mRNA, protein, and activity) were higher in milkfish acclimated to FW (Lin et al., 2003, 2006) and HSW (unpublished data) than SW-acclimated individuals. This indicates that this marine species maintains a comparatively low level of NKA expression in their primary natural habitats. Similarly, some euryhaline teleosts responded to FW with higher gill NKA expression, e.g., European seabass (*Dicentrarchus labrax*; Lasserre, '71), mullets (*Mugil cephalus*; Ciccotti et al., '94), gilthead Sea Bream (*Sparus auratus*; Laiz-Carrión et al., 2005), black porgy (*Acanthopagrus schlegeli*; Choi and An, 2008). On the contrary, the other euryhaline teleosts responded with higher gill NKA expression following acclimation to SW, e.g., Atlantic salmon (*Salmo salar*; D'Cotta et al., 2000), tilapia

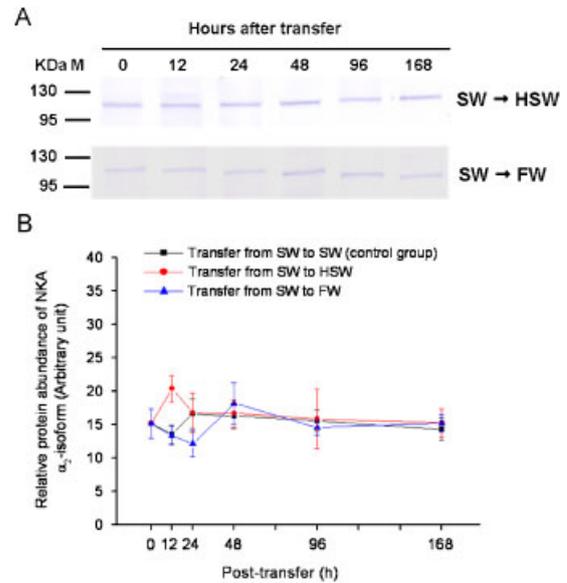


Fig. 5. The summary of  $\text{Na}^+/\text{K}^+$ -ATPase (NKA)  $\alpha_2$ -like protein in gills of milkfish sampled at a series of different times after transfer from SW to HSW or FW. (A) Representative immunoblots of NKA  $\alpha_2$ -like protein probed with a polyclonal antibody revealed single immunoreactive bands at approximately 114 kDa. (B) The relative protein abundance following transfer from SW to HSW or FW indicated that NKA  $\alpha_2$ -like protein expression was not significantly different following salinity transfer compared to the 0 hr. Values were mean  $\pm$  SEM ( $n = 6$ ). The asterisks indicated significant differences ( $P < 0.05$ ) compared with the 0 hr using Dunnett's test following a one-way ANOVA. M, marker; SW, seawater; HSW, hypersaline water.

(*Oreochromis mossambicus*; Lee et al., 2003), and spotted green pufferfish (*Tetraodon nigroviridis*; Lin et al., 2004). Different patterns of salinity-dependent branchial NKA expression may result from diverse primary natural habitats of euryhaline teleosts with differing mechanisms to respond to ambient salinity changes. Furthermore, branchial NKA proteins isolated from FW- and SW-acclimated rainbow trout (*Oncorhynchus mykiss*) exhibited different biochemical properties. It was suggested that diverse NKA isoforms might be involved in ionoregulation of environments with different salinities (Pagliarani et al., '91).

Characterization of rat NKA  $\alpha$ - and  $\beta$ -isoforms in insect cell lines demonstrated that  $\text{Na}^+$  and  $\text{K}^+$  affinity varied among NKA isoform combinations with a rank order of  $\alpha_2\beta_1 > \alpha_1\beta_1 > \alpha_3\beta_1$  and  $\alpha_1\beta_1 > \alpha_2\beta_1 > \alpha_3\beta_1$ , respectively (Blanco and Mercer, '98). The biochemical or kinetic properties of NKA were thus strongly influenced by different isoforms of catalytic  $\alpha$ -subunit to match physiological demands. Different affinities of gill NKA for  $\text{Na}^+$  and  $\text{K}^+$  were reported in FW- and

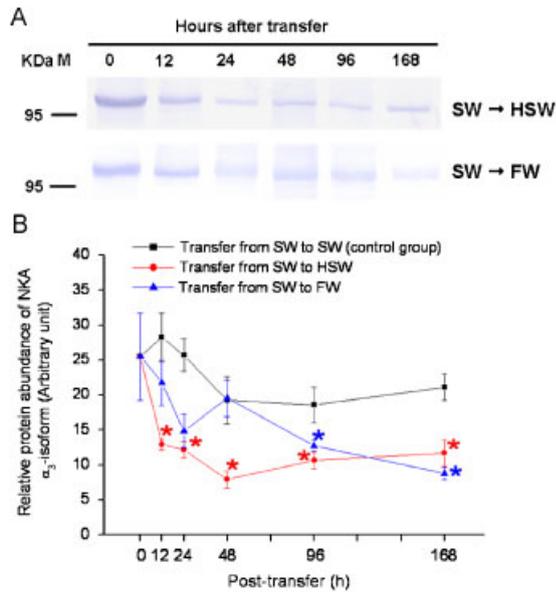


Fig. 6. The summary of  $\text{Na}^+/\text{K}^+$ -ATPase (NKA)  $\alpha_3$ -isoform in gills of milkfish sampled at a series of different times after transfer from SW to HSW or FW. (A) Representative immunoblots of NKA  $\alpha_3$ -isoform probed with a polyclonal antibody revealed single immunoreactive bands at approximately 105 kDa. (B) The relative protein abundance following transfer from SW to HSW indicated that NKA  $\alpha_3$ -isoform decreased significantly after 12 hr post-transfer. On the other hand, following transfer from SW to FW, changes in the relative protein abundance of NKA  $\alpha_3$ -isoform levels declined gradually during 48 hr post-transfer and significantly decreased at 96 and 168 hr post-transfer. Values were mean  $\pm$  SEM ( $n = 6$ ). The asterisks indicated significant differences ( $P < 0.05$ ) compared with the 0 hr using Dunnett's test following a one-way ANOVA. M, marker; SW, seawater; HSW, hypersaline water.

SW-acclimated milkfish (Lin and Lee, 2005). Therefore, alteration of NKA activity in gills of fish following salinity changes may result from different NKA isoforms.

There is accumulating evidence to presume that NKA  $\alpha$ -isoforms may change in teleosts to cope with the environmental fluctuations, e.g., salinity or temperature. The mRNA expression patterns of gill NKA  $\alpha_1a$  and  $\alpha_1b$  examined in various salmonid species were found to express differentially in FW- and SW-acclimated individuals (Richards et al., 2003; Bystriansky et al., 2006, 2007a,b; Nilsen et al., 2007). Changes in mRNA expression are often assumed to parallel changes in protein abundance. The lack of correlation between mRNA and protein of branchial NKA  $\alpha$ -isoforms was reported (Morrison et al., 2006). This lack of correlation between mRNA and protein suggested the correlation with posttranscriptional regulation (Scott et al., 2004). This study,

therefore, investigates the effect of environmental salinity on the protein expression of branchial NKA  $\alpha$ -isoforms-like proteins in milkfish.

The amino acid identities of individual isoforms across species are higher than the identities of different isoforms within one species (Blanco and Mercer, 1998). Therefore, the heterologous antibodies to NKA  $\alpha$ -isoforms (polyclonal antibodies directed against the rat  $\alpha$ -specific isoforms) were used in Atlantic salmon (*Salmo salar*) (D'Cotta et al., 2000) as well as in this study. Among the isoform-specific heterologous antibodies used in milkfish, mouse monoclonal antibody a6F to the avian NKA  $\alpha_1$ -isoform and goat polyclonal antibody sc-16052 to human NKA  $\alpha_3$ -isoform had been applied in teleosts previously (tilapia, *Oreochromis mossambicus*; Lee et al., 1998; Antarctic fish, *Trematomus bernacchii*; Brauer et al., 2005). This study identified three NKA  $\alpha$ -isoform-like proteins in milkfish gills with these isoform-specific antibodies by immunoblotting analysis (Figs. 1A, 2A, and 3A). Three NKA  $\alpha$ -isoform-like proteins were also found in the gills of Antarctic fish (Guynn et al., 2002), while only  $\alpha_1$  and  $\alpha_3$  were reported in tilapia gills (Lee et al., 1998; Feng et al., 2002). The immunoblots of milkfish gill lysates probed with each isoform-specific antibody revealed single immunoreactive bands at approximately 105–115 kDa (Figs. 1A, 2A, and 3A), similar to those recognized in tilapia (Lee et al., 1998) and Antarctic fish (Guynn et al., 2002; Brauer et al., 2005).

This study revealed that the SW-acclimated group was significantly higher than the FW- or HSW-acclimated groups in the levels of NKA  $\alpha_1$ - and  $\alpha_3$ -like proteins (Figs. 1 and 3). Expression pattern of NKA  $\alpha_2$ -like protein in the gills were not salinity-dependent (Fig. 2B). It was suggested that  $\alpha_1$ - and  $\alpha_3$ -like proteins might play the role for osmo- or ionoregulation but  $\alpha_2$ -like protein probably performed the other physiological function. The affinity of gill NKA for sodium increased significantly in warm-acclimated Antarctic teleost (*T. bernacchii*; Morrison et al., 2006) that is opposite to milkfish, which has lower sodium affinity in SW than FW (Lin and Lee, 2005). Upregulation of gill  $\alpha_1$ - and  $\alpha_2$ -like proteins influenced sodium affinities and fulfilled some of the requirements for the subtly moderated enzyme behaviors (e.g., elevated NKA activity) in warm-acclimated *T. bernacchii* (Morrison et al., 2006), while the changes in gill NKA  $\alpha_1$ - and  $\alpha_3$ -like proteins may play the potential role for reduction of sodium affinities to decrease NKA activity in SW-exposed milkfish. In addition, the role of NKA

$\alpha 1$ -like protein in milkfish gills was similar to the NKA  $\alpha 1b$  mRNA reported in salmonid fishes as the major SW-responsive NKA  $\alpha$ -isoform.

Upon acute challenge with environmental salinity, urgent and efficient response of the regulatory mechanisms is required to carry out physiological processes to maintain osmotic and ionic balance in fish body fluids (Marshall et al., '99; Lin et al., 2004, 2006). Acute responses of three  $\alpha$ -isoform-like proteins in milkfish were also investigated in this study. The relative abundance of branchial NKA  $\alpha 1$ -like protein decreased immediately upon transfer from SW to HSW, resulted in 4-fold decrease at 96 hr post-transfer, and sustained to 168 hr (Fig. 4). Moreover, the abundance of NKA  $\alpha 3$ -like protein decreased rapidly during the first 12 hr post-transfer (Fig. 6). These results suggested that NKA  $\alpha 3$ -like protein may perform more efficient and earlier regulation than  $\alpha 1$ -like protein when milkfish are transferred from SW to HSW. As with the acclimation experiment, no significant difference was found in NKA  $\alpha 2$ -like protein abundance at each time point after transfer to HSW (Fig. 5). On the other hand, the relative abundance of NKA  $\alpha 1$ - and  $\alpha 3$ -like proteins reduced significantly at 96 hr following transfer from SW to FW (Figs. 4 and 6). According to the time-course experiments, it was presumed that euryhaline milkfish adapted to various environmental salinities (i.e., transfer from SW to HSW or FW) with the crucial regulated responses of NKA  $\alpha$ -isoforms triggered at 96 hr.

In this study, three NKA  $\alpha$ -isoform-like proteins ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) were identified in milkfish gills and relative changes in protein levels in different salinity acclimation were examined. The time-course experiment showed that highly efficient changes in the relative abundance of  $\alpha$ -isoform-like proteins illustrated the outstanding osmoregulatory ability of the euryhaline milkfish. The patterns of branchial NKA  $\alpha$ -isoforms protein abundance did not concert to pan- $\alpha$  ( $\alpha 5$ ) antibody. The existence of the  $\alpha$ -isoforms other than  $\alpha 1$ , 2, and 3 reacting on osmoregulation in the gills of this euryhaline species is the possible reason. Further study is needed to determine whether the other  $\alpha$ -isoforms exists in gills of this euryhaline species and to discuss the independent roles of different isoforms.

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