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The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water☆

Y.M. Lin, C.N. Chen, T.H. Lee*

Department of Life Sciences, National Chung-Hsing University, 250, Kuo-Kuang Road, Taichung 402, Taiwan

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Abstract

Juvenile milkfish *Chanos chanos* (Forsskål, 1775) were transferred from a local fish farm to fresh water (FW; 0‰), brackish water (BW; 10‰, 20‰) and seawater (SW; 35‰) conditions in the laboratory and reared for at least two weeks. The blood and gill of the fish adapted to various salinities were analyzed to determine the osmoregulatory ability of this euryhaline species. No significant difference was found in plasma osmolality, sodium or chloride concentrations of milkfish adapted to various salinities. In FW, the fish exhibited the highest specific activity of Na, K-ATPase (NKA) in gills, while the SW group was found to have the lowest. Relative abundance of branchial NKA α-subunit revealed similar profiles. However, in contrary to other euryhaline teleosts, i.e. tilapia, salmon and eel, the naturally SW-dwelling milkfish expresses higher activity of NKA in BW and FW. Immunocytochemical staining has shown that most Na, K-ATPase immunoreactive (NKIR) cells in fish adapted to BW and SW were localized to the filaments with very few on the lamellae. Moreover, in FW-adapted milkfish, the number of NKIR cells found on the lamellae increased significantly. Such responses as elevated NKIR cell number and NKA activity are thought to improve the osmoregulatory capacity of the milkfish in hyposaline environments.

Keywords: Fresh water; Brackish water; Seawater; Osmoregulation; Gill; Mitochondrion-rich cell; Na, K-ATPase; Milkfish

1. Introduction

Although ionoregulation in fish is mediated by a group of structures including the gastrointestinal epithelium and kidney, the gill is the major site in the balance of ion movement between diffusional gains or losses (Evans, 1993). In the branchial epithelium, mitochondrion-rich cells (MR cells, i.e. chloride cells) are the main sites for active ion transport. They secrete ions in seawater (SW)-adapted fish and, in freshwater (FW)-adapted fish, absorb ions and maintain acid-base balance (Wood and Marshall, 1994).

Na, K-ATPase (NKA) is a universal membrane-bound enzyme that actively transports Na^+ out of and K^+ into animal cells. It is important not only for sustaining intracellular homeostasis but also for providing a driving force for many transporting systems in a variety of osmoregulatory epithelia including fish gills (McCormick, 1995). 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). Immunocytochemical data provide evidence that NKA is located mainly in gill MR cells of SW- or FW-

☆ YML and CNC contributed equally to this paper.

*Corresponding author. Tel.: +886-4-2285-6141; fax: +886-4-2287-4740.

E-mail address: thlee@dragon.nchu.edu.tw (T.H. Lee).

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adapted teleosts (Dang et al., 2000; Lee et al., 2000).

Salinity adaptation by euryhaline teleosts is a complex process involving a set of physiological responses to milieus with differing ionoregulatory requirements. The mechanisms of ionoregulation are reasonably well understood (for reviews, see Evans, 1993) and most investigators agree that salinities, which differ from the internal osmotic concentration of the fish, must impose energetic regulatory costs for active ion transport. However, teleost models of metabolic and cellular adaptation to varying environmental salinity have long been based on studies conducted on a restricted number of euryhaline species. Dominating the species studied are diadromous fish such as salmonids (salmon) and anguillids (eel), and non-diadromous fish, e.g. freshwater cichilids, *Oreochromis* spp. Most studies on these 'model' species show a similar positive correlation between environmental salinity and gill NKA activity (reviewed by Sakamoto et al., 2001) which forms the well established 'diadromid paradigm' (Marshall and Bryson, 1998; Lee et al., 2000). Yet, other studies have shown a negative correlation between NKA activity and external salinity in several euryhaline species of non-estuarine marine teleosts such as flounder, sea bream, and mullet (for reviews, see Marshall and Bryson, 1998) which does not support the paradigm.

The milkfish (*Chanos chanos*) is a very well-suited subject for a study of salinity adaptation. It is widely distributed throughout the tropical and subtropical Indo-Pacific (Bagrinao, 1994). The milkfish is a marine inhabitant and an efficient osmoregulator (Ferraris et al., 1988). It demonstrates extremely euryhaline behaviors throughout its life history although it does not appear to require a freshwater environment for any part of its life cycle (Bagrinao, 1994). Milkfish occur naturally and are commercially cultured in fresh, brackish and oceanic waters as well as in hypersaline lagoons (Crear, 1980). Farming of the milkfish is an important aquaculture industry in Indonesia, the Philippines and Taiwan.

In light of the scarcity of current data on marine fish hyperosmoregulation, further investigation of NKA expression in gills of euryhaline marine teleosts in varying salinities is warranted. The present set of experiments were conducted to characterize the adaptive response of NKA in gills of the milkfish acclimated to salinities ranging from a hyperosmotic environment of 35‰ (SW) down to a hyposmotic environment of 0‰ (FW) and to determine whether the NKA expression of this non-estuarine marine species deviates from the diadromid paradigm.

2. Materials and methods

2.1. Fish and experimental environments

Juvenile milkfish (*C. chanos*) with 31.0 ± 11.8 g body weight and 12.5 ± 1.3 cm standard length were obtained from a local fish farm. Experimental environments of different salinities were made from local tap water with proper amounts of synthetic sea salt (Instant ocean). The fish used in the present study were maintained in 10‰ brackish water (BW) at 27 ± 1 °C for more than one month and then adapted to either fresh water (FW; 0‰), BW (10 and 20‰), or seawater (SW; 35‰) with a daily 12 h photoperiod for at least 2 weeks. One week is enough for salinity-adaptation in milkfish (Ferraris, 1988). The water was continuously circulated through fabric-floss filters and partially refreshed every 3 days. Fish were fed a daily diet of commercial pellets ad libitum.

2.2. Plasma analysis

Fish 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 until analysis. Plasma osmolality was measured by a Wescor 5520 vapro osmometer. $[Na^+]$ was determined with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer. $[Cl^-]$ was determined by the ferricyanide method (Franson, 1985) using a Hitachi U-2001 spectrophotometer. Plasma osmolality and Na^+, Cl^- concentrations of each fish were calculated as the mean of the replicate samples taken from that fish. Four or five fish were sampled for each salinity treatment.

2.3. Na, K-ATPase (NKA) activity

Gill NKA activity was determined according to Hwang et al. (1989) except that the homogenization medium (HM) was added with protease inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 ml aprotinin; 10 μl

protease inhibitor in 1 ml HM). Each sample was assayed in triplicate.

2.4. Na, K-ATPase antibody

A mouse monoclonal antibody (α5) against the α-subunit of the avian NKA (Takeyasu et al., 1988) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA) and applied in the present study.

2.5. Western blot

The gill scrapings were suspended in the mixture of homogenization medium and protease inhibitor (v/v: 100/1). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a motorized Teflon pestle at 600 rpm for 30 s. The homogenate was then centrifuged at 13 000 $\times g$, 4 °C for 20 min. The supernatants were used for determination of protein and for immunoblotting. The procedures of immunobloting were performed as described by Wu et al. (2003) with some modification. Briefly, aliquots of gill homogenates and pre-stained molecular weight standards (Invitrogen) were heated at 37 °C for 15 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from unstained gels to PVDF (Poly-Screen, NEN) using a tank transfer system (Bio-Rad, Mini Protean 3). Blots were preincubated for 2 h in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% (vol/vol) Tween 20, pH 7.4) containing 5% (wt/vol) non-fat dried milk to minimize non-specific binding, then incubated at room temperature with primary antibody (α5) diluted in PBST (1:4000) for 2 h. The blot was washed in PBST, followed by a 2-h incubation with AP-conjugated secondary antibody (Jackson) diluted 2500$\times$ in PBST. Blots were visualized after incubation with a NBT/BCIP kit (Chemecon). Immunoblots were photographed by a digital video (SONY TRV900), imported as JPG files into a commercial software package (Kodak Digital Science 1D, 1995). The results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands. Western blot analysis for each salinity group was repeated four times using different sample fishes.

2.6. Immunocytochemical detection of NKIR cells

For identification of branchial NKIR cells, the first gill arch was removed, fixed in 4% paraformaldehyde for 24 h at 4 °C, dehydrated in ethanol and embedded in paraffin. Serial sections (7 μm) were cut parallel to the long axis of the filament and mounted on slides coated with poly-L-lysine. Sections were immunocytochemically stained with the monoclonal antibody (α5) to NKA α-subunit followed by a commercial kit (PicTure™, Zymed). The immunostained sections were counterstained with Hematoxylene. Negative control experiments, in which PBS was used instead of the primary antibody, were conducted (data not shown) to confirm the above positive results.

To quantify the distribution of 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 OCT, fixed samples were washed with PBS, and the gill arch and one side of the filament were removed. The cryosection was processed according to Wu et al. (2003). Cryosections were immunostained as described in the previous paragraph. Our preliminary observations have indicated that most NKIR cells in gills of the milkfish were distributed in the interlamellar epithelium near the afferent side covering the cartilage of the filament. 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. The basal region of the lamella is composed of two parts. These areas can be 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.

2.7. Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) (Tukey's pair-wise method). Values were expressed as the means $\pm$ S.E.M.

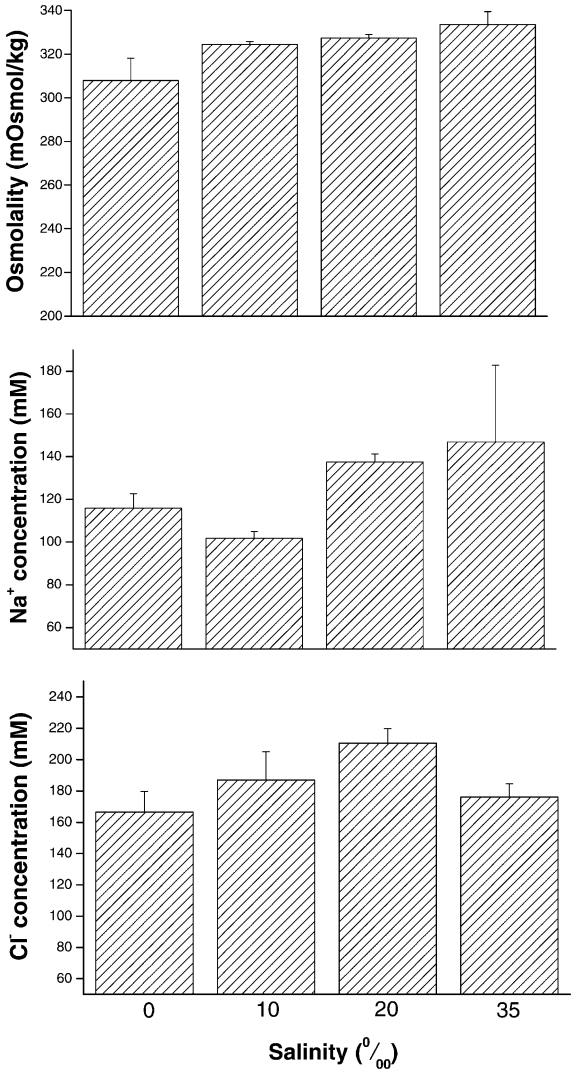

Fig. 1. Plasma osmolality, sodium and chloride concentrations of milkfish adapted to water of different salinities. $N=4$ for all groups. No significant difference was found among various groups of fish in either osmolality, $[Na^+]$, and $[Cl^-]$. Values are indicated by means ± S.E.M.

(the standard error of the mean) unless stated otherwise.

3. Results

3.1. Plasma analysis

There was no significant difference in plasma osmolality among milkfish adapted to fresh water (FW; 0‰), brackish water (BW; 10 and 20‰), and seawater (SW; 35‰) (Fig. 1). Also, no significant difference was seen in serum sodium or chloride concentrations among the fish groups (Fig. 2).

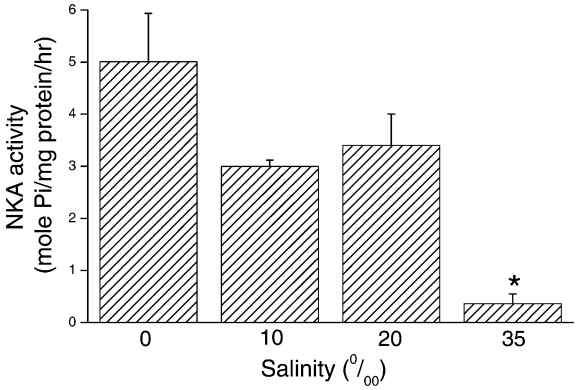

Fig. 2. Na, K-ATPase activity of gill epithelium in milkfish adapted to water of different salinities. The asterisk indicates that the activity of the fish from seawater was significantly lower ($P<0.05$). $N=5$ for all groups. Values are shown as means ± S.E.M.

3.2. Gill NKA expression

The specific activity of gill NKA of milkfish adapted to either FW or BW were significantly higher than that of fish adapted to SW (Fig. 2). Branchial NKA activity in the FW and BW groups were approximately 7 and 5 times higher than that of the SW group. The immunoreactive band rec-

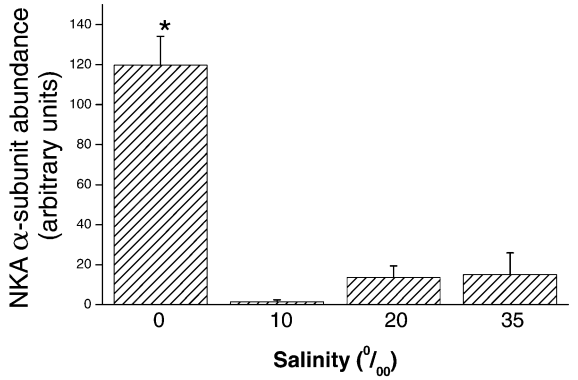

Fig. 3. Corresponding relative intensity of immunopositive bands showing the α-subunit of Na, K-ATPase ($N=4$, means ± S.E.M) expressed in gills of milkfish adapted to water of different salinities. The monoclonal antibody ($\alpha 5$) was specific for NKA α-subunit and the abundance was significantly higher (asterisk) in fish of the FW group than the other groups ($P<0.05$).

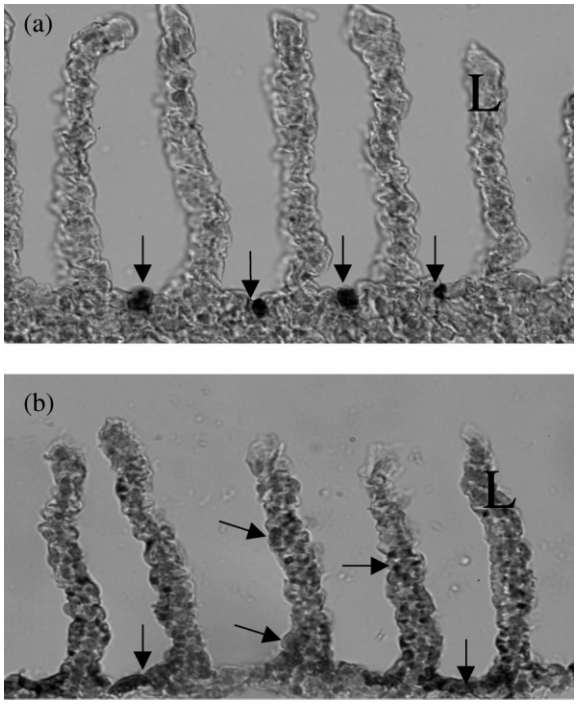

Fig. 4. Representative immunostaining of Na, K-ATPase immunoreactive (NKIR) cells in longitudinal sections of gill filaments from SW-(a), and FW-(b) adapted milkfish. The objective lens was 60×. Arrows indicated that NKIR cells occurred on lamellae (finger-like projections) and/or interlamellar regions (basal to and between lamellae). More NKIR cells exhibited in the gill lamellae of FW-adapted milkfish (b) than SW-adapted fish (a).

ognized by the monoclonal antibody to NKA α-subunit appeared to be more intense in fish of the FW group (Fig. 3). The image analysis of immunoblots indicates that compared to the groups from BW and SW the average amount of gill Na, K-ATPase expressed in FW group was approximately six times higher. No significant difference was found between groups from SW and BW (Fig. 3).

3.3. Immunohistochemistry of gill NKA

Changes in the distribution of gill NKA-immunoreactive (NKIR) cells associated with salinity were visualized using immunolocalization of the α-subunit on paraffin sections (Fig. 4). Staining for NKA was restricted to relatively large cells (presumably MR cells) and was hardly found on respiratory pavement cells (Fig. 4). The number of NKIR cells quantified by immunostaining of cryosections followed a trend similar to NKA protein abundance adapted to (Table 1): FW milkfish had more NKIR cells than BW and SW milkfish due to a substantial increase in lamellae (Fig. 5). No difference of NKIR cell number was found in the interlamellar regions of filaments (Fig. 5). In the lamellae, the number counted within the 10‰ groups was less than the other groups (Fig. 5).

Table 1
Mean values of gill NKIR cell numbers in filaments (F; interlamellar regions), lamellae (L) and basal lamellar regions (B) of milkfish adapted to environments of different salinities

Salinity (‰)	Mean values			
	F	L	B	Sum
0	1.53	2.60*	0.91[a]	5.04
10	1.35	0.04	0.25[b]	1.64
20	1.18	0.20	0.60[a]	1.98
35	1.64	0.23	0.61[a]	2.48

The asterisk indicates that in lamellae the number of the 0‰ group is significantly higher. Different letters show that in basal lamellar regions there are significant differences.

4. Discussion

Evans (1984) has estimated that 95% of teleost species are stenohaline, living entirely in either

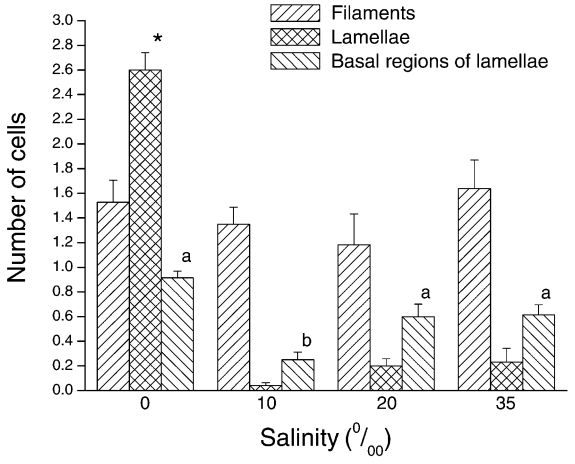

Fig. 5. NKIR cell numbers in filaments (interlamellar regions), lamellae, and basal regions of lamellae of the gills of milkfish adapted to water of different salinities. No significant difference was found in interlamellar regions between SW- or FW-adapted fish. In lamellae, the number of NKIR cells in FW group is significantly higher than in the other groups (asterisks). In the basal regions of lamellae, NKIR cell numbers are significantly lower in the 10‰ groups as indicated by dissimilar letter. Values were means ± S.E.M. ($N=4$).

FW or SW. The remaining 5% 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. This capacity to evolve euryhalinity may be one reason that teleosts can be found in almost all aquatic habitats. Among them, milkfish are marine residents but are extremely euryhaline throughout their life history (i.e. non-diadromous; Bagrinao, 1994). The present study confirms the capacity of juvenile milkfish to tolerate abrupt changes in salinity and the fish osmoregulate well over a wide range of salinities. These characteristics make milkfish a very good model fish for research in osmoregulation.

Plasma osmolalities and Na^+ and Cl^- levels (Fig. 1) observed in FW-, BW- and SW-adapted milkfish are well within the ranges reported for *C. chanos* (Ferraris et al., 1988; Swanson, 1998) and other marine teleosts (*Sparus aurata*: Mancera et al., 1993; *Mugil cephalus*: Ciccotti et al., 1994; *Platichthys flesus*: Gaumet et al., 1995; *Pomacanthus imperator*: Woo and Chung, 1995; *Dicentrachus labrax*: Jensen et al., 1998; *Mylio macrocephalus*: Kelly et al., 1999). Salinity-induced alterations in the circulating electrolytes of euryhaline fish are well documented. The plasma $[Na^+]$ and $[Cl^-]$ of most marine teleosts studied so far responded to changes in salinity in the same way as they responded to osmolality following a minimum of 10 days acclimation period (Mancera et al., 1993; Ciccotti et al., 1994; Gaumet et al., 1995; Woo and Chung, 1995; Jensen et al., 1998; Kelly et al., 1999). Acclimation of euryhaline marine fish to low salinity regimes usually results, when given enough time, in minor differences between pre- and post-transfer electrolyte levels (Mancera et al., 1993; Woo and Chung, 1995; Kelly et al., 1999). Similar to tilapia, *Oreochromis mossambicus* (Lee et al., 2000), a FW euryhaline species, no significant difference was found in plasma osmolality, Na^+ and Cl^- concentrations of milkfish adapted to either FW, BW or SW for two weeks (Fig. 1). Ferraris et al. (1988) described that abrupt transfer of the milkfish from SW to FW results in significant decreases of plasma osmolality but eventually it returns to the level equal to that of the control fish in 1 week. This suggests that the milkfish were fully adapted to their new environment and explains our results of no difference in osmolality among various groups of 2-week-adapted fish.

Since the first work of Epstein et al. (1967) and Kamiya and Utida (1968) on killifish (*Fundulus spp.*) and eels *Anguilla japonica*, the significant role played by branchial NKA in ion transport has been confirmed through years and species (McCormick, 1995). The generally accepted diadromid paradigm model of branchial NKA response to SW adaptation is an increase in its activity (review in Sakamoto et al., 2001). However, contradictions do occur in the literature. Several species of teleosts (*Chelon labrosus*, *Dicentrarchus labarax*, Lasserre, 1971; Jensen et al., 1998; *Liza ramada*, Gallis and Bourdichon, 1976; *P. flesus*, Stagg and Shuttleworth, 1982; Gaumet et al., 1995; *Opsanus beta*, Mallery, 1983; *Macqcuaria novemaculata*, Langdon, 1987; *M. cephalus*, Ciccotti et al., 1994; *P. imperator*, Woo and Chung, 1995; *M. macrocephalus*, Kelly et al., 1999; *Fundulus heteroclitus*, Katoh et al., 2002) including the milkfish, *C. Chanos*, exhibit a response that questions the applicability of a single model. That is, higher or similar levels of gill NKA activity were exhibited in FW- or low salinity-acclimated fish than in SW fish. From an ecophysiological viewpoint, it may be of furthermore importance to note that, of the fish exhibiting this 'alternative' NKA response, the majority are either marine or estuarine-dependent marine species. This signifies that adaptation by marine fish in low salinity environments may in fact be a response considerably different from the diadromid paradigm.

In milkfish the in vitro activity of gill NKA is at a minimum in SW (Fig. 2). Throughout their life, milkfish spend most their time in coastal or marine environments, but will actively forage in estuaries or lagoons (Crear, 1980; Bagrinao, 1994). Euryhaline teleosts usually minimize the energetic cost of osmoregulation for adaptation in the juvenile phase, i.e. juveniles maintain ion-osmotic homeostasis with lowest possible NKA activity allowing more available energy for growth (Langdon, 1987). Thus, the lowest levels of gill NKA expression found in SW-adapted individuals may be energetically favorable for the marine-resident milkfish. It suggests that the fish may normally maintain a comparatively low level of NKA activity in their natural habitats.

The protein abundance as well as the activity of branchial NKA is at a maximum in FW-adapted milkfish and at a minimum in SW-adapted individuals (Fig. 4). However, discrepancies were found in BW-adapted fish. Measured abundance of gill

NKA protein in tilapia adapted to SW, showed no significant difference when compared with BW-adapted fish (Fig. 2), while a significant difference in NKA activity was observed between the two groups (Fig. 3). The increase in NKA activity in a BW environment reflects the physiological demand for adaptation. An insignificant difference of NKA protein abundance found in SW- and BW-adapted individuals indicates the protein levels are sufficient for efficient homeostatic control (e.g. homeostasis of plasma osmolality). Using the same antibody to NKA α-subunit, our immunocytochemical data mirror the change of the protein abundance, i.e. significant expression only in FW milkfish (Table 1 and Fig. 5). In FW-adapted milkfish, concurrent elevation of NKA activity and protein abundance (Figs. 2 and 3) evidently came from the increased numbers of Na, K-ATPase immunoreactive (NKIR) cells (Table 1). Such an increase is necessary for marine-resident milkfish to maintain a constant level of plasma osmolality in FW. In the euryhaline tilapia, *O. mossambicus*, Lee et al. (2000, 2003) have shown similar salinity-dependent patterns of NKA activity and the amounts of NKA protein in gills. However, to the best of our knowledge, no published report described the abundance of NKA protein of marine fish in varying salinities. Clearly, further work on the duality of such a response is warranted.

Immunocytochemical studies on gill sections as well as biochemical studies on isolated mitochondrion-rich (MR) cells have unambiguously demonstrated that these epithelial cells contain most NKA activity and protein in gills. It is generally thought that this enzyme is critically involved in ionoregulatory functions of these cells (reviewed by Sakamoto et al., 2001). Hence, NKIR cells represent MR cells. In milkfish, epithelial NKIR cells occur mostly on the interlamellar region near the afferent side of the filament but rarely appear on the afferent region of the filament (Lee et al., unpublished data). MR cells in the gill filament epithelium are abundant in both FW and SW teleosts. However, in stenohaline FW fish (Lee et al., 1996) or FW-adapted euryhaline fish (Sakamoto et al., 2001), MR cells also appear on the lamellar epithelium. In milkfish, NKIR cells are also found in lamellae. Our results indicated that in milkfish, the elevation of gill NKA activity and protein amounts are related to the increase in NKIR cell number (primarily on lamellae of the FW fish) (Table 1 Fig. 5) as well as in the quantity of functioning NKA per cell (i.e. in the BW fish) (Figs. 2 and 3). The occurrence of lamellar NKIR cells was thought to meet the physiological requirement of ion-uptake in some freshwater-adapted euryhaline teleosts (Uchida et al., 1996; Sasai et al., 1998; Hirai et al., 1999; Versamos et al., 2002) but not in others (Laurent and Perry, 1990; Lin and Sung, 2003). MR (NKIR) cells are effective at eliminating ions in hypertonic SW as well as absorbing ions in hypotonic FW (Evans et al., 1999; Chang et al., 2001). However, whether there is a functional differentiation between lamellar and filamental MR cells is still under debate.

In summary, juvenile milkfish in various fixed salinities from full strength SW to FW maintained their plasma osmolality and ionic concentrations within very narrow limits. In BW-adapted fish, a decline in salinity induces a substantial rise in NKA activity only due to the recruitment of latent pumps. Alternatively, in FW-adapted individuals, elevated NKA activity attributed to parallel increase of NKIR cell numbers as well as newly synthesized NKA protein amounts enable the marine-resident milkfish to be extremely euryhaline.

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