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Differential responses in gills of euryhaline tilapia, *Oreochromis mossambicus*, to various hyperosmotic shocks

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ABSTRACT

Euryhaline tilapia (*Oreochromis mossambicus*) survived in brackish water (BW; 20‰) but died in seawater (SW; 35‰) within 6 h when transferred directly from fresh water (FW). The purpose of this study was to clarify responses in gills of FW tilapia to various hyperosmotic shocks induced by BW or SW. In FW-acclimated tilapia, scanning electron micrographs of gills revealed three subtypes of MR cell apical surfaces: wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III). Density of apical surfaces of mitochondrion-rich (MR) cell in gills of the BW-transfer tilapia decreased significantly within 3 h post-transfer due to disappearance of subtype I cells, but increased from 48 h post-transfer because of increasing density of subtype III cells. SW-transfer individuals, however, showed decreased density of MR cell openings after 1 h post-transfer because subtype I MR cell disappeared. On the other hand, relative branchial Na^+/K^+-ATPase (NKA) α1-subunit mRNA levels, protein abundance, and NKA activity of the BW-transfer group increased significantly at 6, 12, and 12 h post-transfer, respectively. In the SW-transfer group, relative mRNA and protein abundance of gill NKA α1-subunit did not change while NKA activity declined before dying in 5 h. Upon SW transfer, dramatic increases (nearly 2-fold) of plasma osmolality, $[Na^+]$, and $[Cl^-]$ were found prior to death. For the BW-transfer group, plasma osmolality was eventually controlled by 96 h post-transfer by enhancement of NKA expression and subtype III MR cell. The success or failure of NKA activation from gene to functional protein as well as the development of specific SW subtype in gills were crucial for the survival of euryhaline tilapia to various hyperosmotic shocks.

1. Introduction

The ability of Mozambique tilapia (*Oreochromis mossambicus*) tolerating salinities up to 120‰ (Stickney, 1986) makes them a good model organism for studies on ionic and osmotic acclimation in euryhaline teleosts. Tilapia gill, as in other euryhaline species, is a major extra-renal organ to balance between diffusional ion gains or losses (Evans et al., 2005). Functional and structural differentiation of the gill is influenced considerably by environmental salinity. In the gill epithelium, mitochondrion-rich cells (MR cells, i.e., chloride cells) are the main place of ion secretion in seawater (SW) fish, and ion uptake in freshwater (FW) fish (Evans et al., 2005). Branchial MR cells of tilapia change in size, density, morphology (ultrastructure), and distributions of ion transporters in response to alterations of environmental salinity (Uchida et al., 2000; Lee et al., 2000; Wilson et al., 2000; Lee et al., 2003; Tang et al., 2008). In FW-acclimated tilapia, scanning electron micrographs of gills revealed three subtypes of MR cells: wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III), named according to the appearance of their apical surfaces (Lee et al., 1996; van der Heijden et al., 1997; Hiroi et al., 2005). Each subtype of MR cell was found to be dominant in a medium of particular ionic concentrations, whereby the prevalence of each cell-subtypes varied with the ionic compositions of the water (Lee et al., 2000; 2003; Lin and Hwang, 2001; Lin et al., 2004; Hiroi et al., 2005; Tang et al., 2008). On the other hand, in SW-acclimated tilapia, only one subtype of MR cells with significant apical crypts (subtype III) was found in the gill epithelium (Kültz et al., 1995; van der Heijden et al., 1997; Lee et al., 2000; 2003; Hiroi et al., 2005). Reversibility of MR cell apical surface subtypes was observed in tilapia within several hours after transfer to a new medium of different salinity (Lee et al., 1996; Lin et al., 2004; Hiroi et al., 2005) or hyposmotic ionic compositions (Chang et al., 2003; Lin and Hwang, 2004; Tang et al., 2008). Studies showed that different phenotypes of MR cells with various compositions of ion transporters corresponded with the functions of different ions transporting (Lee et al., 2000; Wilson and Laurent, 2002; Chang et al., 2001, 2003; Perry et al., 2003; Wu et al., 2003; Hiroi et al., 2005).

Na^+/K^+-ATPase (NKA) is a universal membrane-bound enzyme that provides a driving force for many transport systems in a variety of osmoregulatory epithelia, including fish gills (Evans et al., 2005). Immunocytochemical studies demonstrated that NKA was located

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mainly in MR cells of gill epithelia in euryhaline teleosts (reviewed by Hwang and Lee, 2007) including tilapia (Uchida et al., 2000; Wilson et al., 2000; Lee et al., 2003; Hiroi et al., 2005; Tang et al., 2008). This heterodimeric integral membrane protein is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex. The catalytic α-subunit with four isoforms (α1–α4) has a molecular weight of about 100 kDa, while the glycosylated β-subunit with three isoforms (β1–β3) is a smaller one of approximate 60 kDa. NKA α1 expressed mainly in the transporting epithelia in higher vertebrates functions primarily in a housekeeping capacity by maintaining osmotic balance and cell volume regulation (Scheiner-Bobis, 2002). The full-length cDNA encoding of tilapia NKA α1-isoforms showed high homology (more than 85% identity) at the amino acid level to other animals (Feng et al., 2002). Moreover, the abundance of NKA α1-mRNA and α1-protein in tilapia gill tissues increased with environmental salinity (Lee et al., 1998; Feng et al., 2002), identical to the pattern of relative amounts of NKA α-protein (Lee et al., 2003).

Most studies on branchial NKA activity of tilapia revealed a pattern of expression proportional to environmental salinity (Hwang et al., 1989; Morgan et al., 1997; Uchida et al., 2000; Lee et al., 2000, 2003; Lin et al., 2004). In several euryhaline teleosts, e.g., eel, sea bass, trout, striped bass, and gilthead seabream (Forrest et al., 1973; Jensen et al., 1998; Madsen and Naamansen, 1989; Seidelin et al., 2000; Tipsmark et al., 2004; Laiz-Carrión et al., 2005), gill NKA activity did not increase until 3–10 days after transfer from FW to SW; while in mullet and killifish, gill NKA activity elevated rapidly within 3 h after transfer from FW to brackish water (BW) or SW (Hossler, 1980; Mancera and McCormick, 2000; Towle et al., 1977). Time-course studies on different euryhaline species revealed that the source of changing NKA activity upon salinity challenge might be alterations on mRNA level (Scott et al., 2004; Seidelin et al., 2000; Singer et al., 2002), or protein level (Tipsmark et al., 2002; Lee et al., 2000, 2003; Lin et al., 2003), or both levels (D'Cotta et al., 2000; Lin et al., 2004).

SW-tilapia was found to survive direct transfer to FW without pre-acclimation to BW (Lin et al., 2004). SW-tilapia gills responded to FW transfer by reducing NKA activity as well as modifying densities of MR cell subtypes within 3 h. NKA α1-subunit mRNA behaved similarly as did the amounts of NKA α1-subunit protein within 6 h post-transfer. The time-course changes in NKA expression that paralleled modifications of MR cell subtype were thought to improve the osmoregulatory capacity of tilapia, when acclimating from SW to FW (Lin et al., 2004). On the other hand, FW tilapia survived direct transfer to BW (20‰), but died during SW (35‰) transfer (Hwang, 1987). Since most studies have focused on responses of euryhaline teleosts during the processes of successful acclimation to hyperosmotic shocks, it is intriguing to elucidate the physiological responses for acclimation to various hyperosmotic shocks (BW or SW) of FW tilapia. When the gill epithelial MR cell morphology was changed, the variation of the physiological responses to hyperosmotic shocks was evaluated by measuring plasma osmolality, and Na^+ and Cl^- ion concentrations. The osmoregulatory mechanisms of tilapia upon hyperosmotic challenge can be interpreted through the alterations in abundance of branchial NKA from gene, to protein, and then functional enzyme (activity) levels.

2. Materials and methods

2.1. Experimental animals

Tilapia (*O. mossambicus*) weighing 5–9 g were obtained from laboratory stocks. Fish were reared in a tank with 300 L aerated local tap water (fresh water; FW) at 27 ± 1 °C with a daily 12 h photoperiod. Water was continuously circulated through fabric-floss filters and was partially refreshed every 3 days. Fish were fed a daily diet of commercial pellets *ad libitum*. Seawater (35‰; SW) and brackish water (20‰; BW) were prepared from local tap water with proper amounts of the synthetic sea salt "Instant Ocean" (Aquarium Systems, Mentor, OH, USA). The parameters of the water were identical to our previous experiments (Lin et al., 2004).

2.2. Acclimation experiments

After reared in FW for one month, tilapia were separated into two groups to detect the responses of gills in fish exposed to different hyperosmotic shocks: (1) FW-seawater (35‰; SW) group — FW tilapia were transferred to SW directly and sampled every hour before death; (2) FW-brackish water (20‰; BW) group — FW tilapia were transferred to BW directly and sampled at 3, 6, 12, 24, 48, 96 h. One fish was sampled at each time point. Gills or blood were sampled for the following molecular and biochemical analyses, as well as ultrastructural examination. Fish used for collecting blood were anesthetized in buffered MS-222, while fish used for collecting gills were killed by spinal section and pithing of the brain before sampling. The number of individual fish used in different experiments was described in the figure legend.

2.3. Subtypes and densities of mitochondrion-rich (MR) cells

Excised gill filaments were examined by scanning electron microscopy according to Lee et al. (1996). In brief, tissues were fixed at 4 °C in phosphate-buffered 4% paraformaldehyde plus 5% glutaraldehyde (pH 7.2) for 12 h followed by fixation in 1% osmium textroxide (pH 7.2) for 1 h at 4 °C. Tissues were dehydrated in ascending concentrations of ethanol from 50% to absolute, then in 100% acetone, and were dried using a Hitachi HCP-2 critical-point drier (Tokyo, Japan). After sputter-coating with a gold-palladium complex for 3 min using an Eiko 1B-2 vacuum evaporator (Tokyo, Japan), specimens were examined and photographed with a Hitachi S-2500 scanning electron microscope (Tokyo, Japan).

Different subtypes of MR cells were identified under SEM observation according to size and morphology of the apical surfaces (Lee et al., 1996). Areas on the trailing edge of the filaments were chosen at random for counting at 1250× magnification. Two areas (4000 μm^2 each) were counted on each of five gill filaments from each fish. Averages of ten areas were obtained from each fish per group.

2.4. Plasma analysis

Blood was then collected from caudal veins in the caudal peduncle using heparinized 1 mL syringes with 27 G needles and injected into heparinized 0.5 mL centrifuge tubes. After a 10 min, 4 °C, 1000 g centrifuge, the plasma was stored in a refrigerator before analyses. Plasma osmolality was determined with a WESCOR 5520 VAPRO™ Vapor Pressure Osmometer (Logan, UT, USA). $[Na^+]$ was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan). $[Cl^-]$ was evaluated by the Ferricyanide method (Franson, 1985) using a Hitachi U-2001 spectrophotometer (Tokyo, Japan).

2.5. Molecular analysis

2.5.1. Total RNA extraction and reverse transcription

Total RNA was extracted from the excised gill epithelium by using RNeasy Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA integrity was verified by 0.8% agarose gel electrophoresis. Extracted RNA samples were stored at −80 °C after isolation. First-strand cDNA was synthesized by reverse transcribing 9 μL of the total RNA (5 μg) using a 1 μL Oligo(dT) primer and a 1 μL PowerScript™ Reverse Transcriptase (Clontech, Franklin Lakes, NJ, USA) following the manufacturer's instructions.

2.5.2. Primers used for real-time PCR

The cDNA sequence of Na^+/K^+-ATPase (NKA; GenBank Accession No. AAD11455) and GAPDH (glyceraldehydes 3-phosphate dehydrogenase) (unpublished data) were aligned and compared with the sequences of other species from the database. Primers were designed using Primer Express software (version 2.0.0, Applied Biosystems, Foster City, CA, USA). α-1 isoform gene specific primer sequences were as follows (5′ to 3′): forward — GCCAACTGCCACACAGACAC and

reverse — TCCCAATAAAACATTTCCACTG. GAPDH primer sequences were as follows (5′ to 3′): forward — CATCGAGGAGGCACTCATGA and reverse — TCAATGGTAAGCTGACAGGCAT (Lin et al., 2004).

2.5.3. Real-time PCR analysis

NKA α1-subunit mRNA was quantified by a real-time PCR (ABI PRISM 7000 Sequence Detection System (SYBR Green II) real-time quantitative PCR, Applied Biosystems). For methods of quantifying mRNA by real-time PCR, refer to Johnson et al. (2000). PCR reactions contained 8 μL of cDNA (500× dilution), 2 μL of NKA α1-isoform primer mixture (l00 nM) or GAPDH primer mixture (100 nM), and 10 μL of SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR reactions were performed as follows: 1 cycle of 50 °C for 3 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. All samples were run in triplicate. Reactions for quantifying GAPDH copy number were performed exactly as described above except for the use of different probes and primers. NKA mRNA values were corrected for the values obtained for GAPDH from the same DNA samples to obtain the values reported. For each unknown sample, the corresponding NKA and GAPDH values were read using linear regression analyses from their respective standard curves (data not shown). A relative NKA expression value was obtained using the following formula: $2^{\wedge}[(Ct_{NKA,n}-Ct_{GAPDH,n})-(Ct_{NKA,0}-Ct_{GAPDH,0})]$, where Ct corresponded to the threshold cycle number.

2.6. Protein analysis

2.6.1. Preparation of membrane fractions

Membrane fractions were prepared according to the method modified from Stanwell et al. (1994). Gills of the fish were excised, quickly washed in phosphate buffered saline (PBS) to remove the clotted blood, and blotted dry. The gill epithelia were immediately scraped off the underlying cartilage with a scalpel. All procedures were performed on ice. 10 μL of proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) was added to 1 mL of buffer A or B. Gill scrapings were suspended in 1 mL of buffer A (20 mM Tris-base, 2 mM $MgCl_2{\cdot}6H_2O$, 2 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 250 mM sucrose, proteinase inhibitor, pH 7.4). Homogenization was performed in a glass Potter–Elvehjem homogenizer with a motorized Teflon pestle at maximum speed for 20 strokes. The homogenate was then centrifuged at 100,000 g for 1 h at 4 °C. The pellet was suspended in 200 μL of buffer B (20 mM Tris-base, 2 mM $MgCl_2{\cdot}6H_2O$, 5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 5 mM NaF, 0.1% Triton X-100, proteinase inhibitor, pH 7.5) and vortexed every 10 min during a 1 h incubation period at 4 °C. Dissolved pellet was centrifuged again at 100,000 g for 1 h at 4 °C. The supernatant, referred to as the membrane fractions, was stored at −80 °C. Protein concentrations of the supernatant were identified by reagents from the Protein Assay Kit (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard.

2.6.2. Immunoblot of NKA

Immunoblotting procedures were carried out as described by Lee et al. (2000) with little modification. A mouse monoclonal antibody 6F raised against the α1-isoform of the avian NKA (Takeyasu et al., 1988), demonstrated to be α1-isoform specific and to show broad species specificity (Arystarkhova and Sweadner, 1996), was used as the primary antibody in this study. The secondary antibody was an alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Baltimore Pike, PAa, USA). Proteins of the membrane fractions were divided by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels (30 μg of protein/lane). The separated proteins were then transferred to PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting. After preincubation for 2 h in PBST buffer containing 5% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 2 h in primary antibody (6F) diluted in 1% BSA and 0.05% sodium azide in PBST (1:5000 dilution), washed in PBST, and reacted for 2 h with secondary antibody (1:5000 dilution). Blots were developed after incubation with BCIP/NBT kit (Zymed, South San Francisco, CA, USA). Immunoblots were photographed and imported as TIF files into the ID image analysis software package (Kodak digital Science ID, 1995). Results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

2.7. Functional analysis

2.7.1. Preparation of gill homogenates

Gills prepared as described above were suspended in 1 mL of homogenization solution (100 mM imidazole–HCl, 5 mM Na_2EDTA, 200 mM sucrose, 0.1% sodium deoxychlolate, pH 7.6) with 10 μL proteinase inhibitor. Homogenization was performed in a glass Potter–Elvehjem homogenizer with a motorized Teflon pestle at maximum speed for 20 strokes. The homogenate was then centrifuged at 13,000 g at 4 °C for 20 min. Protein concentrations of the supernatant were determined as described above.

2.7.2. Specific activity of NKA

NKA activity was assayed by adding aliquots of suspended gill homogenates to the reaction mixture (100 mM imidazole–HCl, 125 mM

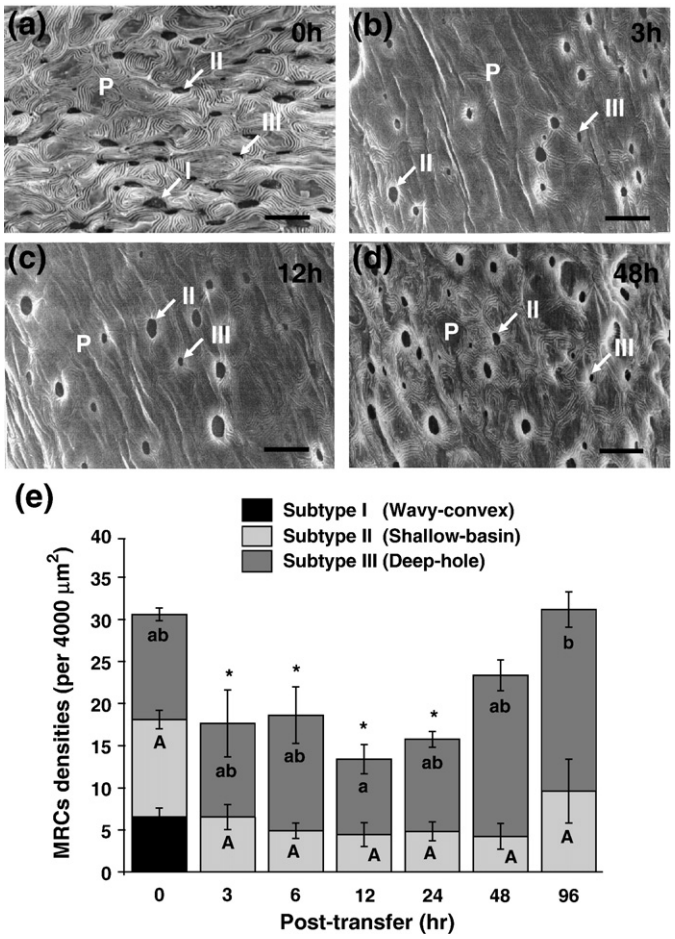

Fig. 1. Scanning electron micrographs show the effects of direct transfer, from FW to BW, on tilapia gill mitochondrion-rich (MR) cell phenotype and the densities of various subtypes ($n=4$ for each time point). (a–d) The apical openings of MR cells, including subtypes I, II, and III, were indicated by arrows. Subtype I (wavy-convex) MR cells were found only in 0 h (FW) individuals. (e) The asterisks showed significant differences in densities of total number of MR cell opening crypt between the experimental time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Different letters indicated significant differences in densities of subtypes II (in capital case) and subtype III (in lower case) MR cells ($p<0.05$, one-way ANOVA, Tukey's test). Densities of MR cell apical openings decreased significantly within 3 h post-transfer because subtype I MR cells disappeared. Apical crypt densities increased again after 48 h due to the proliferation of subtype III MR cells. P, pavement cells. Scale bar: 10 μm. Values are means ± SEM.

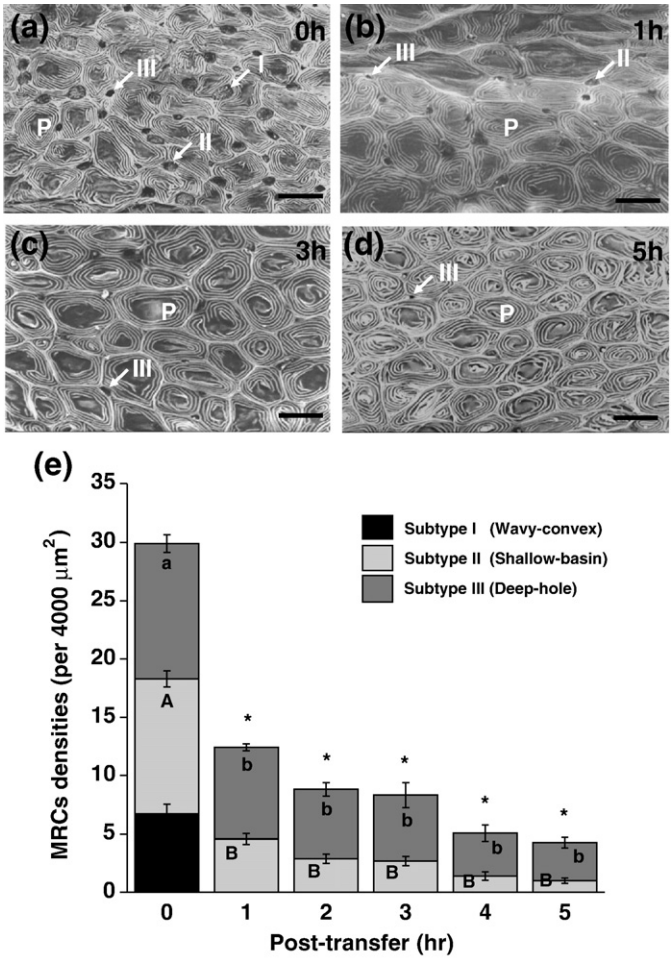

Fig. 2. Scanning electron micrographs show the effects of direct transfer, from FW to SW, on tilapia gill MR cell phenotype and the densities of various subtypes ($n=7$ for each time point). (a–d) The apical openings of MR cells, including subtypes I, II, and III, were indicated by arrows. Subtype I (wavy-convex) MR cells were found only in 0 h (FW) individuals. (e) The asterisks showed significant differences in densities of total number of MR cells opening crypt between the experimental time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Different letters indicated significant differences in densities of subtypes II (in capital case) and subtype III (in lower case) MR cells ($p<0.05$, one-way ANOVA, Tukey's test). Densities of MR cell apical openings decreased significantly due to the disappearance of subtype I cells within 1 h post-transfer continuing to that of only one seventh compared to FW levels before death. P, pavement cells. Scale bar: 10 μm. Values are means±SEM.

NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, 5 mM Na_2ATP, pH 7.6). The reaction was run at 37 °C for 30 min and then stopped by addition of 200 μL of ice-cold 30% trichloroacetic acid. The inorganic phosphate concentration was determined by the method of Peterson (1978). The enzyme activity of NKA was defined as the difference between the inorganic phosphates liberated in the presence and absence of 3.75 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

2.8. Data analysis

Values were expressed as means±SEM. Time-course results were analyzed using a one-way ANOVA followed by *a posteriori* comparisons (Tukey's or Dunnett's multiple-comparisons test) and $p<0.05$ was set as the level of significance.

3. Results

3.1. Responses to hyperosmotic shocks of gill MR cells

Tilapia transferred directly from FW to BW can survive with no mortality. When tilapia were transferred, apical openings of gill MR cell

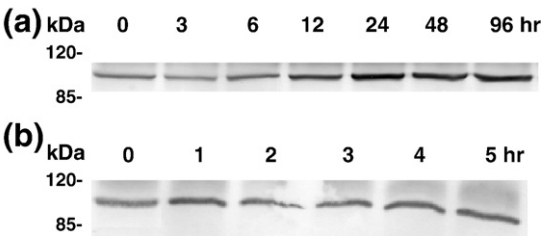

Fig. 3. Representative immunoblots of the Na, K-ATPase (NKA) α1-subunit in gills of tilapia transferred from FW to BW (a) or SW (b). The immunoreactive band of the NKA α1-subunit became more evident from 12 h post-transfer in BW-transfer group (a), while in the SW-transfer group, no obvious change occurred during the time-course (b).

densities declined significantly from 3 h post-transfer due to the disappearance of wavy-convex (subtype I) mitochondrion-rich (MR) cells (Fig. 1). Then, a significant increase in densities of deep-hole (subtype III) MR cells at 96 h post-transfer compared to 12 h post-

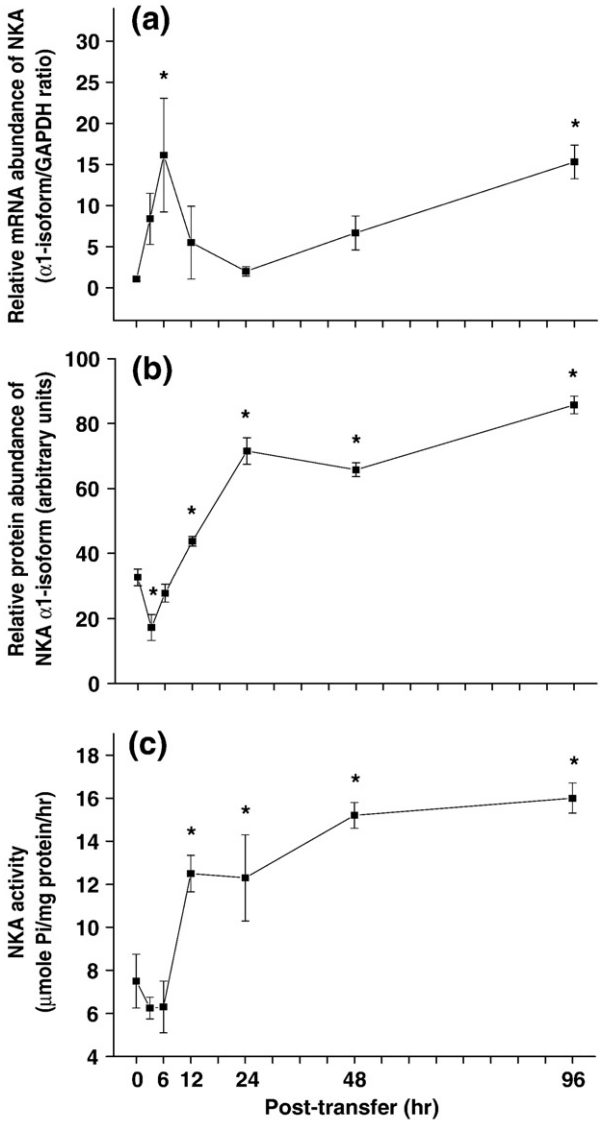

Fig. 4. Effects of direct transfer from FW to BW on mRNA abundance (a), protein amounts (b), and activity (c) of NKA of tilapia gills ($n=8$ for each time point). The asterisks indicate significant differences between the test time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Values are means±SEM. After transfer, NKA α1-subunit mRNA levels increased within 6 h (a) and thereafter, followed by a significant elevation in protein amounts (b) and activity (c).

transfer gave apparent augmentation to total MR cell densities similar to those of FW samples (Fig. 1). In contrast, tilapia died at 5–6 h when transferred directly from FW to SW. When tilapia were transferred to SW, gill MR cell apical-surface densities declined significantly from 1 h post-transfer due to the disappearance of wavy-convex (subtype I) and a reduction of shallow-basin (subtypes II) and deep-hole (subtype III) MR cells (Fig. 2). At 5 h post-transfer, the remaining densities of subtype II and III were declined to about one seventh of FW samples (Fig. 2).

3.2. Immunoblots of Na^+/K^+-ATPase (NKA)

Immunoblots on the membrane fractions of tilapia gills performed using the monoclonal antibody of the NKA α1-subunit revealed single immunoreactive bands with relative molecular masses of about 100 kDa (Fig. 3).

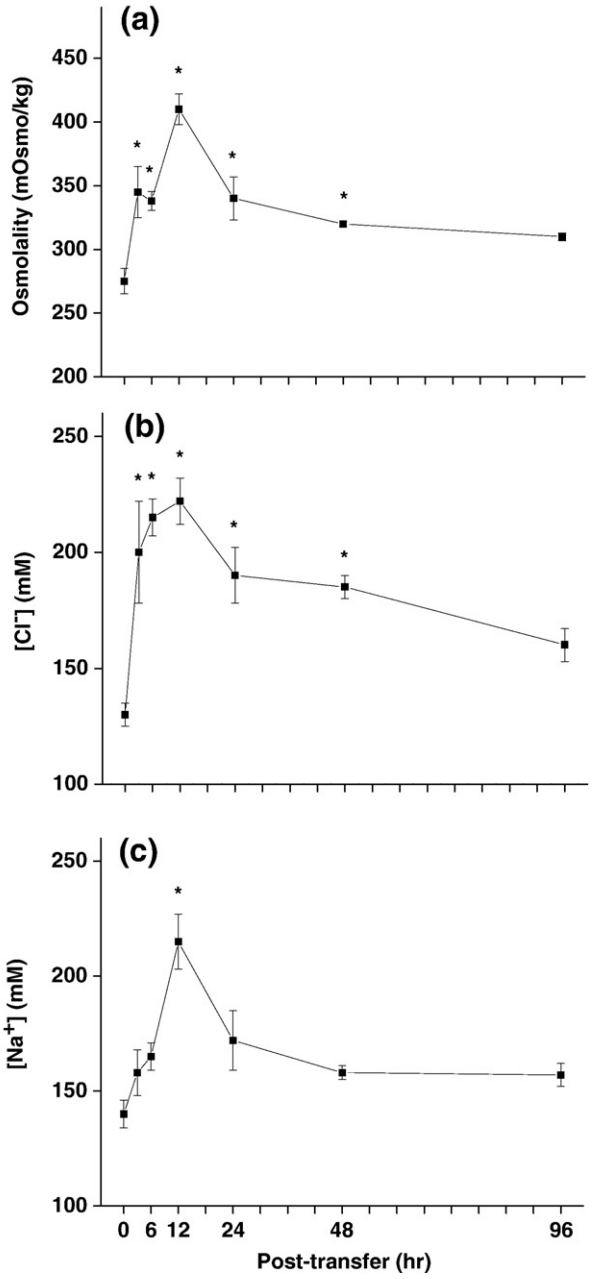

Fig. 5. Effects of direct transfer of tilapia from FW to BW on plasma osmolality (a), $[Cl^-]$ (b), and $[Na^+]$ (c) ($n=8$ for each time point). The asterisks indicate significant differences between the test time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Values are means±SEM. Significant increases in osmolality and $[Cl^-]$ occur within 3 h.

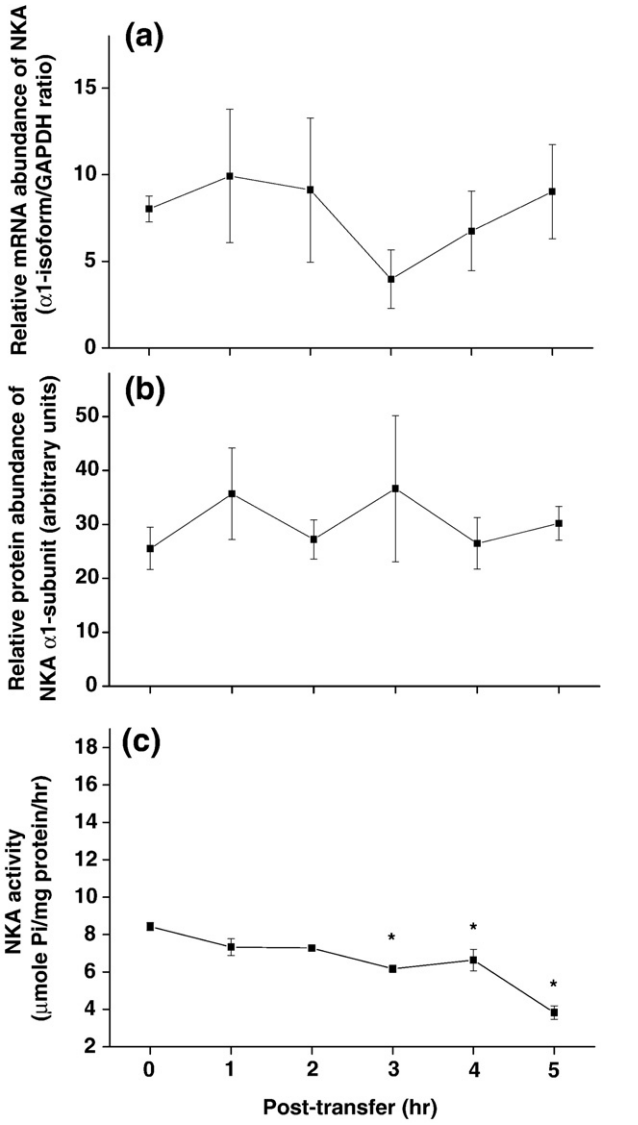

Fig. 6. Effects of direct transfer of tilapia from FW to SW on mRNA abundance (a), protein amounts (b), and activity (c) of NKA of tilapia gills ($n=8$ for each time point). The asterisks indicate significant differences between the test time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Values are means±SEM. No significant change in relative mRNA and protein abundance of the NKA α1-subunit was found (a and b). NKA activity, however, decreased significantly within 3 h post-transfer (c).

3.3. Physiological responses to hyperosmotic shocks

When tilapia was transferred from FW to BW, fish behaved normally. In the BW group, NKA α1-mRNA abundance first increased to a peak at 6 h post-transfer, but then declined to near initial levels at 24 h, and then resumed increasing until the end of the observed period (Fig. 4a). Both NKA α1-subunit protein abundance and NKA activity elevated significantly from 12 h post-transfer (Figs. 3a, 4b, and c). Meanwhile, osmolality and Cl^- concentration of the plasma increased significantly within 3 h post-transfer but declined to levels similar to FW samples by 96 h post-transfer (Fig. 5a and b). Plasma Na^+ concentrations peaked at 12 h post-transfer but dropped to the level of FW samples thereafter (Fig. 5c). In our results, the control group in which FW fish were transferred to FW showed no significant change of NKA activity and plasma osmolality among all time point (data not shown).

When tilapia was transferred from FW to SW, fish became quiescent and inactive, opening and closing their mouths near the water surface. No significant change in NKA α1-mRNA abundance occurred in gills of tilapia transferred from FW to SW (Fig. 6a). A similar result was observed

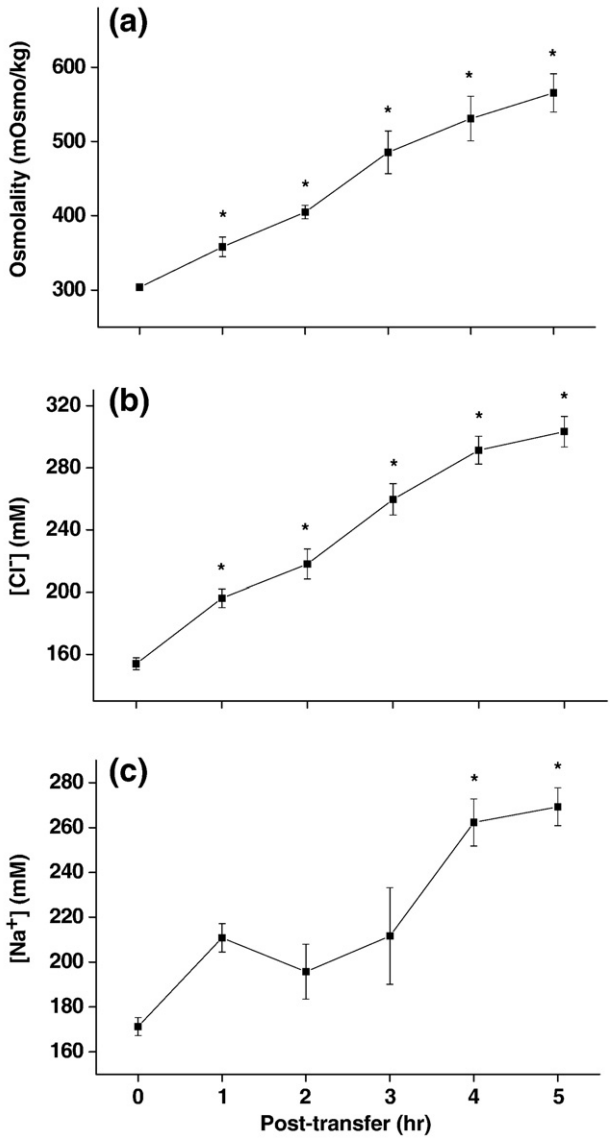

Fig. 7. Effects of direct transfer of tilapia from FW to SW on plasma osmolality (a), [Cl^-] (b), and [Na^+] (c) ($n=8$ for each time point). The asterisks indicate significant differences between the test time-points and the 0 h point ($p<0.05$, one-way ANOVA, Dunnett's test). Values are means±SEM. Before death, the osmolality, [Na^+], and [Cl^-] increased significantly to nearly 2-fold compared to that of FW tilapia.

in protein levels of the gill NKA α1-subunit (Fig. 6b), as shown by image analyses of the immunoblots (Fig. 3b). Following transfer, NKA activity gradually decreased, with a significant reduction to about half the original value by 5 h post-transfer (Fig. 6c). Meanwhile, osmolality and Cl^- concentration of the plasma increased significantly within 1 h post-transfer and by 5 h post-transfer, concentrations elevated nearly 2-fold compared to that of FW samples (Fig. 7a and b). Plasma Na^+ concentration increased after 3 h post-transfer (Fig. 7c).

4. Discussion

Physiological studies on Cl^- influx suggested that the major roles of wavy-convex type (subtype I) were Cl^- uptake while deep-hole type (subtype III) were Cl^- secretion (Chang et al., 2001; 2003; Lin and Hwang, 2001). Moreover, subtype II MR cells with shallow-basin apical surfaces were thought to be responsible for [Ca^{2+}] uptake because of apparent relationship between ion influx and cell density (Tsai and Hwang, 1998). Most branchial MR cells of SW tilapia were deep-hole MR cells (Kültz et al., 1995; van der Heijden et al., 1997; Lee et al., 2000, 2003; Uchida et al., 2000; Hiroi et al., 2005) contained basolateral NKA, NKCC ($Na^+/K^+/2Cl^-$ cotransporter), and apical CFTR (cystic fibrosis transmembrane conductance regulator), and accompanied chloride-secreting activity (Kaneko and Shiraishi, 2001; Wu et al., 2003; Hiroi et al., 2005). In gills of rainbow trout, there is accumulating evidence for two distinct populations of MR cell subtypes with different components of ion transporters (Goss et al., 2001; Galvez et al., 2002; Richards et al., 2003). Various distribution or components of ion transporters implicated that the major roles of each subtype of MR cells were different.

Tilapia gill MR cells in this study were found to respond to BW. Tilapia responded to the elevation of plasma [Cl^-] induced by higher [Cl^-] in BW with disappearance of wavy-convex (subtype I) MR cells within 3 h post-transfer (Fig. 1) which might be due to the internalization of the apical membrane (Lin and Hwang, 2004) and the "close" reaction of adjacent pavement cells as reported in gills of mudskipper (*Periophthalmus modestus*, Sakamoto et al., 2000) and killifish (Daborn et al., 2001; Katoh and Kaneko, 2003), then led to a significant decrease in MR cell apical-surface densities (Fig. 1). And the proliferation of deep-hole subtype MR cells responsible mostly for Cl^- secretion. At 96 h post-transfer, the number of deep-hole subtype was significantly higher than 12 h post-transfer (Fig. 1). The results showed that deep-hole MR cell regulated plasma osmolality and [Cl^-] at 96 h post-transfer more efficiently (Fig. 5). Our findings agreed with the results of Hiroi et al. (2005) that type IV (SW-type) ion secretory MR cells showed a remarkable increase in number between 24 and 48 h. No significant difference in total MR cell apical-surface densities between FW tilapia and BW individuals (48 and 96 post-transfer) was found but the constitution and density of MR cell subtypes changed (Fig. 1). Similarly, within 3 h after transfer of striped bass to SW induced adaptive changes in ultrastructures of MR cells, correlating with an increased branchial Cl^- efflux (King and Hossler, 1991) and the compensation of deflections in plasma [Na^+] by 24 h (Tipsmark et al., 2004). On the other hand, killifish responded to hyposmotic shock-induced rapid changes in plasma osmolality and alterations of MR cell morphology (subtypes) and densities accompanied by CFTR disappearance within 24 h after transfer (Katoh and Kaneko, 2003). In tilapia gills, SW deep-hole MR cells replaced FW wavy-convex MR cells as a long-term adaptive response to elevated external salinity in BW. In SW-hyperosmotic shock, however, functional MR cell densities declined significantly from 1 h post-transfer (Fig. 2). Unlike that found in BW-shock, serious ion imbalance were found in SW and decrease of ion-secretory deep-hole MR cells, so the MR cells in gills did not provide physiological functions necessary for survival in SW of tilapia. Hence, rapid and reversible modifications of branchial MR cell types/subtypes with different functions of ionoregulation should be the strategies used by some euryhaline teleosts during FW- or SW-acclimation.

During SW transfer, some euryhaline teleosts increased their gill NKA activity to facilitate ion secretion against a concentration gradient (Marshall, 2002). Increase in gill NKA activity during salinity acclimation of euryhaline fish might be attributed to (i) the modulation of the hydrolytic rate of the enzyme, as reported in the gills of Atlantic cod (*Gadus morhua*, Crombie et al., 1996) and striped bass (Tipsmark et al., 2004); or (ii) precedence of or accompaniment of increased NKA α-subunit mRNA and/or protein abundance. Previous studies reported that the gill NKA α- or α1-subunit mRNA levels increased during salinity transfer in tilapia (Hwang et al., 1998; Feng et al., 2002). In this study, BW-hyperosmotic shock triggered the increase of tilapia gill NKA α1-mRNA, α1-protein abundance, and its activity, at 6, 12, and 12 h post-transfer, respectively (Fig. 4). These results implicated that, upon salinity challenge, activation of genomic DNA transcription occurred and preceded an increase in the abundance of the NKA α1-mRNA, peaked at 6 h post-transfer. Subsequent increases in translation of NKA α1-protein were observed after 12 h post-transfer (Fig. 4). Similarly, killifish gill NKA α_{1a}-mRNA as well as activity responded in a short time when exposed to SW (Mancera and McCormick, 2000; Scott

et al., 2004). However, in the gills of brown trout (*S. trutta*, Madsen et al., 1995; Nielsen et al., 1999; Seidelin et al., 2000), European sea bass (Jensen et al., 1998), and Atlantic salmon (D'Cotta et al., 2000; Singer et al., 2002), NKA mRNA levels did not significantly increase until 24 h, followed by a time-lag of several days for the ensuing rise in α-protein abundance and NKA activity. In these species, an increase in NKA mRNA expression preceding elevated enzyme activity by several days suggested a hyperosmotic response different from that of tilapia or killifish. Unlike the response of striped bass to SW-transfer that gill NKA activity increased (after 3 days in SW) without parallel changes in NKA mRNA and protein abundance (Tipsmark et al., 2004), significant decrease was found in gill NKA activity of tilapia at 3 h post SW-transfer (Fig. 6). Early activation of the regulatory mechanism in gills, i.e., NKA, at the gene level, was thus crucial to survival of tilapia when exposed to abrupt changes of ambient salinity.

Upon hyperosmotic shock in BW, the osmoregulatory mechanism of tilapia is readily activated. Elevated gill NKA activity from 12 h post-transfer and the subsequent plasma [Na^+] decrease indicating an efficient regulatory mechanism of plasma [Na^+], rather than [Cl^-]. Both protein abundance and activity of NKA in gills of tilapia were upregulated following BW exposure. The difference in NKA contents observed between SW- and FW-subtype MR cells suggested that, the increase in NKA transcription and translation might be due to changes in MR cell subtypes and densities upon salinity challenge (Uchida et al., 2000; Lee et al., 2003). Hence, following a 12 h post-transfer crisis period, tilapia attained a regulatory period to maintain homeostasis of plasma osmolality (Fig. 5). The hyperosmotic shock of SW, in contrast to that of BW, is lethal to tilapia. A significant increase in plasma [Na^+] from 4 h post-transfer corresponded to the breakdown of the regulatory mechanisms, i.e., NKA activity, from 3 h post-transfer (Fig. 7). Failure to increase NKA α1-mRNA and protein abundance which accompanied with a dramatic decrease of functional MR cell apical-surface densities within 1 h after transfer might explain the dysfunction of NKA in SW-shock. Serious ionic imbalance in plasma indicated a failure of induction of the above osmoregulatory mechanisms during the crisis period. Consequently, the fish died.

Euryhaline teleosts acclimated to higher salinity environments experienced two periods: (i) a crisis period in which there was a rapid increase in gill ion exchange accompanied by elevated plasma ions and osmolality, followed by (ii) a regulatory period in which an increase in gill Na^+/K^+-ATPase (NKA) activity together with a proliferation and/or development of functional mitochondrion-rich (MR) cells, net sodium and chloride efflux increases, and plasma ion balances were restored (see review of Evans et al., 2005). In this study, hyperosmotic shock in brackish water (BW) induced a typical "crisis-then-regulation" pattern in tilapia: plasma osmolality and [Cl^-] increased by more than 20% within 3 h but approached original levels by 96 h post-transfer; while plasma [Na^+] peaked at 12 h but decreased (regulated) from 24 h post-transfer (Fig. 5). Although the time scales from crisis to regulation were different (from hours to days), the "two phases" (crisis-then-regulation) in salinity acclimation were reported in many euryhaline teleosts, e.g., the long-jawed mudsucker (Yoshikawa et al., 1993), killifish (Zadunaisky et al., 1995), European sea bass (Jensen et al., 1998), silver sea bream (Kelly and Woo, 1999), striped bass (Tipsmark et al., 2004), gilthead sea bream (Sangiao-Alvarellos et al., 2005), and milkfish (Lin et al., 2006). This signified a well-developed ability to rapidly activate osmoregulatory mechanisms after transfer between environments of different salinities within the range of tissue tolerance. On the other hand, unlike the BW group, tilapia challenged with SW-hyperosmotic shock experienced serious plasma ionic imbalance during the crisis period, where osmolality and [Cl^-] increased by more than 80% (Fig. 7a and b). But rainbow trout were acclimated to full strength SW from FW directly with little or no mortality (Hawkings et al., 2004). It showed that no regulation was found in this group because the change of salinity was greater than the range of withstanding in the crisis period and regulatory mechanisms were not activated in time before death.

Taken together, acclimation of the euryhaline tilapia upon hyperosmotic BW or SW shocks was determined mainly by rapid modulation of densities and subtypes of MR cells with different directions of ion transport correlated to NKA at transcriptional/translational levels in gills. Expression of branchial Na^+ and Cl^- related transporters of the euryhaline tilapia in response to hyperosmotic shocks will be performed in future works, to illustrate more detailed mechanisms of salinity-acclimation.

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References

Arystarkhova, E., Sweadner, K.J., 1996. Isoform-specific monoclonal antibodies to Na, K-ATPase α subunits. J. Biol. Chem. 271, 23407–23417.
Chang, I.C., Lee, T.H., Yang, C.H., Wei, Y.Y., Chou, F.I., Hwang, P.P., 2001. Morphology and function of gill mitochondria-rich cells in fish acclimated to different environments. Physiol. Biochem. Zool. 74, 111–119.
Chang, I.C., Wei, Y.Y., Chou, F.I., Hwang, P.P., 2003. Stimulation of Cl^- uptake and morphological changes in gill mitochondria-rich cells in freshwater tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. 76, 544–552.
Crombie, H.J., Bell, M.V., Tytler, P., 1996. Inhibition of sodium-plus-potassium-stimulated adenosine triphosphatase (Na^+,K^+-ATPase) by protein kinase C activators in the gills of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B 113, 765–772.
Daborn, K., Cozzi, R.R.F., Marshall, W.S., 2001. Dynamics of pavement cell-chloride cell interactions during abrupt salinity change in *Fundulus heterclitus*. J. Exp. Biol. 204, 1889–1899.
D'Cotta, H.C., Valotaire, C., Le Gac, F., Prunet, P., 2000. Synthesis of gill Na,K-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels. Am. J. Physiol. 278, R101–R110.
Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.
Feng, S.H., Leu, J.H., Yang, C.H., Fang, M.J., Huang, C.J., Hwang, P.P., 2002. Gene expression of Na^+–K^+-ATPase α1 and α3 subunits in gills of the teleosts *Oreochromis mossambicus*, adapted to different environmental salinities. Mar. Biotechnol. 4, 379–391.
Forrest Jr., N.J., Cohen, A.D., Schon, D.A., Epstein, F.H., 1973. Na transport and Na–K-ATPase in gills during adaptation to seawater: effects of cortisol. Am. J. Phyiol. 224, 709–713.
Franson, M.A.H., 1985. Standard Methods for Examination of Water and Waste Water, 16th ed. American Public Health Association, Washington DC.
Galvez, F., Reid, S.D., Hawkings, G., Goss, G.G., 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. Am. J. Physiol. 282, R658–R668.
Goss, G.G., Adamia, S., Galvez, F., 2001. Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. Am. J. Physiol. 281, R1718–R1725.
Hawkings, G.S., Galvez, F., Goss, G.G., 2004. Seawater acclimation causes independent alterations in Na^+/K^+- and H^+-ATPase activity in isolated mitochondria-rich cell subtypes of the rainbow trout gill. J. Exp. Biol. 207, 905–912.
Hiroi, J., McCormick, S.D., Ohtani-Kaneko, R., Kaneko, T., 2005. Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. J. Exp. Biol. 208, 2023–2036.
Hossler, F.E., 1980. Gill arch of the mullet, *Mugil cephalus* III. Rate of response to salinity change. Am. J. Physiol. 238, R160–164.
Hwang, P.P., 1987. Tolerance and ultrastructural responses of branchial chloride cells to salinity changes in the euryhaline teleost *Oreochromis mossambicus*. Mar. Biol. 94, 643–649.
Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A 148, 479–497.
Hwang, P.P., Sun, C.M., Wu, S.M., 1989. Changes of plasma osmolality, chloride concentration and gill Na–K-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. Mar. Biol. 100, 295–299.
Hwang, P.P., Fang, M.J., Tsai, J.C., Huang, C.J., Chen, S.T., 1998. Expression of mRNA and protein of Na^+–K^+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*). Fish Physiol. Biochem. 18, 363–373.
Jensen, M.K., Madsen, S.S., Kristiansen, K., 1998. Osmoregulation and salinity effects on the expression and activity of Na^+, K^+-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). J. Exp. Zool. 282, 290–300.

Johnson, M.R., Wang, K., Smith, J.B., Heslin, M.J., Diasio, R.B., 2000. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal. Biochem. 278, 175–184.
Kaneko, T., Shiraishi, K., 2001. Evidence for chloride secretion from chloride cells in the yolk-sac membrane of Mozambique tilapia larvae adapted to seawater. Fish. Sci. 67, 541–543.
Katoh, F., Kaneko, K., 2003. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. J. Exp. Biol. 206, 4113–4123.
Kelly, S.P., Woo, N.Y.S., 1999. Cellular and biochemical characterization of hyposmotic adaptation in a marine teleost, *Sparus sarba*. Zool. Sci. 16, 505–514.
King, J.A.C., Hossler, F.E., 1991. The gill arch of the striped bass (*Morone saxatilis*). Alteration in the ultrastructure of chloride cell apical crypts and chloride efflux following exposure to seawater. J. Morphol. 209, 165–176.
Kodak Digital Science ID (1995) Kodak ID image analysis software. Windows Versions 2.0.3. Eastman Kodak Company, Rochester, NY.
Kültz, D., Jürss, K., Jonas, L., 1995. Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*). Cell Tissue Res. 279, 65–73.
Laiz-Carrión, R., Guerreiro, P.M., Fuentes, J., Canario, A.V.M., Martín Del Río, M.P., Mancera, J.M., 2005. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. J. Exp. Zool. 303A, 563–576.
Lee, T.H., Hwang, P.P., Lin, H.C., Huang, F.L., 1996. Mitochondria-rich cells in the branchial epithelium of the teleost, *Oreochromis mossambicus*, acclimated to various hypotonic environments. Fish Physiol. Biochem. 15, 513–523.
Lee, T.H., Tsai, J.C., Fang, M.J., Yu, M.J., Hwang, P.P., 1998. Isoform expression of Na^+–K^+-ATPase α-subunit in gills of the teleost, *Oreochromis mossambicus*. Am. J. Physiol. 275, R926–R932.
Lee, T.H., Hwang, P.P., Shieh, Y.E., Lin, C.H., 2000. The relationship between 'deep-hole' mitochondria-rich cells and salinity adaptation in the euryhaline teleost, *Oreochromis mossambicus*. Fish Physiol. Biochem. 23, 133–140.
Lee, T.H., Feng, S.H., Lin, C.H., Hwang, Y.H., Huang, C.L., Hwang, P.P., 2003. Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. Zool. Sci. 20, 29–36.
Lin, L.Y., Hwang, P.P., 2001. Modification of morphology and function of integument mitochondria-rich cells in tilapia larvae (*Oreochromis mossambicus*) acclimated to ambient chloride levels. Physiol. Biochem. Zool. 74, 469–476.
Lin, L.Y., Hwang, P.P., 2004. Mitochondria-rich cell activity in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae acclimatized to different ambient chloride levels. J. Exp. Biol. 207, 1335–1344.
Lin, Y.M., Chen, C.N., Lee, T.H., 2003. The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp. Biochem. Physiol. A 135, 489–497.
Lin, C.H., Huang, C.L., Yang, C.H., Lee, T.H., Hwang, P.P., 2004. Time-course changes in the expression of Na, K-ATPase and the morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. J. Exp. Zool. 301A, 85–96.
Lin, Y.M., Chen, C.N., Yoshinaga, T., Tsai, S.C., Shen, I.D., Lee, T.H., 2006. Short-term effects of hyposmotic shock on Na^+/K^+-ATPase expression in gills of the euryhaline milkfish, *Chanos chanos*. Comp. Biochem. Physiol. A 143, 406–415.
Madsen, S.S., Naamansen, E.T., 1989. Plasma ionic regulation and gill Na^+/K^+-ATPase changes during rapid transfer to seawater to yearling rainbow trout, *Salmo gairdneri*: time course and seasonal variation. J. Fish Biol. 34, 829–840.
Madsen, S.S., Jensen, M.K., Nøhr, J., Kristiansen, K., 1995. Expression of Na^+, K^+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. Am. J. Physiol. 269, R1339–R1345.
Mancera, J.M., McCormick, S.D., 2000. Rapid activation of gill Na+, K+-ATPase in the euryhaline teleosts *Fundulus heteroclitus*. J. Exp. Zool. 287, 263–274.
Marshall, W.S., 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J. Exp. Zool. 293, 264–283.
Morgan, J.D., Sakamoto, T., Gordon, G.E., Iwama, G.K., 1997. Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. Comp. Biochem. Physiol. A 117, 391–398.
Nielsen, C., Madsen, S.S., Bjornsson, B.T.H., 1999. Changes in branchial and intestinal osmoregulatory mechanisms and growth hormone levels during smolting in hatchery-reared and wild brown trout. J. Fish Biol. 51, 215–219.
Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid–base regulation. J. Exp. Zool. 300A, 53–62.
Peterson, G.L., 1978. A simplified method for analysis of inorganic phosphate in the presence of interfering substances. Anal. Biochem. 84, 164–172.
Richards, J.G., Semple, J.W., Bystriasky, J.S., Schulte, P.M., 2003. Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol. 206, 4475–4486.
Sakamoto, T., Yokota, S., Ando, M., 2000. Rapid morphological oscillation of mitochondrion-rich cell in estuarine mudskipper following salinity changes. J. Exp. Zool. 286, 666–669.
Sangiao-Alvarellos, S., Arjona, F.J., Martín del Río, M.P., Míguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291–4304.
Scheiner-Bobis, G., 2002. The sodium pumps — its molecular properties and mechanics of ion transport. Eur. J. Biochem. 269, 2424–2433.
Scott, G.R., Richards, J.G., Forbush, B., Isenring, P., Schutle, P.M., 2004. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol. 287, C300–C309.
Seidelin, M., Madsen, S.S., Blenstrup, H., Tipsmark, C.K., 2000. Time-course changes in the expression of Na^+,K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. Physiol. Biochem. Zool. 73, 446–453.
Singer, T.D., Clements, K.M., Semple, J.W., Schutt, P.M., Bystriansky, J.S., Finstad, B., Fleming, I.A., McKinley, R.S., 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Can. J. Fish. Aquat. Sci. 59, 125–135.
Stanwell, C., Gescher, A., Bradshaw, T.D., Pettit, G.R., 1994. The role of protein kinase C isoenzymes in the growth inhibition caused by bryostatin 1 in human A549 lung and MCF-7 breast carcinoma cells. Int. J. Cancer 56, 585–592.
Stickney, R.R., 1986. Tilapia tolerance of saline waters: a review. Progr-Fish Cult. 48, 461–470.
Takeyasu, K., Tamkum, M.M., Renaud, K.J., Fambrough, D.M., 1988. Ouabain-sensitive (Na^++K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. J. Biol. Chem. 263, 4347–4354.
Tang, C.H., Chang, I.C., Chen, C.H., Lee, T.H., Hwang, P.P., 2008. Phenotypic changes of mitochondrion-rich cells and responses of Na^+/K^+-ATPase in gills of tilapia exposed to deionized water. Zool. Sci. 25, 205–211.
Tipsmark, C.K., Madsen, S.S., Seidelin, M., Christensen, A.S., Cutler, C.P., Cramb, G., 2002. Dynamics of Na, K, 2Cl co-transporter and Na, K-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). J. Exp. Zool. 293, 106–118.
Tipsmark, C.K., Madsen, S.S., Borski, R.J., 2004. Effects of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J. Exp. Zool. 301A, 979–991.
Towle, D.W., Gilman, M.E., Hempel, J.D., 1977. Rapid modulation of gill Na^++K^+-ATPase activity during acclimation of the killifish *Fundulus heteroclitus* to salinity change. J. Exp. Zool. 202, 179–186.
Tsai, J.C., Hwang, P.P., 1998. The wheat germ agglutinin binding sites and development of the mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*). Fish Physiol. Biochem. 19, 95–102.
Uchida, K., Kaneko, T., Miyazaki, H., Hasegawa, S., Hirano, T., 2000. Excellent salinity tolerance of Mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater. Zool. Sci. 17, 149–160.
van der Heijden, A.J.H., Verbost, P.M., Eygensteyn, J., Li, J., Wendelaar Bonga, S.E., Flik, G., 1997. Mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*) adapted to fresh water or sea water: quantification by confocal laser scanning microscopy. J. Exp. Biol. 200, 55–64.
Wilson, J.M., Laurent, P., 2002. Fish gill morphology: inside out. J. Exp. Zool. 293, 192–213.
Wilson, J.M., Laurent, P., Tufts, B.L., Benos, D.J., Donowitz, M., Wayne Vogl, A., Randall, D.J., 2000. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. J. Exp. Biol. 203, 2279–2296.
Wu, Y.C., Lin, L.Y., Lee, T.H., 2003. Na^+, K^+, $2Cl^-$-cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. Zool. Stud. 42, 186–192.
Yoshikawa, J.S.M., McCormick, S.D., Young, G., Bern, H.A., 1993. Effects of salinity on chloride cells and Na^+–K^+-ATPase activity in the teleost *Gillichthys mirabilis*. Comp. Biochem. Physiol. A 105, 311–317.
Zadunaisky, J.A., Cardona, S., Au, L., 1995. Chloride transport activation by plasma osmolarity during adaptation to high salinity of *Fundulus heteroclitus*. J. Membr. Biol. 143, 207–217.