The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments

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ABS TRACT

The recent model showed that seawater (SW) mitochondrion-rich (MR) cells with hole-type apical openings secrete Cl⁻ through the transporters including the Na⁺, K⁺-ATPase (NKA), Na⁺, K⁺, 2Cl⁻ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR). The present study focused on the dynamic elimination of the Cl⁻ secretory capacity and illustrated different phases (i.e., acute and regulatory phases) of branchial MR cells in response to hypoosmotic challenge. Time-course remodeling of the cell surfaces and the altered expressions of typical ion transporters were observed in the branchial MR cells of SW-acclimated brackish medaka (*Oryzias dancena*) when exposed to fresh water (FW). On the 1st day post-transfer, rapid changes were shown in the acute phase: the flat-type MR cells with large apical surfaces replaced the hole-type cells, the gene expression of both Odcnfcc1a and Odcfttr decreased, and the apical immunostaining signals of CFTR protein disappeared. The basolateral immunostaining signals of NKCC1a protein decreased throughout the regulatory phase (>1 day post-transfer). During this period, the size and number of NKA-immunoreactive MR cells were significantly reduced and elevated, respectively. Branchial NKA expression and activity were maintained at constant levels in both phases. The results revealed that when SW-acclimated brackish medaka were transferred to hypoosmotic FW for 24 h, the Cl⁻ secretory capacity of MR cells was eliminated, whereas NKCC1a protein was retained to maintain the hypoosmoregulatory endurance of the gills. The time-course acute and regulatory phases of gill MR cells showed different strategies of the euryhaline medaka when subjected to hypoosmotic environments.

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1. Introduction

Euryhaline teleosts exhibit hyperosmoregulatory and hypoosmoregulatory abilities to keep plasma osmolality within narrow physiological ranges in fresh water (FW) and seawater (SW), respectively (Kaneko et al., 2008). The gills exposed to the external medium are the major osmoregulatory organ in euryhaline teleosts. Several review articles have illustrated the ion transporter systems of mitochondrion-rich (MR) cells, also called ionocytes or chloride cells, in gill (Marshall, 2002; Hirose et al., 2003; Evans et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011). The basolateral membranes of MR cells form extensive tubular systems, whereas the primary site of ion transport between MR cells and the external environment is the apical membrane. Typical phenotypes (apical surface morphology) of the gill MR cells were usually found to vary across environments. The apical surfaces of FW MR cells were generally flat with dense microvilli-like projections. In contrast, the SW MR cells exhibited deeply invaginated surfaces with smaller orifices without projections (Hosslér et al., 1979; King and Hossler, 1991; Lee et al., 1996; Perry, 1997; Katoh and Kaneko, 2003). In a recent molecular model of ion transporters, gill MR cells expressed Na⁺, K⁺-ATPase (NKA) on their basolateral membrane. This membrane protein provided the driving force allowing secondary ion transporters to absorb ions in FW and secrete ions into SW (Wood and Marshall, 1994; Evans et al., 1999; Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007; Hwang et al., 2011). In FW teleosts, the ionocytes express apical Na⁺, Cl⁻ cotransporter (NCC), apical Na⁺, H⁺ exchanger 3 (NHE3), and basolateral chloride channel 3 (CLC3). These proteins absorb external Na⁺ and Cl⁻ ions (Yan et al., 2007; Hiroi et al., 2008; Wang et al., 2009; Tang et al., 2010). However, the Cl⁻ secretory capacities in the branchial MR cells of the SW teleosts involve the basolateral Na⁺, K⁺, 2Cl⁻ cotransporter (NKCC) and the apical cystic fibrosis transmembrane conductance regulator (CFTR)
which transport Cl\(^-\) from the blood through the cytosol to the external medium (Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007; Evans, 2008). It is, therefore, essential for euryhaline teleosts to alter the functional transporters and remodel the apical morphologies of MR cells that are acclimated to environments with different levels of salinity.

Numerous euryhaline teleosts can tolerate and survive after transfer from SW to FW or vice versa (Lee et al., 1996; Katoh and Kaneko, 2003; Lin et al., 2003, 2004b; Tse et al., 2006; Tipsmark et al., 2008a,b). Apical morphology remodeling was observed in SW-acclimated killifish (Fundulus heteroclitus, Katoh and Kaneko, 2003) and Mozambique tilapia (Oreochromis mossambicus, Lin et al., 2004a) when transferred to hypoosmotic environments. Meanwhile, in the transfer experiments, changes in NKA-immunoreactive (IR) cell size or numbers among certain euryhaline species were reported (Ayson et al., 1994; Katoh and Kaneko, 2003; Lin et al., 2006). Moreover, changes in the time course of gene expression or the distribution of NKCC and CFTR were also reported in striped bass (Morone saxatilis, Tipsmark et al., 2004; Madsen et al., 2007), tilapia (Hiroi et al., 2008), and killfish (Katoh and Kaneko, 2003; Scott et al., 2005) upon hypoosmotic challenge. Previous studies found two phases of response in fish exposed to hyperosmotic or hypoosmotic stress: an acute (crisis or adverse) phase occurred in the 24 h after transfer to the new environment; the following period was the regulatory phase (Gong et al., 2004; Lin et al., 2004a, 2006; Madsen et al., 2007). However, to this point, no study has integrated the dynamic remodeling of cellular profiles (apical morphologies, cell size, cell numbers) and time-course changes in NKA activity and the two ion transporters involved in Cl\(^-\) secretion (mRNA abundance and protein distribution) to clarify the crisis and regulatory phases in gill MR cells of SW fish when exposed to FW.

The brackish medaka (Oryzias dancena, also designated as O. melastigma; Inoue and Takei, 2002, 2003), is closely related to the Japanese medaka, but it primarily inhabits river mouths and estuaries (Roberts, 1998). Brackish medaka showed better salinity tolerance than Japanese medaka in terms of the survival rates of adult fish and the hatching rates of embryos (Inoue and Takei, 2002, 2003). On the basis of the results reported by Kang et al. (2008), cellular profiles, including the size and numbers of gill MR cells (NKA-immunoreactive cells), were determined in SW- and FW-acclimated brackish medaka. However, the apical morphologies of branchial MR cells in this euryhaline medaka were not yet clear. The time-course experiment used to investigate the transfer from SW to FW illustrated that the retaining expressions of gill NKCC1a protein in gills of the brackish medaka correlated with their hypoosmoregulatory endurance (Kang et al., 2010). The responses of gill MR cells to direct transfer of the brackish medaka from SW to FW, to our knowledge, were less well understood. Therefore, the present study aimed to verify the phases of responses at the cellular and molecular levels in gill MR cells exhibited ion secretory capacities of the brackish medaka when exposed to hypoosmotic challenge. We first observed the morphologies of MR cells in gills of the SW- and FW-acclimated medaka. The dynamic changes in cellular profiles of MR/NKA-IR cells, including apical morphologies, cell size, and cell numbers, were investigated during FW exposure. Meanwhile, the expressions of gill NKA were examined. In addition, two dominant molecules of the Cl\(^-\) secretory mechanism in the gills, NKCC1a and CFTR, were investigated with regard to mRNA abundance and protein distribution when the medaka were transferred from SW to FW to elucidate Cl\(^-\) secretion.

2. Materials and methods

2.1. Fish and experimental condition

Adult brackish medaka (O. dancena) obtained from a local aquarium averaged 2.5 ± 0.3 cm in length. Seawater (35‰; SW) was prepared from local fresh tap water (FW) with proper amounts of RealOcean™ Synthetic Sea Salt (TAAM, Camarillo, CA, USA) for raising fish. The medaka were acclimated to FW or SW for at least three weeks. SW-acclimated medaka transferred directly to FW for time-course experiments. The FW-exposure groups were sampled at 1 h, 3 h, 6 h, 12 h, 24 h (1 day), 48 h (2 days), 72 h (3 days), 96 h (4 days), 168 h (7 days), 14 days and 21 days, respectively, for subsequent observations or analyses to illustrate the time-course changes in gill mitochondrion-rich (MR) cells of brackish medaka in response to hypoosmotic challenges. In the time-course experiment, 4 individuals were sampled for each time point. The water was continuously circulated through fabric-floss filters and partially refreshed every two weeks. The water temperature was maintained at 28 ± 1 °C. The photoperiod cycle was 14 h light: 10 h dark every day. The fish were fed a daily diet of commercial pellets. Following the experiment, the fish were not fed for 2 days and were anaesthetized with MS-222 (100–200 mg/L) before sampling. The use protocol for the experimental fish was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University (IACUC Approval No. 96–48) to T.H.L.

2.2. Scanning electron microscope (SEM)

The gills were fixed at 4 °C in a fixative consisting of 5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) for 12 h. Fixed gills were rinsed for 15 min with three changes of 0.1 M phosphate buffer (PB) at 4 °C, then post-fixed with 1% (w/v) osmium tetroxide in 0.1 M PB for 1.5 h. After post-fixation, the gills were rinsed in PB and dehydrated in ascending concentrations of ethanol, from 30% to absolute. Samples were then critical point dried using liquid CO₂ in a Hitachi HCP-2 (Tokyo, Japan) critical point drier, mounted on aluminum stubs with silver paint, and sputter coated for 3 min with a gold-palladium complex in a Pt Coater (JFC-1600, JEOL, Tokyo, Japan). The coated gill filaments were observed with a scanning electron microscope (JSM-6700F, JEOL). Furthermore, afferent regions (10,000 μm²) of three gill filaments from each medaka were randomly selected and ratios of three phenotypes of MR cells with different apical surfaces in the selected regions were analyzed.

2.3. Antibodies

Primary antibodies used in this study include: (1) a mouse monoclonal antibody (α5; Developmental Studies Hybridoma Bank) raised against the α-subunit of the avian Na⁺, K⁺-ATPase (NKA); (2) a mouse monoclonal antibody (MAB25031; R&D Systems, Boston, MA, USA) directed against 104 amino acids at the C-terminus of the human cystic fibrosis transmembrane conductance regulator (CFTR); (3) a mouse monoclonal antiserum (T4; Developmental Studies Hybridoma Bank) raised against the C-terminus of human Na⁺, K⁺, 2Cl⁻ co-transporter (NKCC); (4) a rabbit monoclonal antibody (EP1845Y; Abcam, Cambridge, UK) raised against the N-terminus of human NKA α-subunit. The secondary antibody for immunoblots was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA). For double immunofluorescence staining, the secondary antibodies were Dylight-488-conjugated goat anti-Rabbit IgG and Dylight-549-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore Pike, PA, USA).

2.4. Immunohistochemical detection and analysis of NKA immunoreactive (NKA-IR) cells

The gills were excised and fixed in Bouin’s solution at room temperature for 48 h. The samples were dehydrated through a graded ethanol series, infiltrated with xylene, and embedded in paraffin. Cross sections of the gills were cut at 5 μm thickness and mounted on poly-L-lysine-coated glass slides. The deparaffinized sections
were immunohistochemically stained with a monoclonal antibody (α5) to the NKA α-subunit followed by a commercial kit (PicSure™, Zymed, South San Francisco, CA, USA) for visualization of the immunoreaction. The immunostained sections were then counterstained with Hematoxylin (Merck, Hohenbrunn, Germany) and observed with a microscope (Olympus BX50, Tokyo, Japan). According to the methods of Kang et al. (2008), the number and size of the NKA-IR cells in the first and secondary pairs of the medaka gills were quantified. The average values of the cell size and number were determined from 20 gill filament. These values for different groups of fish were then calculated and compared.

2.5. Immunoblotting of NKA

Four pairs of gills from the medaka were excised and blotted dry. The gill samples were suspended in a mixture of homogenization medium (100 mM imidazole-HCl, 5 mM Na2EDTA, 200 mM sucrose, 0.1% sodium deoxycholate, pH 7.6) and proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) (vol/vol: 50:1). Homogenization was performed in microcentrifuge tubes with a Polytron PT1200E (Lucerne, Switzerland) at the maximal speed for 25 rotations on ice. The homogenates were then centrifuged at 5500×g and 4 °C for 20 min. Protein concentrations of the supernatant were determined by reagents from the Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. The pre-stained protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). Aliquots containing 30 μg of the gill homogenates were heated at 60 °C for 25 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from the unstrained gels to PVDF (Millipore, Bedford, MA, USA) using a tank transfer system (Bio-Rad, Mini Protein 3). Blots were preincubated for 2 h in PBST (phosphate buffer saline with Tween 20) buffer (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.2% (vol/vol) Tween 20, pH 7.4) containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding; they were then incubated at 4 °C with primary antibody (α5) diluted in PBST (1:4000) overnight, followed by a 1 h reaction with horseradish peroxidase-conjugated antibody (1:10,000 dilution). Blots were developed using the ECL kit (Pierce). Immunoblots were photographed and imported as TIFF files into the ID image analysis software package (MCID Analysis Evaluation 7.0). Results were converted to numerical values in order to compare relative intensities of immunoreactive bands.

2.6. Na+/K+-ATPase (NKA) activity

The gill NKA activity of brackish medaka was measured according to Kang et al. (2008) based on the NADH-linked methods. ADP derived from the hydrolysis of ATP by ATPase was enzymatically coupled to the oxidation of reduced NADH using lactate dehydrogenase (LDH) and pyruvate kinase (PK). The four pairs of gills from each sample were dissected quickly and stored in a microcentrifuge tube at −80 °C before use. The samples of six time-points (0, 1, 3, 7, 14, 21 days) were rapidly homogenized for detection of the NKA activity at the same time.

2.7. Total RNA extraction and reverse transcription

Each total RNA sample from the gills was extracted from four medaka with the RNA-Bee™ (Tel-Test, Friendwood, TX, USA) according to the manufacturer’s instructions. The RNA pellet was dissolved in 50 μL DEPC-H2O and treated with the RNA clean-up protocol from the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA) according to the manufacturer’s instructions to eliminate genomic DNA contamination. RNA integrity was verified by 0.8% agarose gel electrophoresis. Extracted RNA samples were stored at −80 °C after isolation. First-strand cDNA was synthesized by reverse transcribing 5 μg of the total RNA using 1 μL of Oligo(dT) (0.5 μg/μL) primer and 1 μL of PowerScript™ Reverse Transcriptase (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions.

2.8. Quantitative real-time PCR (qPCR)

The expression of Odnkcc1a mRNA was quantified with the MiniOpticon real-time PCR system (Bio-Rad) using the following gene primers. The Odnkcc1a primers were sequences as follows (5′ to 3′): forward−CTCTCCACTTCA GCCATCG and reverse−ACCCATACTGGCAAACGC. The Odftr primers were sequences as follows (5′ to 3′): forward−GTAGAGTGGC AGTGTGCC and reverse−GAGTGGAGCCGGTGGACAT. The β-actin primer sequences were as follows (5′ to 3′): forward−CTTGA CTTGAGAGAGAT and reverse−AGAAGAAGGCTTGAAGAG. The PCR reactions contained 8 μL of cDNA (1000×), 2 μL of either a 5-μM gene-specific primer mixture or a 5-μM β-actin primer mixture and 10 μL of 2× SYBR Green PCR MasterMix (Bio-Rad). The mRNA values were normalized using the expression of the β-actin mRNA from the same cDNA samples. The occurrence of secondary products and primer-dimers was inspected by melting curve analysis and electrophoresis to confirm that the amplification was specific. One identical control sample was used as the internal control among different groups. For each unknown sample, the comparative Ct method with the formula $2^{-\Delta\Delta Ct}=2^{-(Ct_{target\ gene}-Ct_{\beta-actin})- (Ct_{target\ gene}-Ct_{\beta-actin})_{control}}$ was used to obtain the corresponding Odnkcc1a, Odftr, and β-actin values, where Ct corresponded to the threshold cycle number.

2.9. Cryosectioning

The gills of the brackish medaka from different post-transfer time-points were fixed in neutral formalin (pH 7.2) in 4 °C for 3 h. After washing in PBS, the samples were permeated with methanol. Before cryosectioning, the samples were stored in methanol at −20 °C for an additional 2 h. Samples were washed with PBS, equilibrated with O.C.T. (Sakura Tissue-Tek, Torrance, CA, USA) overnight at 4 °C and then mounted for cryosectioning. Sections of 7 μm thickness were cut with a cryostat (Leica CM3050S, Nussloch, Germany) at −25 °C. The cut sections were placed on 0.03% poly-L-lysine (Sigma)-coated slides and kept in slide boxes at −20 °C before immunofluorescence staining.

2.10. Double immunofluorescence staining and the quantification of NKCC1a-IR/NKA-IR and CTR-IR/NKA-IR cells

The cryosection slides were rinsed in PBS and incubated in 5% bovine serum albumin (Sigma) and 2% Tween-20 (Merck, Hohenbrunn, Germany) in PBS for 30 min. Samples were then incubated with anti-NKCC antibody (diluted with 1% bovine serum albumin, 1: 100 dilution) or anti-CFTR antibody (1:50 dilution) at room temperature. After washing three times with PBS, incubated with Dylight-549 goat anti-mouse IgG at room temperature for 1 h and washed several times more with PBS. After the first staining, the samples were incubated with anti-NKA rabbit antibody (Abcam, 1:200) for 2 h at room temperature. The samples were then washed several times with PBS and incubated with Dylight-488 goat anti-rabbit IgG at room temperature for 1 h, which was followed by several PBS washes. The samples were then mounted in Clearmount™ (Zymed) and examined using a fluorescent microscope (BX50, Olympus) with a digital camera (Nikon COOLPIX 5000). Only those cross-sections of filaments that were approximately 175 ± 25 μm in length were randomly selected to minimize the effects of cutting angles on the sections. The percentages represented the average cell numbers of NKCC- or CTR-positive NKA-IR cells divided by total NKA-IR cells in the first and secondary pairs of the medaka gills. The proportions were determined from 15
filaments. These values were then compared among different groups of the fish.

2.11. Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) (Dunnett’s pair-wise method), and \( P<0.05 \) was set as the significant level. Values were expressed as the mean ± S.E.M. (the standard error of the mean) unless stated otherwise.

3. Results

3.1. Remodeling of \( \text{Na}^+\text{,K}^+\text{-ATPase-} \) (NKA-) immunoreactive (IR)/ mitochondrial-rich (MR) cell profiles

The apical openings of the seawater (SW) MR cells (diameters ranged from 0.5 to 2 μm) were smaller than those of freshwater (FW) MR cells (diameter: 3–20 μm). The morphologies of the apical surface were observed by scanning electron microscopy, which showed that SW MR cells were hole-type and FW MR cells were flat-type. In addition, only the apical surfaces of FW cells exhibited numerous microvilli-like projections (Fig. 1A and B). In the micrographs of immunohistochemical staining, smaller orifices were found in the apical regions of SW MR/NKA-IR cells than in the FW cells (Fig. 1C and D). When the medaka were transferred from SW to FW, the flat-type MR cells were classified into two groups, the small (3–7 μm) group and the large (8–20 μm) group, to elucidate correlations between apical sizes of flat-type MR cells and low environmental salinities (Fig. 2). The average frequencies of three types, including the hole-, small flat-, and large flat-type MR cells were quantified from samples obtained at ten time points post-transfer (Fig. 3). In gills of the SW medaka transferred to FW for 24 h, the frequency of the hole-type MR cells quickly decreased to zero, whereas the frequency of large flat-type MR cells increased to 80% (Fig. 3). At 24 h post-transfer, the ratio of small flat-type cells increased to 50%, and that of large flat-type cells decreased to 30% until 96 h. In addition, the hole-type MR cells occurred again in the 72, 96, and 168-h post-transfer groups (Fig. 3). When SW-acclimated fish were transferred to FW for 1/4 days (6 h), the apical structure of MR cells compared with that of FW fish (Fig. 1B) was flat-type with scattered projections (Fig. 4A). The apical surface extended to the maximal diameter (approximately 20 μm) at 1 day (24 h) after transfer (Fig. 4C). There were short projections on the apical surfaces of the MR cells in the gills of SW-acclimated medaka exposed to FW for 1/2, 1, and 3 days (Fig. 4B, C, D). Until the 4-day timepoint, long and dense projections (microvilli-like) similar to those of the FW individuals were observed on the apical surfaces of gill MR cells (Fig. 4E). The size and number of MR cells were determined by NKA-immunoreactive staining in the cross-sections of gill filaments from fish in the time-course groups (Fig. 5). The representative immunostaining pictures of 3 (Fig. 5A) and 14 (Fig. 5B) days-post transfer groups were shown. Smaller size and more number of NKA-IR cells were found in the 14 days-post transfer group compared to the 3 days group. Compared with the size of the gill NKA-IR cells in the control group (0 days/SW; approximately 90–80 μm²), after exposure to FW for 14 and 21 days, the cell size decreased significantly (approximately 70–76 μm²) (Fig. 5C). Moreover, the numbers of NKA-IR cells gradually increased when the fish were exposed to FW, and they significantly increased >7 days post-transfer. The numbers in the 14- and 21-days post-transfer groups were approximately 1.5- and 2-fold higher than that of the control (0 day) group, respectively (Fig. 5D).

3.2. Alterations of ion transporters involved in the \( \text{Cl}^- \) secretory capacities of MR cells

When SW medaka were exposed to FW, there was no difference in the expression of NKA α-subunits or NKA activities. However, the abundance of gill \( \text{Odcftr} \) gene expression decreased >3 h post-transfer. The
mRNA levels in the 3 and 12 h post-transfer groups were approximately 2- and 10-fold lower than those of the control (0 day) group, respectively (Fig. 7). In the cellular distributions of CFTR protein (Fig. 8), the ratio of NKA-IR cells with apical CFTR-IR signals (16%) in the 12 h post-transfer group was significantly lower than the control and the 3 and 6 h post-transfer groups (75–78%). No CFTR-IR signal was observed in the groups at 24 and 48 h post-transfer (Fig. 8C and D). Meanwhile, the abundance of gill Odnkcc1a gene reduced significantly >1 h post-transfer. The Odnkcc1a mRNA levels of the 1 and 12 h post-transfer groups decreased to approximately 1/2- and 1/10-fold of the level in the control (0 day) group, respectively (Fig. 9). The basolateral NKCC1a signals of T4 antibody were identified by colocalization to NKA-IR signals. At 7 and 14 days after transfer to FW, the percentages of basolateral NKCC1a-IR/NKA-IR cells in gill filaments reduced to approximately 1/3- and 1/10-fold, respectively, of those observed in the SW (0 day) group (Fig. 10).

4. Discussion

The current morphological classification of mitochondrion-rich (MR) cells was proposed based on their apical structures or distinct ion transporters (Perry, 1997; Wilson and Laurent, 2002; Evans et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011). Previous review articles depicted that generally the apical morphologies of gill MR cells were flat-type with microvilli in the FW-adapted teleosts and hole-type in the SW individuals (Perry, 1997; Evans et al., 2005; Kaneko et al., 2008; Hwang et al., 2011). The apical morphologies of MR cells of brackish medaka in FW and SW conformed to typical phenotypes of MR cells in the other teleosts (Fig. 1). A deeply invaginated apical membrane was found in gill MR cells of the SW-acclimated fish. In contrast, the flat-type MR cells exhibited numerous projections, similar to microvilli, in gills of the FW individuals. The immunohistochemical staining in this study revealed that the Na⁺, K⁺-ATPase (NKA)-immunoreactive (IR)/ MR cells of SW medaka had oriﬁces in the apical region Fig. 1C, but those of the FW fish did not (Fig. 1D). Identical structures were reported in previous studies on the other euryhaline species using transmission electron microscopy (TEM) (Lee et al., 1996; Perry, 1997; Katoh and Kaneko, 2003). According to the results of Kang et al. (2008), the size

Fig. 2. SEM micrographs showed the effects of direct transfer of brackish medaka from SW to FW on phenotypic changes in branchial MR cells. The afferent regions of gill filaments were observed (A) 0, (B) 1, (C) 3, (D) 6, (E) 12, (F) 24, (G) 48, (H) 72, (I) 96, and (J) 168 h after transfer. Due to the varying diameters of the apical openings, three phenotypes of MR cells were classified: hole type, 0.5–2 μm (blue arrows); two groups of the flat types, 3–7 μm (yellow arrowheads) and 8–20 μm (red asterisks). Scale bars, 30 μm.

Fig. 3. Average frequencies of three types of MR cells were quantified in the afferent regions of gill filaments at different time-points (n = 4). The hole-type MR cells (0.5–2 μm; gray region) disappeared at 24 h after transfer from SW to FW but reappeared at 72 h. The large flat-type cells (8–20 μm; black region) and small flat-type cells (3–7 μm; white region) were the dominant types in the 48-h and 96-h groups, respectively.
of branchial NKA-IR/MR cells was bigger in the SW medaka than in the FW fish. Therefore, the larger MR cells with hole-type apical openings (SW-type) and the smaller MR cells with flat-type apical openings (FW-type) may play roles in ion secretion and ion absorption, respectively, in the gills of brackish medaka.

This study verified the salinity effects of gill MR cells on the remodeling of apical morphology and alterations of the Cl− secretory capacity in the brackish medaka upon hypoosmotic challenge. Our results dynamically revealed that the SW-type MR cells exhibited acute and regulatory phases in the gills of brackish medaka upon hypoosmotic challenge (Fig. 11). On the basis of the various diameters of the apical openings, three types of MR cells were identified in the gills of brackish medaka when exposed to FW (Figs. 2 and 3): the smallest hole type (0.5–2 μm) and the small (3–7 μm) and large (8–20 μm) flat-type cells. In the gills of the brackish medaka that were acclimated to SW, the major and minor types of MR cells were hole-type (90%) and small flat-type (10%), respectively. A low ratio of recruited MR cells was found (14.7%) in gills of the killifish >3 days post-transfer (Katoh and Kaneko, 2003; Choi et al., 2011). Within 1 day after the transfer of brackish medaka from SW to FW (the acute period), the hole-type MR cells disappeared rapidly; meanwhile, the number of large flat-type MR cells increased. Thus, the large flat-type MR cells might be transformed from the hole-type MR cells but not newly differentiated cells during this period. The hole-type MR cells were observed in the gills of killifish exposed to FW for 3 days (Katoh and Kaneko, 2003). The hole-type cells that reappeared in the gills of the brackish medaka >3 days post-transfer might be newly differentiated MR cells. Therefore, the number of NKA-IR/MR cells increased >7 days post-transfer (Fig. 5D). Previous studies reported that the numbers of gill MR cells increased upon hypoosmotic challenge in black sea bream (Mylio macrocephalus, Kelly et al., 1999), sea bass (Lateolabrax japonicus, Hirai et al., 1999; Dicentrarchus labrax, Versamos et al., 2002), and milkfish (Chanos chanos, Lin et al., 2003, 2006) due to cell proliferation. Within 3 days of FW exposure, the hole-type MR cells would mature to be flat-type, similar to the FW individuals. In the following period (3–14 days post-transfer), the large flat-type MR cells would be transformed from hole-type MR cells or small flat-type MR cells. In addition, the microvilli structures on the apical surface in flat-type MR cells of brackish medaka exhibited diverse reactions when exposed to FW (Fig. 4). Six hours after transfer from SW to FW, the apical structure of the hole-type cells was transformed and enlarged to the flat type with poorly developed microvilli. The short projections associated with the apical surfaces of MR cells were found in the medaka.
>12 h (1/2 days) post-transfer. In flat-type MR cells of the FW-exposed medaka, the time-course formation of microvilli was similar to that of the killifish (Katoh and Kaneko, 2003). Hence, the formation of microvilli in the apical surfaces of MR cells was hypoosmotic-dependent and was thought to be involved in ion absorption. However, the time-course changes in the size of NKA-IR/MR cells indicated that the size of MR cells was constant after transfer to FW for 7 days and then decreased >14 days post-transfer (Fig. 5C). A similar pattern was found in the gill MR cells of tilapia after transfer from SW to FW (Hiroi et al., 2005). The pre-existing MR cells (SW-type) did not reduce in size; thus the reduced pattern of cell size was caused by the recruitment of differentiated MR cells (FW-type) that were small in size. Taken together, the results from the present study indicated that the MR cells of the SW medaka could remodel the apical structures but

![Fig. 5. Micrographs of NKA-IR cells in cross sections of gills of the brackish medaka transferred from SW to FW for 3 (A) and 14 (B) days. The Na⁺/K⁺-ATPase immunoreactive (NKA-IR) cell (red) were mainly distributed in the afferent epithelium of the filament. The yellow diagram illustrated the outline of each NKA-IR cell. Scale bars, 50 μm. The average size (C) and number (D) of NKA-IR cells in the cross-sections of gill filaments in the brackish medaka transferred from SW to FW for 3, 7, 14, and 21 days were analyzed using ImagePro PLUS software (n=4 for all groups). Smaller NKA-IR cells were found in the 14- and 21-day groups. The numbers of NKA-IR cells were significantly greater than in the control group at 7, 14, and 21 days after transfer. The asterisk indicates a significant difference (P<0.05) at the given time-point compared with 0 days as determined using one-way ANOVA with Dunnett's test. The values shown represent the mean±S.E.M.](image-url)
maintain consistent cell size in the acute period after transfer to FW, followed by the appearance of more differentiated small-sized MR cells to replace the pre-existing MR cells in the regulatory phase.

In euryhaline teleosts, the lowest levels of NKA expression and activity were generally found in individuals acclimated to environments with salinity similar to their natural habitats (Jensen et al., 1998; Feng et al., 2002; Scott et al., 2004a,b; Lin et al., 2006; Hwang et al., 2011). Kang et al. (2008) reported that the protein abundance and enzyme activity of gill NKA were expressed at similar levels in the FW- and SW-acclimated brackish medaka. In the present study, branchial NKA expression and activity of the brackish medaka was demonstrated to be stable in response to hypoosmotic challenge (Fig. 6). This pattern was similar to that observed in killifish, the BW-residing fish (Marshall et al., 1999; Katoh et al., 2002). Several studies indicated that the composition of the various isoforms of the NKA α-, β-subunit was salinity dependent (Richards et al., 2003; Bystriansky et al., 2006; Nilsen et al., 2007). The highest value of the mRNA abundance of NKA α1a was found in the FW medaka (Kang et al., 2008). Because the mRNA pattern was different compared with the patterns of protein expression and NKA activity, the results implied that expression of the other NKA isoforms might increase in SW to supply relevant levels of gill NKA activity sufficient for ionoregulation.

Shen et al. (2011) reported that Cl– effluxes of MR cells in the yolk membranes of medaka larvae decreased at 1 h after transfer to hypoosmotic media. The cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl– channel, mainly appears in branchial MR cells of SW fish, indicating its role in Cl– secretion upon salinity challenge (Evans et al., 2005; Hwang and Lee, 2007; Kaneko et al., 2008). Tse et al. (2006) reported higher expression of the cftr gene in MR cells than in the pavement cells of Japanese eel (Anguilla japonica). In addition, our unpublished data identified the full-length sequence of the Odcftr gene (JQ728537) and found that the abundance of the Odcftr gene was significantly higher in gills of the SW medaka than in the FW fish. The present study determined that the level of branchial Odcftr gene expression decreased to its lowest level at 12 h after transfer to FW (Fig. 7). According to Scott et al. (2005), the mRNA levels of cftr in gills of FW-exposed killifish were down-regulated. To adapt to FW, killifish may rapidly reduce the level of cftr gene expression in the gills. Similar to the study in killifish (Marshall et al., 2002), a human-CFTR antibody was used in this study. CFTR signals were detected in the apical regions of NKA-IR cells of the SW medaka but not in FW fish (Kang et al., unpublished data). Previous studies indicated that apical distributions of CFTR protein disappeared in MR cells of killifish and tilapia when exposed to hypoosmotic environments for 24 h (Marshall et al., 2002; Katoh and Kaneko, 2003; Hiroi et al., 2005). In the present study, the disappearance of apical CFTR in MR cells of the SW-acclimated medaka upon FW exposure was similar to the patterns observed for the other species described above (Fig. 8). Sharma et al. (2004) reported that the CFTR was ubiquitinated in human primary respiratory epithelia. The ubiquitinated CFTR was redirected from recycling endosomes to lysosomes for degradation. Moreover, the abundance of membrane CFTR was reduced by ubiquitination in gills of the killifish when exposed to SW with arsenic (Shaw et al., 2010). These results showed that the apical CFTR of MR cells played a quick role in the elimination of the capacity for Cl– secretion through protein ubiquitination during the acute phase in euryhaline teleosts.

Na+, K+, 2Cl− cotransporter 1 (NKCC1) is thought to be the secretory isoform of NKCC, a member of the SLC12A family. Two isoforms, nkcc1a and nkcc1b, were identified in the European eel (A. anguilla; Cutler and Cramb, 2002), tilapia (Hiroi et al., 2008), and brackish

![Graph A: NKA α-subunit abundance](image)

![Graph B: NKA activity](image)

**Fig. 6.** Protein amounts of the NKA α-subunit (A) and levels of NKA activity (B) were determined in gills of SW-acclimated brackish medaka exposed to FW for 0, 1, 3, 7, 14, and 21 days (n=8). There was no significant difference compared with the 0-day SW fish (P>0.05) when using one-way ANOVA with Dunnett’s test. The values shown represent the mean±S.E.M.

![Graph C: Levels of Odcftr gene expression](image)

**Fig. 7.** Levels of Odcftr gene expression were reduced in gills of SW-acclimated brackish medaka after FW exposure. The abundances were relative to identical control samples and normalized to the expression of β-actin (n=5). Significant differences were found from 3 to 48 h. The asterisks indicate significant differences (P<0.05) compared with the 0-h SW fish determined using one-way ANOVA with Dunnett’s test. The values shown represent the mean±S.E.M.
medaka (Kang et al., 2010). In the gills of these species, the major expressed isoform is encoded by the nkkc1a gene. Furthermore, using whole-mount in situ hybridization, the odnkcc1a gene was shown to be localized in NKA-IR/MR cells of medaka gills, which supports the immunocytochemical data of previous studies (Kang et al., 2010). The expression of gill NKCC1a was salinity dependent. The level of Odnkcc1a gene expression in the gills of the brackish medaka was reduced at 1 h after transfer from SW to FW (Fig. 9). The reduced profile of the gill nkkc1a gene was also found in the gills of the killifish, tilapia, and striped bass (Scott et al., 2004a,b; Tipsmark et al., 2004; Hiroi et al., 2008). The results suggested that euryhaline teleosts could exhibit downregulatory mechanisms to immediately reduce the level of Odnkcc1a gene as well as Odcftr gene expression in the gills.

Hiroi et al. (2008) noted that the monoclonal antibody T4 (Developmental Studies Hybridoma Bank) recognized the apical Na⁺,Cl⁻ cotransporter (NCC) and basolateral NKCC1a in type II and type III/IV MR cells, respectively, in tilapia. Similar to tilapia, the apical signals and basolateral signals of antibody T4 were found in the FW-type and SW-type MR cells of brackish medaka, respectively (Kang et al., 2010).

The apical signals of NCC were enhanced to involve in ion absorption in the NKA-IR/MR cells of the brackish medaka exposed to FW (Fig. 10). However, it was difficult to distinguish the apical signals of NCC in the NKA-IR cells with the basolateral signals of NKCC1a. The T4 antibody was not suitable to examine exactly the dynamic expression of NCC signals in all NKA-IR/MR cells. Therefore, the present study focused on the alteration in the basolateral NKCC1a signals of NKA-IR/MR cells when medaka were transferred from SW to FW (Fig. 10). The MR cells with basolateral NKCC1a in tilapia (Hiroi et al., 2008) and saifin molly (Poecilia latipinna, Yang et al., 2011) were observed after transfer from SW to FW for 7 days, and then the proportion of the NKCC1a-IR/NKA-IR cells decreased gradually, as observed for type III/IV cells in tilapia. The sequential pattern of NKCC1a-IR/NKA-IR cells in the gills of FW-exposed brackish medaka was similar to the profiles of the other species and corresponded to the reduced abundance of NKCC1a in gills as determined by immunoblotting with the same antibody (Kang et al., 2010). On the basis of the reduced abundance of Odnkcc1a gene products, the translation of Odnkcc1a gene was suppressed to produce new NKCC1a protein. Therefore, the half-life of the NKCC1a protein was longer than that of CFTR in gill MR cells of medaka upon hypoosmotic challenge. Hoffmann et al. (2007) reported that NKCC expression in epithelial cells was inhibited by dephosphorylation with Ser/Thr protein phosphatases (PP1 and PP2A) and activated through phosphorylation by Sterile 20 protein-related proline alanine-rich kinase (SPAK) and oxidative stress response 1 kinase (OSR1). When the SW medaka was exposed to FW, the ion transporting function of branchial NKCC1a in preexisting MR cells might also be inhibited by PP1 and PP2A until 7 days post-transfer. In the acute phase, pre-existing MR cells exhibited basolateral NKCC1a signals and would transform to FW-type cells. The controversy regarding whether MR cells with basolateral NKCC1a signals pre-existed in the regulatory phase will be explored in future studies. Therefore, the present study suggested that the ratio of MR cells with inactive NKCC1a was correlated with the maintenance of hypoosmoregulatory endurance in the two phases of response by the brackish medaka upon hypoosmotic challenge.

In conclusion (Fig. 11), the acute phase showed short-term changes: apical surfaces of MR cells started to convert the hole-type to the flat-type; the levels of Odnkcc1a and Odcftr transcripts decreased, and the apical immunostaining signals for CFTR protein disappeared. In the regulatory phase (after 1 day post-transfer), the basolateral immunostaining signals of NKCC1a protein decreased gradually. During this period, the size and number of NKA-IR/MR cells were significantly reduced and increased, respectively. The results revealed that MR cells

![Fig. 8. Immunofluorescent micrographs of gill sections in brackish medaka transferred from SW to FW for 6 (A), 12 (B), or 24 (C) h. Gill filaments were double-stained with anti-CFTR protein (red) and anti-NKA (green) antibodies. The arrows indicated apical CFTR-IR/NKA-IR cells. Scale bars, 20 μm. (D) The average percentages of apical CFTR-IR/NKA-IR cells. Percentage of CFTR-IR/NKA-IR cells per section of gill filaments (%). The asterisk indicates a significant difference (P<0.05) compared with the data for the 0-h SW fish determined using one-way ANOVA with Dunnett’s test. The values shown represent the mean ± S.E.M.](image)

![Fig. 9. Levels of Odnkcc1a gene expression were decreased in gills of SW-acclimated brackish medaka after FW exposure. Expression levels were compared with identical control samples and normalized to the expression of β-actin (n = 5). Significant differences were found from 1 to 48 h. The asterisk indicates a significant difference (P<0.05) compared with the 0-h SW fish determined using one-way ANOVA with Dunnett’s test. The values shown represent the mean ± S.E.M.](image)
no longer exhibited the capacity for Cl\(^–\) secretion when CFTR and NKCC1a were not expressed, and MR cells retained branchial NKCC1a protein for maintaining hypoosmoregulatory endurance in the gills of the SW medaka when exposed to FW. Both acute and regulatory phases exhibited constant branchial NKA expression and activity. The present study illustrated that different performances of ion transporters and morphologies in the acute and regulatory phases of gill MR cells made the euryhaline medaka capable of survival in hypoomotic environments. However, whether MR cells with basolateral expression of NKCC1a pre-existed in gills of the SW medaka during exposure to FW is still controversial. It will be intriguing to identify the T4 antibody as a marker to trace the fate of SW-type MR cells when the fish are subjected to FW.

In addition, the detection of immunocytochemical staining with antibody T4 could be used as a marker to trace the fate of SW-type MR cells when the fish are exposed to FW. To our knowledge, there was no relevant marker to identify the FW-type MR cells. It will be intriguing to identify a marker for FW-type MR cells in future studies to verify the different regulatory mechanisms of various types of MR cells in the gills of the brackish medaka.

Acknowledgements

The monoclonal antibodies, T4 and α5, were purchased from the Developmental Studies Hybridoma Bank (DSHB) maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD 2120521205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under Contract N01-HD-6-2915, NICHD, USA. This study was supported by a grant from the Academia Sinica of Taiwan to P.P.H. and T.H.L. (AS-98-TP-B08).

References


![Fig. 10. Immunofluorescent micrographs of gill cryosections in SW-acclimated brackish medaka exposed to FW for 4 (A), 7 (B), or 14 (C) days. Gill filaments were double-stained with anti-NKCC1a protein (red) and anti-NKA (green) antibody. Scale bars, 20 μm. The white asterisks indicate basolateral NKCC1a-IR / NKA-IR cells. (D) Average percentages of basolateral NKCC1a-IR / NKA-IR cells were determined in gill filaments at different time-points after transfer (n=4). Significant differences were found in the fish at 7 and 14 days. The asterisks indicate significant differences (P<0.05) compared with the data for the 0-day SW fish determined using one-way ANOVA with Dunnett’s test. The values shown represent the mean ± S.E.M.](image)

![Fig. 11. The integrated model of time-course changes of SW MR cells during exposure to FW in gills of brackish medaka. On the basis of the model, the dynamic elimination of the capacity to secrete Cl\(^–\) in branchial MR cells exhibited two phases: rapid changes were seen in the acute phase within 1 day post-transfer (24 h), and the regulatory phase was observed >1 day post-transfer.](image)


