Comparative Biochemistry and Physiology, Part A 194 (2016) 19–26

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

FXYD11 mediated modulation of Na^+/K^+-ATPase activity in gills of the brackish medaka (*Oryzias dancena*) when transferred to hypoosmotic or hyperosmotic environments

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ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form 13 January 2016
Accepted 14 January 2016
Available online 19 January 2016

Keywords:
NKA
FXYD11
Oryzias dancena
Salinity
Gills

ABSTRACT

FXYD proteins regulate Na^+/K^+-ATPase (NKA), which is a primary active pump that provides the driving force that triggers osmoregulatory systems in teleosts. To explore the regulatory mechanisms between FXYD and NKA in euryhaline teleosts, the expression of NKA (mRNA, protein, and activity) and FXYD11 and their interaction were examined in the gills of brackish medaka (*Oryzias dancena*) when transferred from brackish water (BW; 15‰) to fresh water (FW) or seawater (SW; 35‰). The mRNA expression of *Odfxyd11* and *Odnka*-α was elevated 48 h post-hypoosmotic transfer. Moreover, FXYD11 protein and NKA activity were upregulated 12 h after transfer to FW. When transferred to SW, the protein abundance of FXYD11 and the NKA α-subunit did not show apparent changes, while *Odfxyd11* and *Odnka*-α mRNA expression and NKA activity increased significantly 12 h and 1 h post-transfer, respectively. To clarify the FXYD11 mechanisms involved in modulating NKA activity via their interaction, co-immunoprecipitation was further applied to *O. dancena* gills. The results revealed that the levels of protein–protein interaction between branchial NKA and FXYD11 increased acutely 12 h after the transfer from BW to FW. However, immediate upregulation of NKA activity 1 h following post-exposure to SW, without the elevation of protein–protein interaction levels, was found. Hence, branchial NKA activity of *O. dancena* was suggested to be rapidly regulated by FXYD11 interaction with NKA when acclimated to hypoosmotic environments. To the best of our knowledge, this is the first study that focuses on the efficacy of interactions between FXYD11 and NKA in the gills of euryhaline teleosts.

1. Introduction

Euryhaline teleosts utilize excellent osmoregulatory functions to maintain their plasma osmolality within a fixed range when exposed to different salinities. The gill is one of the major organs for osmoregulation in teleosts and mitochondrion-rich cells in gill epithelia play a crucial role in maintaining homeostasis. Moreover, Na^+/K^+-ATPase (NKA) is a primary active transporter that hydrolyses one molecule of ATP to antiport two extracellular K^+ and three intracellular Na^+ ions, which provide the primary driving force for the ion-transporting systems of the mitochondria-rich cells (Hwang and Lee, 2007; Hwang et al., 2011). Changes in the branchial NKA activities facilitate euryhaline teleosts to adapt to various external salinities, which increased NKA activity effects ion/osmoregulatory mechanism to maintain fish body fluid homeostasis (Hwang and Lee, 2007).

Several studies indicated that FXYD proteins were novel regulators of NKA in mammals (Geering, 2006). The FXYD protein family contains at least twelve members that have the extracellular FXYD motif, transmembrane domain, and intracellular domain. In mammals, members of the FXYD family include FXYD1 (phospholemman, PLM), FXYD2 (the γ-subunit of NKA), FXYD3 (mammary tumor marker Mat-8), FXYD4 (corticosteroid hormone-induced factor, CHIF), FXYD5 (dysadherin), FXYD6 (phosphohippolin), and FXYD7. In elasmobranchs, FXYD10 (phospholemman-like protein from shark, PLMS) was first identified in the rectal glands of sharks (*Squalus acanthias*). In addition, studies on sharks reported the functions of FXYD10 via C-ter Cys residue interactions that were associated with the negative regulation of shark NKA activity (Mahmmoud et al., 2003; Cornelius et al., 2005; Mahmmoud et al., 2005). On the other hand, teleostean FXYD proteins

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http://dx.doi.org/10.1016/j.cbpa.2016.01.013
1095-6433/

(FXYD2, 5–9, 11, and 12) have been reported in certain teleosts such as the pufferfish (*Tetraodon nigroviridis*), Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), and brackish medaka (*Oryzias dancena*) (Tipsmark, 2008; Wang et al., 2008; Saito et al., 2010; Yang et al., 2013). Importantly, FXYD11 was specifically expressed in the gills of all studied teleosts (Tipsmark, 2008; Saito et al., 2010; Tipsmark et al., 2010; Tang et al., 2012; Yang et al., 2013), and it shared the highest homology with tetrapod FXYD4, which played a role in upregulating NKA activity (Tipsmark, 2008). In zebrafish, the knockdown of FXYD11 resulted in a significant increase in NKA-immunoreactive (−IR) cells to maintain homeostasis in larval skin (Saito et al., 2010). In addition, salinity-dependent expression of FXYD11 and the NKA α-subunit and their interaction were reported in the gills of the brackish medaka (Yang et al., 2013).

The Japanese medaka (*O. latipes*) is a model fish with a compact genomic database that is conducive to molecular studies (Kasahara et al., 2007). However, *O. latipes* is a euryhaline species with a freshwater preference. In this study, the brackish medaka (*O. dancena*) was used because it lives in estuarine habitats (Robert, 1998), and it is capable of living in higher salinity environments. Furthermore, it can be easily bred in the laboratory, and it is a highly homologous species that can be compared to the Japanese medaka genomic database. The estuary-inhabiting (brackish water, BW) brackish medaka may encounter seawater (SW) and freshwater (FW) in its natural habitat. The brackish medaka and Japanese medaka exhibit different patterns of branchial NKA expression based on mRNA levels, protein amounts, and enzyme activities in FW, BW, and SW, respectively (Kang et al., 2008). The NKA protein and activity of brackish medaka had the lowest expression levels in the original habitats, but both parameters were upregulated to maintain ionic homeostasis in response to hyperosmotic and hypoosmotic challenges (Kang et al., 2008). In our previous study, the FXYD11 protein interacted with the NKA α-subunit to regulate NKA activity in the brackish medaka when acclimated to different salinity environments (Yang et al., 2013). This study illustrated the time-course effects of hypoosmotic and hyperosmotic stress on branchial FXYD11 and NKA expression in gills. Furthermore, the efficiency of the interaction between FXYD11 and NKA activity to maintain ionic homeostasis in brackish medaka was examined. Our data suggested that FXYD11 protein regulates NKA activity through protein–protein interactions in gills of euryhaline teleosts.

2. Materials and methods

2.1. Experimental animals

Adult brackish medaka (2.50 ± 0.3 cm standard length) were inbred and raised in the laboratory. BW (15 ‰) and SW (35 ‰) were prepared from local tap water (FW) with proper amounts of the synthetic sea salt "Instant Ocean" (Aquarium Systems, Mentor, OH, USA). The medaka were acclimated to BW at 28 ± 1 °C with a daily 14 h:10 h light:dark photoperiod for at least four weeks before experiments began. Fresh water or artificial seawater was continuously circulated through fabric-floss filters and partially replaced every four weeks. Fish were not fed for one day, and they were anesthetized with MS-222 (100–200 mg/L) before sampling. The protocol used for the experimental fish was reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 96-48 to THL).

2.2. Experimental design

The BW-acclimated medaka were transferred directly to FW and SW for the time-course experiments. The medaka were sampled at 1, 3, 6, 12, 24, 48, 96, and 168 h and for a long-term period (4 weeks) post-transfer (N = 8 at each time point), to analyze the time-course changes in their gills in response to salinity challenges. Fish in the control group (N = 3 at each time point) were transferred from BW to BW, and were then identically sampled with the study group.

2.3. Total RNA extraction and reverse transcription

Using the method of Kang et al. (2008), total RNA samples from the gills were extracted from four individuals using the RNA-Bee™ (Tel-Test, Friendwood, TX, USA), following the manufacturer's instructions. The RNA pellet was dissolved in 40 μL DEPC-H_2O and treated with the RNA cleanup protocol from the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA) to eliminate genomic DNA contamination, following the manufacturer's instructions. Extracted RNA samples were stored at −80 °C after isolation. The concentration and quality of the extracted RNA were measured using a NanoDrop 2000 (Thermo, Wilmington, CA, USA), total RNA concentrations of all samples were 0.4–0.6 μg/μL, and the A260/A280 ratio (1.8–2.0) of purified RNA was measured. First-strand cDNA was synthesized by reverse transcribing 2 μg of the total RNA using 1 μL oligo(dT) (50 pmol/μL) primer, 2 μL random hexamer primer (600 pmol/μL), and 0.5 μL Roche reverse transcriptase (Roche, Mannheim, Germany), following the manufacturer's instructions.

2.4. Primers used for real-time PCR

The gene sequences of the NKA α-subunit (EU490421), β-actin (EU490422), and FXYD11 (JX624725) of brackish medaka have been reported by Kang et al. (2008) and Yang et al. (2013). The target sequence was used to design the primer sequence for real-time PCR using the Primer3 Plus software (Rozen and Skaletsky, 2000). The primer sequences were as follows (5′ to 3′): NKA α-subunit, forward – GAAC CGTCACCATCCTCTG and reverse – GGCTGCCTCTTCTGATGTC; β-actin, forward – CTGGACTTCGAGCAGGAGAT and reverse – AGGAAGGAAG GCTGGAAGAG; FXYD11, forward – GAAGAGACGCACAGCAGCTA and reverse – GCAAGATTTCAACAGCACTGAG.

2.5. Quantitative real-time PCR (qPCR)

The expression of FXYD11 (*Odfxyd11*) and the NKA α-subunit (*Odnka-α*) was quantified using the MiniOpticon real-time PCR system (Bio-Rad, Hercules, CA, USA). PCR reactions contained 8 μL of cDNA (1000× dilution), 2 μL of either 1 μM gene-specific qPCR primers or 1 μM β-actin primers (as an internal control), and 10 μL of 2× SYBR Green Supermix (Bio-Rad). The conditions for qPCR reactions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. All samples were run in triplicate. The melting curve analysis and the PCR product analysis (using a 1% agarose gel) were performed after each reaction to confirm the presence of a single amplification product. One gill cDNA sample from BW-acclimated medaka was used as the running control. Relative gene expression was analyzed by the comparative Ct method with the formula 2^-[(Ct target gene,n – Ct β-actin,n) – (Ct target gene,control – Ct β-actin,control)], where Ct corresponded to the threshold cycle number.

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

The gill NKA activity of brackish medaka was measured according to NADH-linked methods (Kang et al., 2008). 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). Four pairs of gill arches from each medaka were dissected quickly, immersed in liquid nitrogen, and stored in microcentrifuge tube at −80 °C. The tissues were rapidly thawed and homogenized in 400 μL SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.5) containing protease inhibitor (vol/vol: 25:1; Roche) using a Polytron PT1200E (Lucerne, Switzerland) at the maximal speed for 10 s on ice. The homogenates were centrifuged

at 5000 ×*g* and 4 °C for 2 min. The supernatants were assayed for NKA activity and protein concentration. The NKA activity assay solution (50 mM imidazole, 0.5 mM ATP, 2 mM phosphoenolpyruvate, 0.32 mM NADH, 3.3 U LDH/mL, 3.6 U PK/mL, pH 7.5) was mixed with a salt solution (189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, 50 mM imidazole, pH 7.5) in a 3:1 ratio. The samples from the 10 time points (0, 1, 3, 6, 12, 24, 48, 96, 168 h and the long-term period) were rapidly homogenized for detection of NKA activity. A 10 μL sample from one fish was loaded into a well, and 200 μL of the assay mixture was added with or without 1 mM ouabain. Each sample was assayed in triplicate. The 96-well microplate was detected every 15 s for up to 20 min in the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 340 nm and 28 °C. Protein concentrations of the samples were determined using the protein assay dye (Bio-Rad), and bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) was used as a standard.

2.7. Antibodies

The primary antibodies used in this study included: (1) FXYD11, an affinity-purified rabbit polyclonal antibody (LTK BioLaboratories, Taoyuan, Taiwan) against a specific epitope (NQCARLVRGKRSDSSSA) corresponding to the C-terminus region of the FXYD11 protein from brackish medaka (Yang et al., 2013); (2) NKA, a mouse monoclonal antibody (α5, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of the avian NKA; and (3) actin, a rabbit polyclonal antibody (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against the C-terminus of human actin. The secondary antibodies used for immunoblots were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (#0031430 or #0031460, respectively; Pierce, Rock-ford, IL, USA).

2.8. Immunoblotting

The immunoblotting protocol was modified from Yang et al. (2013). The gills were suspended in SEID medium containing protease inhibitor (vol/vol: 25:1; Roche). The homogenates were then centrifuged at 10,000 ×*g* and 4 °C for 10 min. Protein concentrations of the supernatant were determined using reagents from the Protein Assay Kit (Bio-Rad), and bovine serum albumin (Sigma) was used as a standard. Aliquots containing 40 μg of branchial supernatants were mixed with protein sample buffer, and were then heated at 60 °C for 15 min, which was followed by electrophoresis on a 7.5% or 15% (for NKA and FXYD11, respectively) sodium dodecyl sulfate polyacrylamide gel. The pre-stained protein molecular weight marker was purchased from Fermentas (SM0671, Hanover, MD, USA). Separated proteins were transferred from unstained gels to PVDF membranes (Millipore, Bedford, MA, USA) via electroblotting. After 1 h of preincubation in phosphate buffer saline with 0.05% Tween-20 (PBST), buffer containing 5% (wt/vol) nonfat dried milk was added to minimize non-specific binding. The blots were incubated for 2 h at room temperature with the primary antibody diluted in 1% BSA and 0.05% sodium azide in PBST, and they were then incubated at room temperature for 1 h with HRP-conjugated secondary antibody that was diluted in PBST. The blots were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore), and the images were taken using a universal hood with a cooling-charge-coupled device (CCD) camera (ChemiDoc XRS +, Bio-Rad) and the associated software (Quantity One version 4.6.8, Bio-Rad). Immunoreactive bands were analyzed using the Image Lab software version 3.0 (Bio-Rad) and converted to numerical values to compare the relative intensities of immunoreactive bands. Although the molecular weight of one of the low-intensity immunoreactive bands of the FXYD11 was close to those of the actin (Yang et al., 2013), the PDVF membranes transferred from the 15% gel were cut into two parts, one for immunoreaction of the actin primary antibody, and the other for FXYD11 primary antibody, to prevent the wrong quantification. The gill homogenates from BW-acclimated medaka were used as the internal control among different immunoblots (data not shown). The intensity of the immunoreactive band of the internal control was converted to numerical value of 1 in each immunoblot for normalization. Relative expression of target proteins was calculated using the formula target protein/actin.

2.9. Co-immunoprecipitation (Co-IP)

Total gill lysates were used in this experiment. Immunoprecipitation with 100 μg of branchial supernatants and 1 μg of the NKA antibody (α5) was carried out using the ImmunoCruz™ IP/WB Optima System (sc-45042, Santa Cruz Biotechnology), according to the manufacturer's manual, and the samples were stored at 4 °C before use. The above IP solutions were subjected to FXYD11 immunoblotting, and were then analyzed by electrophoresis on a 15% sodium dodecyl sulfate polyacrylamide gel. The immunoreactive bands were quantified as described above. Relative expression was calculated with the formula FXYD11/NKA, and numerical value of the BW sample was set to be 1 for normalization.

2.10. Statistical analysis

Values are expressed as means ± SEM (standard error of the mean). Results were compared using one-way ANOVA analyses with Dunnett's pairwise method, and $P < 0.05$ was set as the significance level.

3. Results

3.1. Time-course changes in medaka gills in response to the hypoosmotic challenge

When the brackish medaka were transferred directly from BW to FW, the relative mRNA abundance of *Odfxyd11* and *Odnka*-α increased significantly (3- and 4-fold, respectively) after 48 h post-transfer (Fig. 1). However, no significant changes in mRNA abundance of branchial *Odfxyd11* and *Odnka*-α were found between the BW point or any time-course point of the control group (Fig. 1).

The immunoblotting of branchial OdFXYD11 revealed a major immunoreactive band at approximately 11 kDa. The immunoreactive bands of the medaka branchial OdFXYD11 protein transferred from BW to FW for 1, 3, 6, 12, 24, 48, 96, 168 h, and 4 weeks were quantified. Compared to the BW-acclimated medaka, the relative protein abundance of branchial FXYD11 increased significantly (~2-fold) after 12 h post-transfer, and continued to increased significantly at 24, 48, 96, 168 h, and 4 weeks after transfer (Fig. 2A). In addition, quantification of the immunoblotting time-course changes of the branchial NKA α-subunit protein showed a single immunoreactive band at approximately 110 kDa. Compared to BW-acclimated fish, relative protein amounts of the branchial NKA α-subunit increased significantly at 168 h and 4 weeks after transfer (Fig. 2B). No significant changes were observed in the protein abundance of branchial FXYD11 and the NKA α-subunit between these time-course points of the control group (Fig. 2).

The NKA activities increased significantly (approximately 2-fold) 12 h post-transfer compared to those of the BW-acclimated branchial medaka samples, and this was followed by a 3-fold increase at 24, 48, 96, 168 h, and 4 weeks after transfer. No significant changes in NKA activity were observed among the time-course in the control group (Fig. 3).

3.2. Time-course changes in medaka gills in response to the hyperosmotic challenge

When the medaka were transferred directly from BW to SW, the relative mRNA abundance of *Odfxyd11*increased significantly, reaching nearly 3-fold at 12 h and 4 weeks post-transfer (Fig. 4A). The relative mRNA abundance of *Odnka*-α increased significantly at 12 h post-

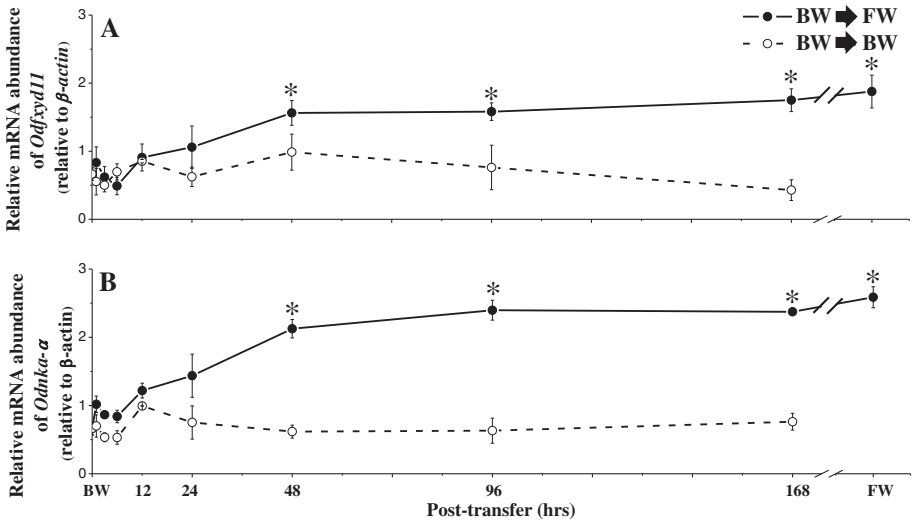

Fig. 1. Relative mRNA abundance of the branchial FXYD11 (A) and NKA α-subunit (B) of brackish medaka after transfer from brackish water (BW) to fresh water (FW) (solid circles) or BW (open circles). The asterisks indicate significant differences as compared to the initial data (N = 4 for all groups, $P < 0.05$). No difference was found between the tested time points and the control group (N = 3 for all groups, $P > 0.05$). Values are means ± S.E.M. resulting from one-way ANOVA with Dunnett's test.

transfer, and it then recovered at 168 h post-transfer (Fig. 4B). No significant changes in mRNA abundance of branchial *Odfxyd11* and *Odnka-α* were found between these time-course points in the control group (Fig. 4).

Regarding the protein levels, the amounts of branchial FXYD11 were not significantly different between the transfer groups and BW fish (Fig. 5A). Compared to BW-acclimated fish, a higher protein abundance of the branchial NKA α-subunit was observed 4 weeks after transfer (Fig. 5B). No significant changes in relative protein abundance of branchial FXYD11 and the NKα-subunit were observed in the control group (Fig. 5). Branchial NKA activities increased significantly at all time points after the transfer. No significant changes were observed in the NKA activities of the control group (Fig. 6).

3.3. The possible regulatory mechanism of FXYD11 for NKA activity after hypoosmotic and hyperosmotic challenges

Based on the changes in mRNA levels, protein abundance, and NKA activity after hypoosmotic and hyperosmotic challenges, we chose the time points when NKA protein abundance was constant and when

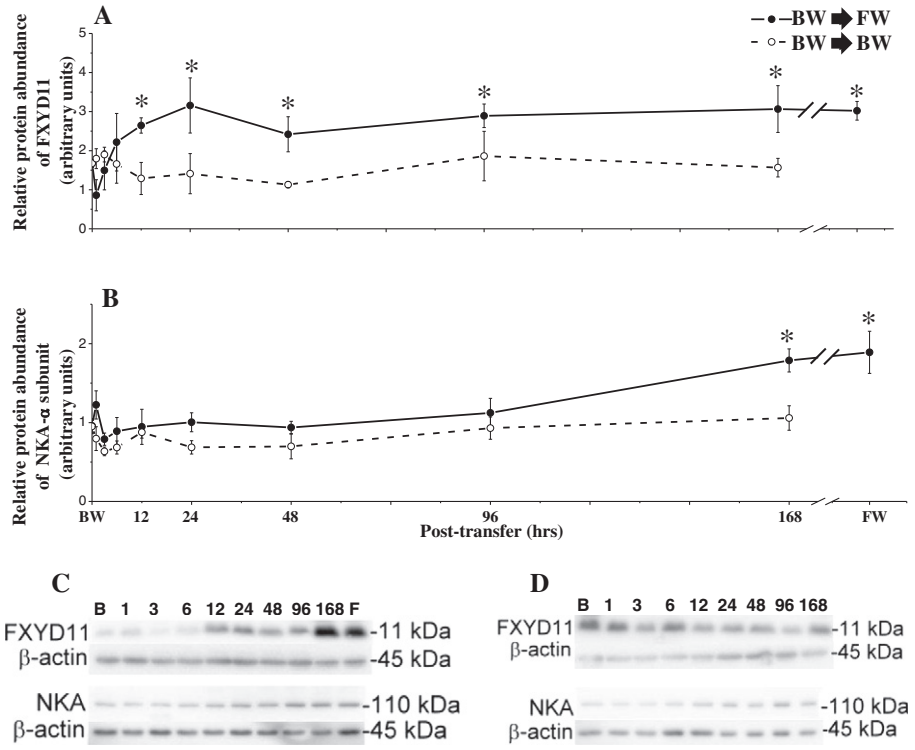

Fig. 2. Relative protein abundance of the branchial FXYD11 (A) and NKA α-subunit protein (B) of brackish medaka after transfer from brackish water (BW) to freshwater (FW) (solid circles) or to BW (open circles). The representative immunoblots of FXYD11 and NKA α-subunit were shown in the experimental (C) and control (D) groups with the time points 1, 3, 6, 12, 24, 48, 96, and 168 h post-transfer. β-actin was used as the loading control. B, Brackish water; F, fresh water. The asterisk indicates a significant difference as compared to the initial data (N = 8 for all groups, $P < 0.05$). No differences were found between the tested time points and the control group (N = 3 for all groups, $P > 0.05$). Values are means ± S.E.M resulting from one-way ANOVA with Dunnett's test.

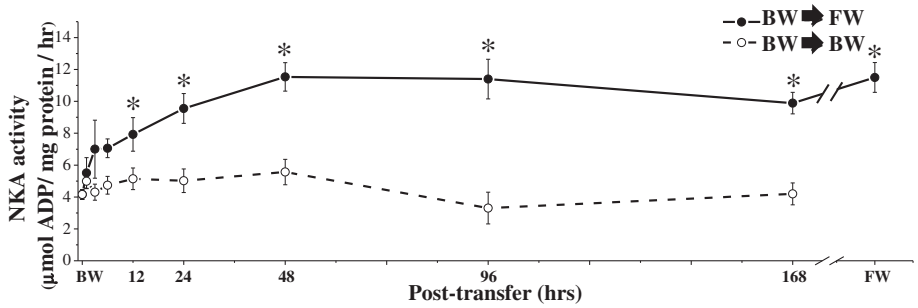

Fig. 3. Changes in branchial NKA activity of brackish medaka after transfer from brackish water (BW) to fresh water (FW) (solid circles) or BW (open circles). The asterisks indicates significant differences as compared to the initial data (N = 4 for all groups, $P < 0.05$). No difference was found among the tested time points and the control group (N = 3 for all groups, $P > 0.05$). Values are means ± S.E.M. resulting from one-way ANOVA with Dunnett's test.

NKA activity increased significantly to further estimate the modulatory effects of FXYD11 on NKA activity. The interaction between FXYD11 and NKA was also examined by Co-IP, and the immunoreactive expression of NKA protein abundance was also a loading control. After the transfer from BW to FW (Fig. 7A), the immunoblotting results indicated an increase in the FXYD11 protein abundance that was found 12 h post-transfer (approximately 3.3-fold) compared with the 6 h-point. Their interaction was then recovered at 24 h post-transfer. Following the hyperosmotic challenge, the interaction between NKA and FXYD11 was not significantly different between 0 and 1 h after the transfer.

4. Discussion

The gill is the major osmoregulatory organ of fish, and branchial NKA act as a primary driving force to affect the activity of branchial ion transporters. In previous studies, different members of FXYD proteins were identified, and they functioned as modulators of NKA activity by interacting with the NKA α-subunit in different mammalian tissues (Geering, 2005; Garty and Karlish, 2006). The fish FXYD protein was first identified in sharks (Mahmmoud et al., 2003). Furthermore, Wang et al. (2008) indicated that the expression of branchial FXYDs and NKA was salinity-dependent, and they exhibited protein–protein interactions in fish. Tipsmark also identified eight members of the FXYD protein family in various Atlantic salmon tissues, and FXYD11 was salinity-dependent and highly expressed in the gills of Atlantic salmon (Tipsmark, 2008; Tipsmark et al., 2010). In the zebrafish (a stenohaline fish), diluted FW induced FXYD11 mRNA expression in gills. Knockdown of FXYD11 may lead to a compensatory response in the upregulation of NKA positive cells in zebrafish embryos (Saito et al., 2010). In other teleosts, FXYD11 was highly expressed in gills of the brackish medaka, Japanese medaka, Japanese eels (*Anguilla japonica*), and spotted scats (*Scatophagus argus*) (Tang et al., 2012; Yang et al., 2013; Hu et al., 2014). Gill FXYD11 expressions of these species were salinity-dependent (Tang et al., 2012; Yang et al., 2013; Hu et al., 2014) which indicated that branchial FXYD11 played an important role in osmoregulation of fish. Although the expression and possible function of branchial FXYD11 were reported following salinity acclimation, effects of sudden salinity changes in gill FXYD expression of euryhaline teleosts remain unclear. In addition, the role of NKA involved in the acclimation of time-course osmotic challenge is also unknown in brackish medaka. In this study, brackish medaka were used to further explore the role of FXYD11 and NKA in the short-term osmoregulation of euryhaline teleosts.

The brackish medaka had the lowest expression of branchial *Odnka*-α and *Odfxyd11* in its natural habitat of BW compared to FW and SW (Robert, 1998; Kang et al., 2010; Yang et al., 2013). Maintaining ionic homeostasis helps prevent passive salt loss in the hypoosmotic environment. The teleosts use NKA, which produces a sodium electrochemical gradient, to provide the major driving force for active ion transport (Hwang et al., 2011). Salinity-dependent *fxyd11* mRNA abundance was found in the gills of larval and adult zebrafish, and it increased by the hypoosmotic challenge (Saito et al., 2010). In the

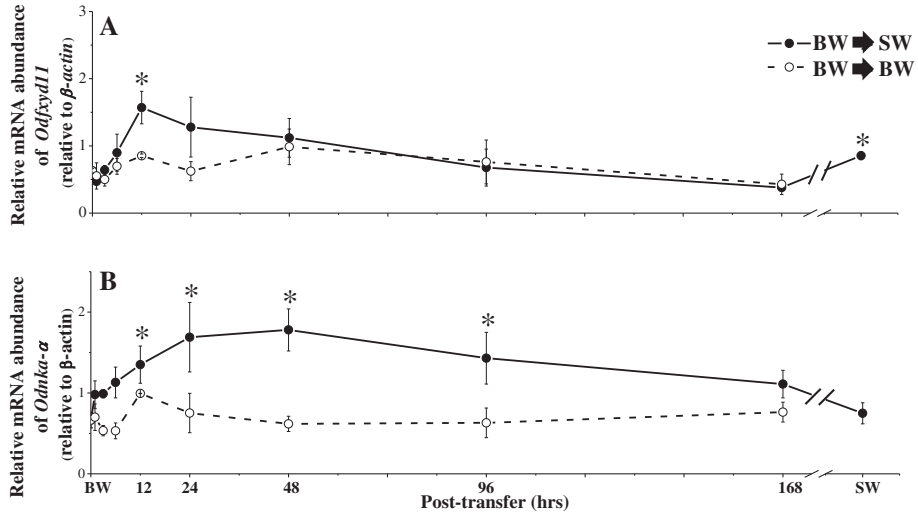

Fig. 4. Relative mRNA abundance of the branchial FXYD11 (A) and NKA α-subunit (B) of brackish medaka after transfer from brackish water (BW) to seawater (SW) (solid circles) or BW (open circles). The asterisks indicates significant differences compared to the initial data (N = 4 for all groups, $P < 0.05$). No difference was found among the tested time points and the control group (N = 3 for all groups, $P > 0.05$) Values are means ± S.E.M. resulting from one-way ANOVA with Dunnett's test.

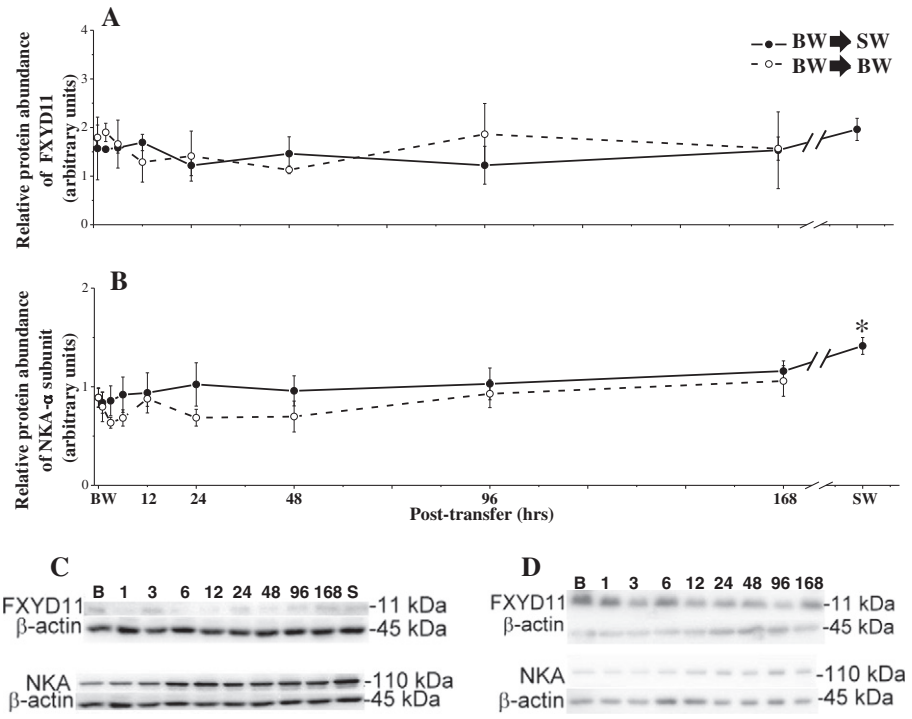

Fig. 5. Relative protein abundance of the branchial FXYD11 (A) and NKA α-subunit protein (B) of brackish medaka after transfer from brackish water (BW) to seawater (SW) (solid circles) or BW (open circles). The representative immunoblots of FXYD11 and NKA α-subunit were shown in the experimental (C) and control (D) groups with the time points 1, 3, 6, 12, 24, 48, 96, and 168 h post-transfer. β-actin was used as the loading control. B, Brackish water; S, seawater. The asterisk indicates a significant difference as compared to the initial data (N = 8 for all groups, $P < 0.05$). No differences were found between the tested time points and the control group (N = 3 for all groups, $P > 0.05$). Values are means ± S.E.M resulting from one-way ANOVA with Dunnett's test.

current model of ionocytes in zebrafish, Na^+–Cl^- cotransporter (NCC), H^+-ATPase (HA)-rich (HR), and NKA-rich (NaR) cells (Hwang et al., 2011) were identified. Saito et al. (2010) reported that the FXYD11 protein was only localized in the NaR cells of the skin of zebrafish larvae. The gills and skin NaR cells were proposed to be associated with the maintenance of calcium homeostasis by Ca^{2+} uptake via epithelial Ca^{2+} channels (Saito et al., 2010). In the present study, the mRNA expression pattern of branchial *Odfxyd11* was the same as *Odnka*-α expression, which increased to the highest level at 48 h after hypoosmotic stress (Fig. 1). In Mozambique tilapia (*Oreochromis mossambicus*), both the expressions of branchial *fxyd11* and *nka*-α1a were simultaneously elevated 48 h after the hypoosmotic challenge (Tipsmark et al., 2011). The α1a form of the α-subunit isoform was the predominant NKA isoform in the gills of FW-acclimated Atlantic salmon (Bystriansky and Schulte, 2011), Mozambique tilapia (Tipsmark et al., 2011), and rainbow trout (*Oncorhynchus mykiss*) (Richards et al., 2003). The NKA mRNA pattern was different compared to that of the protein and NKA activity, and the results suggest that expression of other NKA isoforms may increase in FW to maintain NKA activity for ion homeostasis (Richards et al., 2003; Bystriansky and Schulte, 2011; Tipsmark et al., 2011). In the present study, NKA activity was upregulated 12 h after the hypoosmotic challenge (Fig. 3), and the NKA α-subunit protein was upregulated within 96 h after hypoosmotic challenge (Fig. 2B). Although the expression of OdNKA-α was upregulated at a late stage, OdFXYD11 expression might be an important factor to stimulate NKA activity at 12 h. Therefore, our findings suggested that the FXYD11 protein might regulate NKA activity and help maintaining ionic homeostasis of the brackish medaka ionic homeostasis. Similar findings were also reported in gilthead sea bream; the gill NKA activity was upregulated to maintain plasma osmolality upon exposure to a hypoosmotic environment (Sangiao-Alvarellos et al., 2005). Moreover, Saito et al. (2010) reported that when FXYD11 was knocked down using morpholinos, a compensatory response via elevated NKA positive cell numbers (to maintain NKA activity) was found. Thus, FXYD11 may play a positive role in regulating NKA activity in teleosts. Kang et al. (2008) has reported that NKA activity as well as protein abundance of NKA α-subunit was higher in gills of FW-acclimated brackish medaka rather than those of BW-acclimated fish. However, in the short-term hypoosmotic

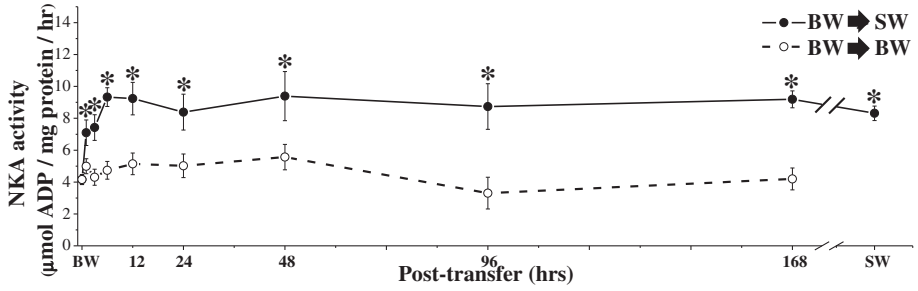

Fig. 6. Changes in branchial NKA activity of brackish medaka after transfer from brackish water (BW) to seawater (SW) (solid circles) or BW (open circles). The asterisks indicate significant differences as compared to the initial data (N = 6 for all groups, $P < 0.05$). No difference was found among the tested time points and the control group (N = 3 for all groups, $P > 0.05$). Values are means ± S.E.M. resulting from one-way ANOVA with Dunnett's test.

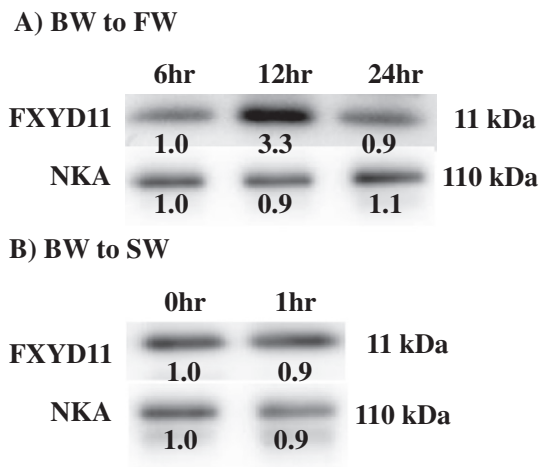

Fig. 7. Co-immunoprecipitation of branchial FXYD11 and NKA α-subunit in brackish medaka after exposure to hypoosmotic or hyperosmotic environments. NKA protein was immunoprecipitated from total gill lysates with a primary antibody (α5), and immunoblotting was then carried out for the FXYD11 and the NKA proteins. Immunoreactive bands for NKA and FXYD11 were detected at 110 and 11 kDa, respectively. Representative immunoblots of FXYD11 and the NKA α-subunit in the gills of brackish medaka post-transfer from BW to FW (A) or SW (B) are shown. The number were expression levels compared at 6 h after transfer from BW to FW with 12 h and 24 h (A), respectively, and the expression levels were compared to 0 h after transfer from BW to SW within 1 h (B).

challenge, the upregulation of NKA activity was not parallel to protein abundance of NKA α-subunit. Rapidly enhanced gill NKA activity (in 12 h post-transfer) might be attributed to increasing levels of interactions between OdFXYD11 and NKA proteins. Taken together, the expression of the NKA protein was not significantly upregulated until 7 days after hypoosmotic stress. Moreover, the brackish medaka might enhance NKA activity within the short-period of time by stimulating OdFXYD11 expression in both transcriptional and translational levels.

Unlike the gill epithelial ionocytes observed in the hypoosmotic challenge, those exposed to the hyperosmotic environment played the role in NaCl secretion. The NKA expressed in the ionocytes provides the primary driving force for the secretion of chloride via the basolateral Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) and the apical cystic fibrosis transmembrane conductance regulator (CFTR) (Hwang et al., 2011). Kang et al. (2008) reported that the branchial NKA activity of brackish medaka was elevated upon the hyperosmotic challenge. Similar findings were also reported in brown trout (*S. trutta*; Seidelin et al., 2000), Japanese eels (Tang et al., 2012), Japanese medaka (Kang et al., 2008; Hsu et al., 2014), striped bass (*Morone saxatilis*; Tipsmark et al., 2004), and Nile tilapia (*O. niloticus*; Velan et al., 2011). In the present study, the branchial NKA activity was quickly upregulated within 1 h after brackish medaka were transferred to SW (Fig. 6), but OdNKA-α and OdFXYD11 protein expression was not change (Fig. 5). There are two hypotheses that may explain this observation. The first possibility is the switching of the NKA α-subunit. Some studies indicated that a transformation of the NKA α-subunit in fish gills occurred during osmotic challenges. The α1a isoform of the α-subunit was the predominant form found in the gills of FW-acclimated Atlantic salmon (Tipsmark et al., 2010), Mozambique tilapia (Tipsmark et al., 2011), and rainbow trout (Richards et al., 2003). When these fish were transferred into SW, *nka*α-1b mRNA increased. In the present study, we had some difficulties clarifying the subtype transcription and translation of the NKA α-subunit because of antibody and gene information limitations. Nevertheless, mRNA expression of the NKA α-subunit was upregulated during SW acclimation, and protein abundance did not change (Fig. 5B). This result implies that the new NKA α-subunit isoform might be transcribed to replace original α-subunit. A similar result was also found in FW-acclimated medaka (Figs. 1 and 2). The isoform-shift of the branchial NKA α-subunit occurred during osmoregulation in euryhaline teleosts. The switching of the α-subunit is thought to be important in salinity adaptation (Tipsmark et al., 2011). Different branchial α-subunit subtypes may have different abilities to interact with FXYD11. In addition, FXYD could interact more efficiently with NKA during SW acclimation. Kang et al. (2008) reported that the NKA-immunoreactive activity of FW-acclimated medaka was 50% higher than SW-acclimated individuals on average, while NKA activity and protein abundance did not significantly change. These results implied that the NKA of SW-acclimated medaka worked more effectively in the long-term acclimation. In the present study, the NKA activity was rapidly regulated during SW than FW acclimation (1 h vs. 12 h). Furthermore, the protein abundance of FXYD11 and NKA did not change during SW acclimation. We also demonstrated that interaction between FXYD11 and NKA α-subunit was not changed within 1 h after brackish medaka were transferred to SW (Fig. 7). The NKA-FXYD complex may be more efficiently and abundantly formed during SW than FW acclimation. Therefore, upon hyperosmotic challenge, medaka could acclimate quickly without the upregulation of OdFXYD11 and OdNKA-α expression.

In teleosts, the interaction of FXYD11 and the NKA α-subunit was first reported in Atlantic salmon using immunoprecipitation and immunohistochemistry (Tipsmark et al., 2010). Moreover, in brackish medaka, Yang et al. (2013) also reported the interaction between FXYD11 and the NKA α-subunit in branchial NKA-IR cells using co-immunoprecipitation and in situ hybridization. The FXYD proteins can regulate NKA activity via phosphorylation and interactive levels of NKA-FXYD complex. In shark rectal glands, phosphorylation of FXYD10 as well as the interaction between the C-terminus Cys^{74} of FXYD10 and Cys^{254} positioned in NKA was shown to inhibit NKA activity (Mahmmoud et al., 2003; Mahmmoud et al., 2005). Moreover, FXYD11 expression (mRNA and protein) was related to hormone treatments in Atlantic salmon (Tipsmark et al., 2010), Japanese eels (Tang et al., 2012), and Mozambique tilapia (Tipsmark et al., 2011). Previous time-course studies reported that *fxyd11* mRNA expression did not correlate with FXYD11 and NKA protein expression or NKA activity. Therefore, in this study, we chose time points when NKA activity was significantly upregulated and where different NKA and/or FXYD11 protein expression patterns were used to determine the possible role of FXYD11 in hypoosmotic and hyperosmotic challenges. Fixed amounts of immunoprecipitation beads and the NKA antibody (α5) were measured to quantify NKA protein abundance. Our results showed that the interactive level between FXYD11 and NKA proteins increased 12 h post-transfer upon hypoosmotic challenge (Fig. 7A), which suggested that the brackish medaka might increase gill NKA activity by elevating the interactive level between FXYD and NKA proteins. However, the branchial NKA activity was quickly induced after the hyperosmotic challenge, while FXYD11 and NKA protein expression as well as the interactive level of FXYD11-NKA complex did not change significantly. These results showed that modulatory mechanisms differed when the brackish medaka were directly transferred from BW to FW or SW. Moreover, there are three putative phosphorylation sites (Ser^{75}, Ser^{77}, and Ser^{79}) found in the OdFXYD11 protein which may function like FXYD10 through phosphorylation to regulate NKA activity (Mahmmoud et al., 2003).

In summary, the present study investigated the time-course expression of FXYD11 in short-term hypoosmotic and hyperosmotic acclimation in euryhaline teleosts. During hypoosmotic acclimation, upregulated FXYD11 protein might increase the interactions with NKA, which then stimulated NKA activity to absorb ions and balance body fluids. NKA and FXYD11 protein abundances, however, were not changed upon hyperosmotic challenge. FXYD11 might interact more efficiently with NKA to overcome hyperosmotic stress, for which the NKA activity was rapidly upregulated to secrete excess ions. To our knowledge, this study is the first to compare the FXYD and NKA expression of teleostean gills under the acute-phase salinity stress. The FXYD11

protein exhibited different patterns of regulating NKA activity upon hypoosmotic or hyperosmotic challenges.

Acknowledgments

This study was supported by a grant from the Ministry of Science and Technology (MOST), Taiwan, to T.-H.L. (103-2311-B-005-004-MY3). The monoclonal antibody α5 was purchased from the Developmental Studies Hybridoma Bank (DSHB) that is 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.

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