

FXYD2c Plays a Potential Role in Modulating Na⁺/K⁺-ATPase Activity in HK-2 Cells Upon Hypertonic Challenge

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Abstract Na⁺/K⁺-ATPase (NKA) is a widely found and important transporter in mammals. The kidney is a major osmoregulatory organ of which the proximal tubules play a crucial role in the maintenance of ionic homeostasis functioning via salt and water reabsorption. FXYD (FXYD domain-containing protein) 2, the γ -subunit of NKA, is the first identified and the most abundant member of FXYD family, affecting the sodium/potassium affinity of NKA in the kidney. Based on DNA microarray analysis, the expression levels of *fxyd2* gene are markedly increased upon hypertonic challenge. Combined with bioinformatic analysis using the NCBI database, we identified an unnamed protein with 145 amino acids,

of which the N-terminus involved the FXYD sequence similar to FXYD2a and FXYD2b, and thus, named as FXYD2c. However, the role of FXYD2c protein in the regulation of NKA expression in the kidney has not been elucidated. In this study, we found that the mRNA and protein levels of FXYD2c were significantly increased upon hypertonic challenge. Immunoprecipitation data revealed that FXYD2c interacts with the NKA $\alpha 1$ subunit. Subsequently, the functional inhibition of *fxyd2c* using short hairpin RNA abrogated NKA activity. Taken together, our study offers novel insight into the potential function of FXYD2c in promoting NKA activity upon hypertonic challenge in HK-2 cells.

Chun-Yu Chang and Cheng-Hao Tang contributed equally to this work.

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Introduction

The mammalian kidney plays a pivotal role in physiological homeostasis including the regulation of blood pH, ionic concentrations, osmotic pressure, and the removal of metabolic wastes (Jeon et al. 2006). On ionic homeostasis, most salts and water are reabsorbed via the proximal convoluted tubule. Upon hypertonic challenge, water passes cell membranes rapidly (Beck et al. 1998), thereby altering intracellular solute concentrations and cell volume. The stabilization of intracellular solute concentrations and cell volume is necessary for cells to perform various functions. The efflux of water caused by hypertonic stress leads to a decrease in cell volume, an increase in ionic strength, macromolecular crowding, DNA breaking, protein oxidation, and cell cycle arrest (Alfieri and Petronini 2007; Beck et al. 1998; Burg et al. 2007). To compensate hypertonicity-induced disturbance, cells have evolved

many responses which are activated immediately to switch the levels of various gene and protein expression.

NKA, belongs to the P-type ATPase family, is a ubiquitous membrane-spanning enzyme that uses the energy of ATP hydrolysis to maintain the transmembrane Na⁺ and K⁺ gradients of animal cells (Geering 2000; Glynn 1993). It is the primary driving force behind the transport of 3 Na⁺ molecules out of the cell and 2 K⁺ molecules into the cell, and it activates other ion transporters to conduct ion regulation. In renal epithelial cells, NKA is confined to the basolateral membrane and being the driving force for Na⁺ reabsorption which is essential for body Na⁺ homeostasis and blood pressure (Glynn 1993). The minimal functional enzyme unit of NKA comprises a catalytic α -subunit containing the cation, the ATP and the phosphate-binding sites, and a glycosylated β -subunit, which is necessary for the structural and functional maturation of the α -subunit (Blanco and Mercer 1998; Geering 2000). Vertebrates have four α (1–4) and three β (1–3) subunits which produce isozymes with different properties of transport. NKA α 1 β 1 has been demonstrated to be the principal isozyme of the kidney, which drives the reabsorption of Na⁺ and water. Modulation of NKA is an important mechanism for animal cells to survive in diverse environments, especially in the compensation of hypertonicity-induced disturbances (Blanco and Mercer 1998; Ohtaka et al., 1996).

The existence of a small membrane protein associated with renal NKA was first postulated by Forbush et al (1978) and later confirmed by the molecular cloning of the mammalian γ -subunit (Mercer et al. 1993). The mammalian γ -subunit (also called FXYP2) has been identified as the most abundant member of the FXYP family that modulates NKA activity in the kidney (Arystarkhova and Sweadner 2005; Cornelius and Mahmmoud 2003; Crambert and Geering 2003; Geering 2006; Therien et al. 1999). The γ subunit (FXYP2) is a small polypeptide of 58 amino acids with an observed molecular weight of 6.5–10 kDa, which co-purifies and co-localizes with the NKA α - and β -subunits of the pig kidney outer medulla (Mercer et al. 1993). In rat kidney, the two identified splice variants, γ a and γ b, are expressed predominantly in the basolateral membrane of the medulla with tissue-specific distribution (Arystarkhova and Sweadner 2005; Therien et al. 1997). Sweadner and Rael (2000) defined the gene family of FXYP proteins based on the invariant amino acids in a signature sequence containing the highly conserved extracellular motif, FXYP (phenylalanine-X-tyrosine-aspartate), two conserved glycine, and a serine residue. Members of the FXYP family are widely distributed in mammalian tissues, possibly with a prominent expression in tissues that perform fluid and electrolyte transport or are electrically excitable (Geering 2006). To date, there are eight members of FXYP protein family reported in mammals, including FXYP1 (or phospholemman, PLM), FXYP2 (or the γ -subunit of NKA), FXYP3 (or mammary tumor marker, Mat-8), FXYP4 (or corticosteroid hormone-

induced factor, CHIF), FXYP5 (or related to ion channel, RIC; or dysadherin), FXYP6 (or phosphohippolin), FXYP7, and FXYP8 (Béguin et al. 2001, 2002; Bogaev et al. 2001; Crambert and Geering 2003, Crambert et al. 2005; Delprat et al. 2007; Geering 2006; Lubarski et al. 2005; Mercer et al. 1993; Studer et al. 2011; Sweadner and Rael 2000).

FXYP protein family regulated NKA via physical interaction (Crambert and Geering 2003; Geering 2006). NKA is modulated by FXYP2 by decreasing the affinity for Na⁺ and K⁺ and increasing the affinity for ATP (Arystarkhova et al. 1999; Lindzen et al. 2003; Wetzel et al. 2004). The FXYP2 isoforms a and b exhibit an inhibitory function in the regulation of the NKA response to hypertonic exposure (Crambert and Geering 2003; Wetzel et al. 2004). Co-localized with NKA, the FXYP2a and FXYP2b are localized in the basolateral membrane of renal epithelial cells (Geering 2006; Sweadner and Rael 2000). In view of the crucial importance of NKA in many physiological and pathophysiological processes, the identification of tissue-specific regulatory mechanisms becomes an important issue. NKA is subjected to short- and long-term regulation by a variety of hormones and neurotransmitters (Lindzen et al. 2003; Ewart and Klip 1995) and can be regulated through the interaction with membrane or cytoplasmic proteins, e.g., the γ -subunit on renal NKA.

By performing a cross-comparison of the two treatments of HK-2 cells, we generated a gene expression profile database of hypertonic versus isotonic conditions from the microarray data. We classified genes according to their potential functions and selected *fxyp2* as the target gene from the category of osmolality-dependent genes. Furthermore, combined with a bioinformatics-based search in NCBI database, a new homolog protein of FXYP2 with 145 amino acids was identified and named with FXYP2c. However, no study focused on the function of FXYP2c in the regulation of NKA upon hypertonic challenge. We, therefore, aimed to assess the potential role of FXYP2c in the regulation of NKA expression, and the molecular mechanism induced upon hypertonic challenge. In this study, we first examined the effects of hypertonicity on the expression of FXYP2c and NKA α 1. We then elucidated the role of protein-protein interactions in regulation of NKA activity. Our study is the first report to demonstrate that FXYP2c plays a potential role in modulating NKA activity of the renal proximal tubule cells upon hypertonic challenge.

Materials and Methods

Cell Culture

HK-2 cells, a human renal proximal tubule cell line (CRL-2190, ATCC, Manassas, VA, USA), were used in the

experiments. The cells were cultured at a density of 5×10^5 cells/ml in 10-cm diameter petri dishes (TPP, Trasadingen, Switzerland) and grown in DMEM/F12 medium (GIBCO, Grand Island, NY, USA) containing 10 % fetal bovine serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin, pH 7.4, at 37 °C in a humidified 5 % CO₂ atmosphere incubator (RCO-3000T, Thermo Scientific Revco, Asheville, NC, USA)

Transfection and Stable Clone Selection

The following experiments were performed in hypertonic medium which was supplied with NaCl solution to reach the osmolality of 600 mOsm/kg H₂O. The osmolality of the medium was assessed using an osmometer (Wescor, South Logan, UT, USA). HK-2 cells were treated in hypertonic conditions, and changes in cell morphology at different time points were observed, as cell volumes were smaller and shrinking, and some cells were floating (Supplementary data online). To confirm the potential function of FXYP2, FXYP2 shRNAs (short hairpin RNA) purchased from the Academia Sinica (Taipei, Taiwan), and the control pLKO.1 scramble (Amp^r) plasmid (a gift from Dr. Jeremy Jian-Wei Chen) were transfected into HK-2 cells. The cloned ID refers to the shRNA target sequences that were listed (Table 1). To purify the high-quality plasmid DNA, a Gene-SpinTM Miniprep Purification Kit (Protech, Taipei, Taiwan) was used, and the extracted DNA was quantified using a NanoDrop 2000 (Thermo, Waltham, MA, USA). The plasmids were transfected into HK-2 cells using jetPEITM (Polyplus Transfection, New York, NY, USA) according to the manufacturer's instructions. For 48–72 h following the transfection, the survival rate of HK-2 cells was confirmed to be more than 90 %. The HK-2 cells stably transfected with shRNA constructs of FXYP2 or specific for FXYP2a and FXYP2b were maintained in medium containing puromycin (final concentration 10 µg/ml) and harvested for subsequent experiments.

Microarray Analysis

Microarray data analysis was performed by the Phalanx Biotech Group (Hsinchu, Taiwan). Human Whole Genome OneArrayTM DNA chips were used for the analysis. Total RNA was isolated from HK-2 cells treated with two

Table 2 Gene expression levels in HK-2 cells under hypertonic exposure

Genes	Description	Folds ^a
<i>fxyd2</i>	FXYP domain containing ion transport regulator 2	1.28
<i>fxyd5</i>	FXYP domain containing ion transport regulator 5	0.80
<i>fxyd7</i>	FXYP domain containing ion transport regulator 7	0.83
<i>clcn3</i>	Chloride channel 3 (CIC-3)	0.85
<i>clcn7</i>	Chloride channel 3 (CIC-7)	1.23
<i>kcnc3</i>	Potassium voltage-gated channel, shaw-related subfamily, member 3	1.21

^a The folds were presented as hypertonic/ isotonic levels

independent stimuli, including isotonic and hypertonic medium. The cells were harvested after a 4 h treatment with RNA-BeeTM reagent (Tel-Test, Friendswood, TX, USA) and then delivered to the Phalanx Biotech Group. By performing a cross-comparison of the two treatments, we generated a gene expression database of hypertonic versus isotonic conditions (data not show). According to the potential roles of the analyzed genes (summary in Table 2), *fxyd2* was selected as the target gene from the category of osmolality-dependent genes of the microarray data.

Bioinformatic Analysis and Production of *fxyd2c* Antibody

To characterize the FXYP2 sequences, we applied the bioinformatic tools available at the National Center for Biotechnology Information (NCBI, [http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The peptide sequences of human FXYP2a and FXYP2b (Accession No. AAG37906; AAG37907) with 66 and 64 amino acids, respectively, were obtained. The alignment of the three FXYP2 isoforms was performed and compared using the UniProtKB website ([http:// www.uniprot.org/uniprot](http://www.uniprot.org/uniprot)), and the specific extracellular region of FXYP2c was identified (Fig. 1). A polyclonal antibody specific for FXYP2c was produced by immunizing a New Zealand white rabbit with a synthetic peptide prepared using peptide-specific affinity purification. In other words, the rabbit polyclonal antibody for FXYP2c was specifically produced against the epitope corresponding to this extracellular region of the cloned FXYP2c amino acid sequence. The synthetic peptide corresponding to amino acid residues 28–45 of human FXYP2c (accession number: BAC04693) shown in the red box in Fig. 1 was designed as the antigen. This region is clearly distinct from that of FXYP2a and FXYP2b. The antigenic sequence of FXYP2c is RTLPEPSGPLPPSSGLSQC, and the additional C was included for carrier protein OVA conjugation (LTK BioLaboratories, Taoyuan, Taiwan). The antiserum was not collected until 0.1 µg antigen in a 1:5,000 dilution

Table 1 The cloned ID for shRNA target sequences of *fxyd2*

Cloned ID	Target sequence
<i>fxyd2</i>	
TRCN0000042949	5'-GACCCGUUCUACUAUGACUAAU-3'
TRCN0000042951	5'-CAUCCUCCUCAGCAGAAGAAU-3'

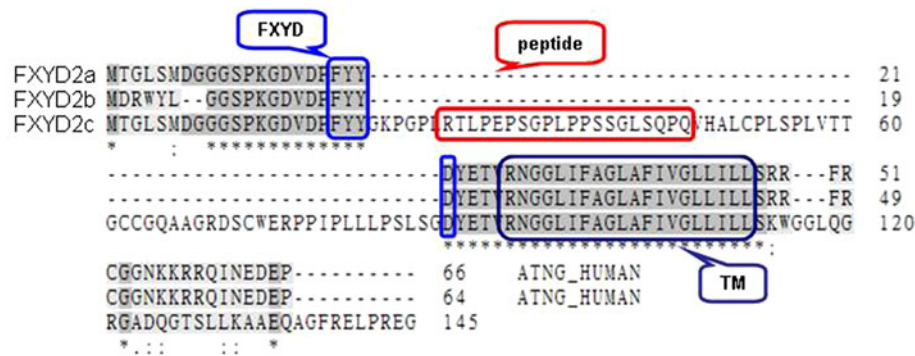


Fig. 1 The antigen was designed according to the results of the FXYP2 isoform alignment. The region (G22-G87) was specifically found in FXYP2c, and the antigen was selected from R28 to Q45 (red

box). The conserved regions were included in the N-terminus, containing the FXYP sequence, the transmembrane domain *TM*, and the C-terminus of these isoforms

was detected via dot blot. The rabbit was injected with antigen six times. The antiserum was further purified using Peptide Specific Affinity Purification to produce the specific antibody. The predicted molecular weight of the FXYP2c was approximately 15 kDa.

Total RNA Extraction and Real-Time PCR

HK-2 cells were exposed to hypertonic conditions for 0, 6, 12, and 24 h. Total RNA was extracted using RNA-BeeTM reagent according to the manufacturer's instructions and dissolved using sterilized deionized water. The RNA was quantified using a NanoDrop 2000 (Thermo, Waltham, MA, USA). First-strand cDNA was synthesized from 2 µg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Each PCR assay contained 100 ng of cDNA, 10 µl of 2 × iQTM SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA), and 10 µM of specific primer pairs. Quantitative real-time PCR was performed using a Mini Opticon real-time PCR system (Bio-Rad) with the thermal cycling conditions as follows: 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The real-time PCR primer sequences are listed in Table 3. Relative quantification was performed using the comparative cycle threshold (*C_T*) method as recommended by the manufacturer. All gene expression quantification data were normalized to the *gapdh* endogenous control. All samples were analyzed in triplicate. The data were analyzed using Bio-Rad CFX manager software (version 1.6; Bio-Rad).

Immunoblotting

The primary antibodies used in this study included (1) mouse monoclonal anti-NKA α1-subunit antiserum (Developmental Studies Hybridoma Bank, Iowa city, IA, USA), 1:5000 dilution; (2) rabbit polyclonal anti-FXYP2c antibody (LTK BioLaboratories), 1:2500 dilution; and (3) mouse monoclonal

anti-α-Tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:8000 dilution. The immunoblotting procedure described by Tang et al. (2010) was used with modification. The cells were exposed to hypertonic conditions and harvested by incubation at 4 °C for 10 min in Cell Culture Lysis Reagent (Promega, Madison, WI, USA) containing 4 % protease inhibitor cocktail (11836145001 Roche, Indianapolis, IN, USA) (v/v: 25/1). The cell lysates were centrifuged at 12,000×g for 10 min at 4 °C. The supernatants were collected, and the protein concentrations were determined by the BCA Protein Assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard. The pre-staining protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). The blots were developed using ImmobilonTM Western (Millipore). The luminescent signals were assessed using a ChemiDoc XRS+ image system (Bio-Rad), and the images were exported using Image lab

Table 3 Primer pairs used for the quantitative real-time PCR

Genes	Primer sequence	Annealing T _m (°C)
<i>fxyp2a</i> and <i>fxyp2b</i>		
Sense	5'-GGACGTGGACCCGTTCTA-3'	60
Anti-sense	5'-TTCTTATTGCCCCACAGC-3'	
<i>fxyp2c</i>		
Sense	5'-AGGACCCCTTCCACCAAG-3'	60
Anti-sense	5'-GGGAGATAAGGGGCACAGA-3'	
<i>nka α1</i>		
Sense	5'-TGTCCAGAATTGCAGGTCTTTG-3'	60
Anti-sense	5'-TGCCCGCTTAAGAATAGGTAGGT-3'	
<i>gapdh</i>		
Sense	5'-GACCTGGCCTACTGTGTGT-3'	60
Anti-sense	5'-CAGCTTGCCTACAACCTGAC-3'	

software (version 3.0; Bio-Rad). α -Tubulin was used as the internal control for all samples.

Na⁺/K⁺ ATPase (NKA) Activity

The assessment of NKA activity was performed according to protocol of Tang et al. (2010). In this experiment, a 96-well microplate was used to measure the inorganic phosphate concentrations to determine the NKA activity levels. The samples were prepared as described above. The final concentrations of the reaction mixture are 100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, and 7.5 mM MgCl₂. Subsequently, 10 μ l of supernatant of the total cell lysate, 100 μ l of 10 mM Na₂ATP, and 50 μ l of 5 mM Ouabain (specific inhibitor of NKA, Sigma) or sterilized deionized water were added to the 340 μ l reaction mixture. Enzyme activity was defined as the difference between the inorganic phosphates liberated in the presence and absence of ouabain in the reaction mixture (μ mol Pi/mg protein/h). The reaction mixture was incubated at 37 °C for 20 min, followed by immediate immersion into an ice bath for 10 min to stop the reaction. The standard curve was prepared with a final concentration of 0, 5, 10, 20, 40, and 80 μ g/ml PO₄³⁻. The reaction mixtures and color reagent (1 % Tween-20 and 1 % ammonium molybdate in 0.9 M H₂SO₄) were mixed 1:1 (v/v) and incubated for 3 min on ice before the samples were assayed using a microplate reader (VERSAmix, Molecular Devices, Sunnyvale, CA, USA) at 405 nm after shaking 10 sec. Each sample was assayed in triplicate.

Immunoprecipitation (IP)

For the IP assays, mouse monoclonal anti-NKA (α 5, Developmental Studies Hybridoma Bank) for detecting all α -subunits was used. The immunoblotting antibodies were used as described above. IP was performed according to the instructions provided with the ImmunoCruzTM IP/WB Optima F System (sc-45043; Santa Cruz) with some modification. In order to confirm the specificity, the FXYD2c antibody was pre-incubated with the synthetic peptide (10 μ g/ml) (Katoh et al. 2003) (i.e., pre-absorbed FXYD2c antibody) and used as the primary antibody for subsequent analysis. The cells were harvested in Cell Culture Lysis Reagent containing 4 % protease inhibitor cocktail. The IP matrix containing 40 μ l suspended (25 % v/v) protein A/G agarose beads, 2 μ g NKA α -subunit antibody, and 500 μ l PBS was incubated at 4 °C on a rotator for 1 h. The pellet matrix was centrifuged at 7500 \times *g* for 1 min at 4 °C, followed by two washes with 500 μ l PBS. After centrifugation, the supernatant (total cellular protein) was added to the IP matrix and incubated at 4 °C on a rotator overnight. After incubation, the IP matrix was centrifuged and washed three times with PBS.

The IP matrix was resuspended in 20 μ l of 4 \times SDS Gel-loading buffer and boiled for 30 min at 37 °C. The IP matrix was spun down quickly to form a pellet; the supernatant was carefully loaded onto the gel, and immunoblotting was performed as described above.

Immunofluorescent Staining (IF)

Cells seeded on coverslips were exposed to isotonic (300 mOsm/kg) and hypertonic (600 mOsm/kg) conditions. The cells were washed twice with pre-warmed (37 °C) 1 \times PBS and fixed with 4 % paraformaldehyde and then permeabilized with 0.1 % Triton X-100 (Sigma). Subsequently, the cells were blocked using 5 % BSA in PBS for 30 min at 25 °C and then incubated with the rabbit polyclonal primary anti-FXYD2c antibody (1:200 dilution) for 1 h at 25 °C. After washing three times with 0.01 % Triton X-100, the coverslips were incubated for 1 h at 25 °C with the secondary antibody, DyLight 488 (green)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Baltimore Pike, PA, USA). For double IF staining, the mouse monoclonal anti-NKA α 1 antibody was subsequently added to the coverslips and incubated for 1 h at 25 °C. The DyLight 549 (red)-conjugated goat anti-mouse IgG was used to detect the primary NKA α 1 antibody. Then coverslips were mounted onto microscopy glass slide with mounting solution containing DAPI (SouthernBiotech, Birmingham, AL, USA). The micrographs were observed using an Olympus fluorescent microscope (Olympus BX50, Tokyo, Japan), and the images were then generated using a digital micro-image cooled CCD camera (Olympus DP72).

Statistical Analysis

The results are shown as the mean \pm S.E.M. (standard error of the mean). For the HK-2 cell (wild type) experiments, statistical significance was determined using the one-way analysis of variance (ANOVA) followed by Dunnett's test. For the shRNA efficiency experiment, statistical significance was compared using Student's *t* test. In the *fxyd2* knockdown experiments under isotonic and hypertonic conditions, different groups were compared at the same time point using one-way ANOVA followed by Tukey's pairwise method. Value *P* < 0.05 was considered statistically significant.

Results

FXYD2c in HK-2 Cells was Selected as a Target Gene to Demonstrate Its Role in Osmotic Homeostasis from the Microarray Data

To identify the candidate genes involved in the response to hypertonic stress in human proximal tubule (HK-2) cells,

DNA microarray analysis was performed in this study to screen up-regulated genes altered during the compensation of hypertonic shock. The analysis showed that the expression level of *fxyd2* gene was elevated following hypertonic exposure (Table 2). Combined with a bioinformatics-based search in NCBI database, a new homolog protein of FXYD2 with a longer amino acid sequence (i.e., 145 amino acids) was identified (accession number: BAC04693) and named as FXYD2c. The conserved regions between FXYD2c and FXYD2a or FXYD2b had high identity (89 %) in the amino acid sequence of the N-terminus involving the FXYD sequence, the transmembrane domain (TM), and the C-terminus. The specific extracellular region of FXYD2c was identified via alignment analysis of the three FXYD2 isoforms (Fig. 1). This region is clearly distinct from FXYD2a or FXYD2b. So it was used to design a specific antiserum against isoform FXYD2c.

Both the mRNA and Protein Levels of FXYD2c in HK-2 Cells Were Increased Upon Hypertonic Challenge

HK-2 cells were exposed to hypertonic (600 mOsm/kg H₂O) conditions, and the relative mRNA levels of the *fxyd2* isoforms were analyzed via real-time PCR. As the nucleotide sequences of the *fxyd2a* and *fxyd2b* are highly conserved, it was difficult to distinguish between these isoforms with the primer pair used for real-time PCR analysis. Among different time points (0, 6, 12, and 24 h) after hypertonic exposure, the highest mRNA abundances of *fxyd2a/2b* and *fxyd2c* were both found at 24 h (Fig. 2). The values were normalized using the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). The relative mRNA levels of *fxyd2a/2b* at 24 h post-exposure were significantly increased approximately

eightfold compared with the isotonic condition (0 h) (Fig. 2a). Moreover, the mRNA levels of *fxyd2c* were significantly increased approximately 10- and 25-fold at 12 and 24 h, respectively (Fig. 2b). The results showed that the induced mRNA abundance of *fxyd2c* was more than *fxyd2a/2b*, and the timing to induce expression of *fxyd2c* was earlier than *fxyd2a/2b* upon hypertonic challenge. The protein abundance of FXYD2c in HK-2 cells upon hypertonic challenge at different time points (0, 6, 12, and 24 h) was also detected. The predicted molecular weight of the protein detected by the polyclonal specific antibody to FXYD2c was approximately 15 kDa. Relative protein levels were normalized by the α -tubulin (Fig. 2c). Based on software analysis (Image Lab, Bio-Rad), the relative protein levels of FXYD2c were significantly increased approximately twofold at 24 h. Our results showed that hypertonic stress induced both of the mRNA and protein expression of FXYD2c in HK-2 cells.

Hypertonic Stress Affected mRNA and Protein Levels of NKA α 1 Subunit and NKA Activity in HK-2 Cells

The primer pair used for the real-time PCR analysis of NKA α 1 subunit expression was designed as described by Murphy et al. (2004). Relative mRNA abundance of the NKA α 1 subunit in HK-2 cells upon hypertonic challenge was significantly increased approximately 1.6- and 3.1-fold at 12 and 24 h, respectively (Fig. 3a). Additionally, the protein levels of the NKA α 1 subunit were significantly elevated approximately 1.5-fold at 24 h (Fig. 3b). The predicted molecular weight of NKA α 1 identified by the monoclonal antibody of was approximately at 100 kDa. These results indicated that hypertonic stress induces the mRNA and protein expression of NKA α 1 subunit in HK-2 cells.

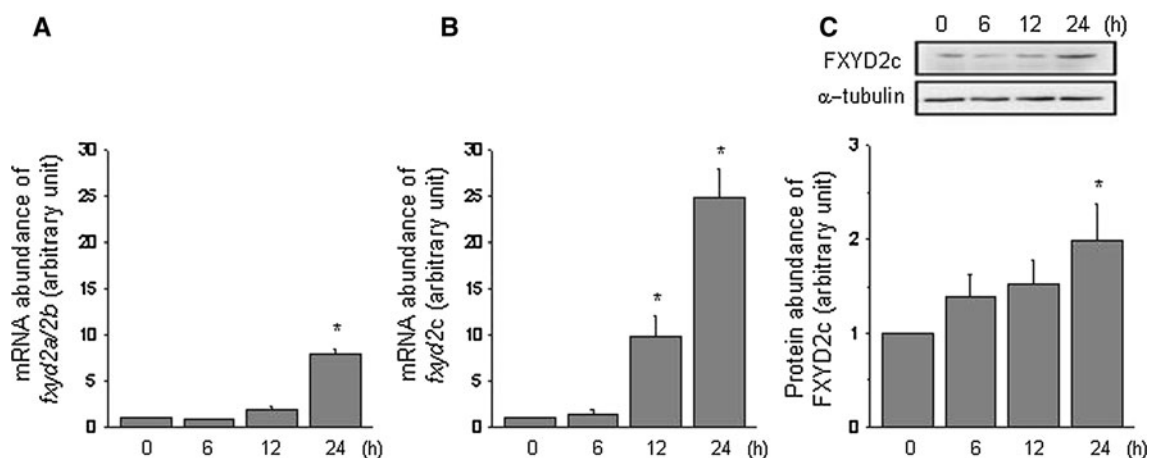


Fig. 2 Relative mRNA abundance of *fxyd2a*, *fxyd2b* (a), and *fxyd2c* (b) in HK-2 cells upon hypertonic challenge. Densitometric analysis demonstrated the relative ratios of the respective mRNA/*gapdh* levels (statistical significance at $*P < 0.05$ compared with 0 h, $n = 6$). The relative protein levels of FXYD2c (c). The upper panel showed the

immunoblots of FXYD2c, and the lower panel showed the quantification values representing relative ratios of the FXYD2c/ α -tubulin ($*P < 0.05$ compared with 0 h, $n = 7$). The data represented the mean \pm SEM. Statistical analysis was performed using one-way ANOVA with the Dunnett's test

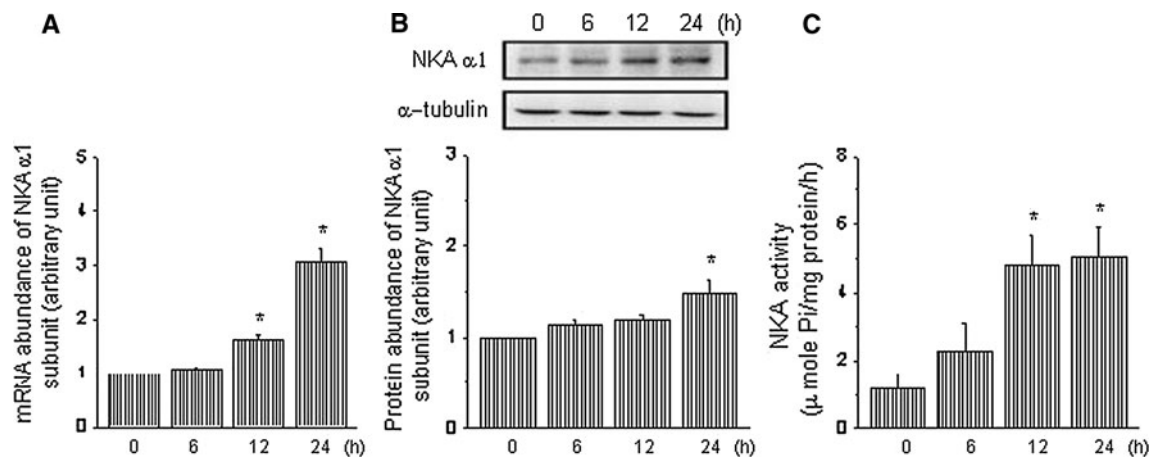


Fig. 3 The mRNA and protein expression levels of the NKA $\alpha 1$ subunit and the specific activity of NKA in HK-2 cells upon hypertonic challenge. The relative mRNA levels of the *nka* $\alpha 1$ subunit (a). Densitometric analysis demonstrated the relative ratios of the *nka* $\alpha 1$ /*gapdh* levels (* P < 0.05 compared with 0 h, n = 6). The relative protein levels of the NKA $\alpha 1$ subunit (b). The upper panel showed the immunoblots of the NKA $\alpha 1$ subunit, and the lower panel showed the quantification values representing the relative ratios of NKA

$\alpha 1$ / α -tubulin levels (* P < 0.05 compared with 0 h, n = 7). Time-course increases in NKA activity in HK-2 cells when exposed to hypertonic environments (c). Activity levels were assayed using the specific inhibitor of NKA (ouabain) and the measurement of Pi concentrations (* P < 0.05 compared with 0 h, n = 5). The data represented the mean \pm SEM. Statistical analysis was performed using one-way ANOVA with the Dunnett's test

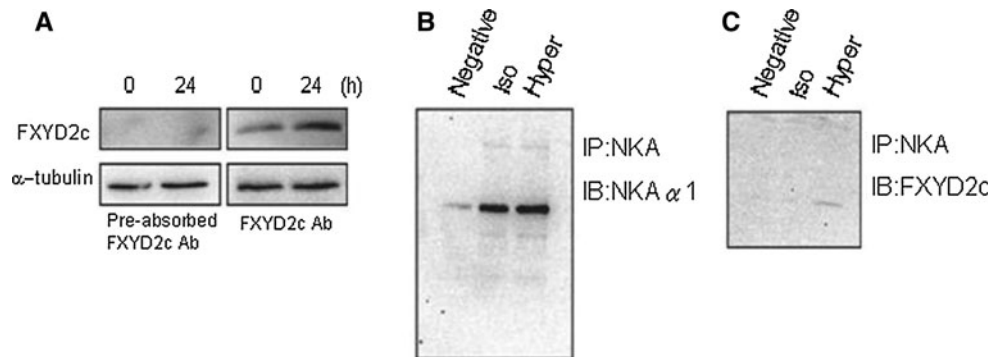


Fig. 4 Immunoprecipitation (IP) showed that hypertonic challenge elevated the specific interaction between FXYD2c and NKA. The specificity of the immunoreactivity was assessed by detecting the FXYD2c antibody and peptide (a). The membrane was incubated with FXYD2c antibody or FXYD2c antibody pre-absorbed with

antigen. The NKA α subunit was IP from HK-2 total cell lysate using primary antibodies, and the immune complex was then analyzed via SDS-PAGE and subsequent immunoblots for the NKA $\alpha 1$ subunit (b), and FXYD2c (c)

Among different time points (0, 6, 12, and 24 h) following hypertonic exposure, the specific activities of NKA were significantly increased approximately 4.8- and 5.0-fold at 12 and 24 h, respectively (Fig. 3c). Interestingly, a positive correlation between the expression patterns of FXYD2c and activity of NKA was found when HK-2 cells were exposed to hypertonic conditions (Figs. 2c, 3c).

FXYD2c Co-Localized With NKA in HK-2 Cells were Both Activated Upon Hypertonic Challenge

NKA is a ubiquitous membrane protein, but the FXYD2 is a kidney-specific membrane protein (Glynn 1993; Forbush

et al. 1978). To address whether FXYD2c is involved in the modulation of NKA, first, we checked the association between FXYD2c and NKA by IP (Fig. 4). The specificity of the FXYD2c antibody was confirmed by pre-incubating the antibody with the synthetic peptide (Fig. 4a). Compared to FXYD2c purified antibody, the immunoreactive band of FXYD2c was disappeared in pre-incubation of the antibody with the antigen (Fig. 4a). The data demonstrated that the antibody specifically recognizes FXYD2c. On the other hand, IP was performed to examine whether FXYD2c modulate NKA through interaction with α subunit. In Fig. 4b, c, the negative lane only consisted of the NKA α subunit and PBS. The IP of the antibody and NKA α subunit

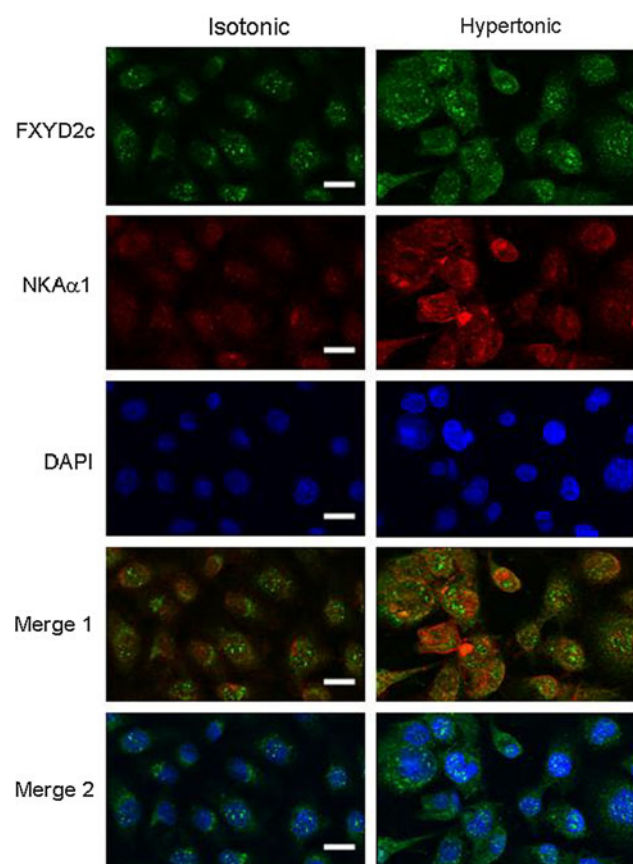


Fig. 5 Immunolocalization of FXYD2c and NKA $\alpha 1$ in HK-2 cells revealed strong staining in the plasma membrane upon hypertonic challenge. The cells were exposed to isotonic (left) or hypertonic (right) condition. The expression level of FXYD2c and NKA $\alpha 1$ was showed. “Merge 1” image was referred to combine the signal of FXYD2c and NKA $\alpha 1$. “Merge 2” image was referred to combine the signal of FXYD2c and DAPI (stained the nuclei). Scale bar = 20 μ m

were then subjected to immunoblotting for the NKA $\alpha 1$ subunit (Fig. 4b) and FXYD2c (Fig. 4c). Furthermore, the immunofluorescent stain (IF) confirmed that the expression levels of FXYD2c and NKA both increased and co-localized to the plasma membrane upon hypertonic challenge (Fig. 5). The signals of immunoreactivity were more intensive in the hypertonic treatment group (for 24 h) (the right column Fig. 5) than the isotonic group (the left column of Fig. 5). Taken together, these data demonstrated that FXYD2c and NKA were co-localized in HK-2 cells both activated upon hypertonic challenge.

Functional Inhibition of FXYD2c With Short Hairpin RNA (shRNA) Declined the Levels of mRNA and Protein Upon Isotonic and Hypertonic Challenge

To clarify the potential function of FXYD2c in regulation of NKA activity, the gene knockdown approach was used in this study. We transfected shRNA constructs (in pLKO.1 expression vectors) against various FXYD2 isoforms into

HK-2 cells. Real-time PCR and immunoblot analyses were conducted to assess the knockdown efficiency of the FXYD2 isoforms. In this experiment, shRNA-A (for knockdown FXYD2a, FXYD2b, and FXYD2c) and shRNA-B (only for knockdown FXYD2a and FXYD2b) constructs were performed to indirectly demonstrate the potential function of FXYD2c in HK-2 cells upon hypertonic challenge. When FXYD2a, 2b, and 2c were all silenced (i.e., shRNA-A), compared with the wild type, the mRNA levels of *fxyd2a/2b* (Fig. 6a) as well as *fxyd2c* (Fig. 6b) were significantly reduced in HK-2 cells at 0 and 24 h. In contrast, when *fxyd2a/2b* were silenced (i.e., shRNA-B), compared with the wild type, the mRNA levels of *fxyd2a/2b* were also significantly reduced at 0 and 24 h (Fig. 6c), but no significant difference was found in mRNA levels of *fxyd2c* in HK-2 cells (Fig. 6d).

The effect of treatment with shRNA-A or shRNA-B on the protein expression of FXYD2c was further analyzed. Representative immunoblots of FXYD2c and α -tubulin were shown in the upper panels of Fig. 7, and the quantification of the immunoblotting results was shown in the lower panels of Fig. 7. After exposing to hypertonic treatment 24 h, the relative protein levels of FXYD2c were significantly increased approximately 2.4-, 1.2-, and 2.1-fold at 24 h in the wild type, shRNA-A, and shRNA-B constructs in HK-2 cells, respectively. Meanwhile, shRNA-A constructs significantly reduced the protein abundance of FXYD2c approximately 0.5- and 3-fold under isotonic conditions and with 24 h hypertonic exposure, respectively, compared with the wild type. Additionally, there was no significant difference between the wild type and shRNA-B constructs (Fig. 7).

Functional Inhibition of FXYD2c Slightly Decreased the Protein Levels of NKA $\alpha 1$ Subunit, but Increased the Activity of NKA Upon Hypertonic Challenge

The effect of FXYD2c knockdown on the expression of NKA $\alpha 1$ subunit was assessed. Immunoblotting was performed to compare the protein levels of the NKA $\alpha 1$ subunit among different groups. The representative immunoblots of the NKA $\alpha 1$ subunit and α -tubulin were shown in the upper panels of Fig. 8a, and the quantification of the immunoblotting results was shown in the lower panels of Fig. 8a. Relative protein levels of NKA $\alpha 1$ subunit were slightly decreased in shRNA-A constructs compared with wild type at isotonic condition (0 h) or the hypertonic shock (24 h). On the other hand, NKA $\alpha 1$ subunit was all increased in the wild type, shRNA-A, and shRNA-B constructs, respectively, upon hypertonic conditions (24 h). No difference was found between the wild type and the shRNA-B constructs in isotonic or hypertonic conditions. Furthermore, the specific activity of NKA was significantly increased approximately

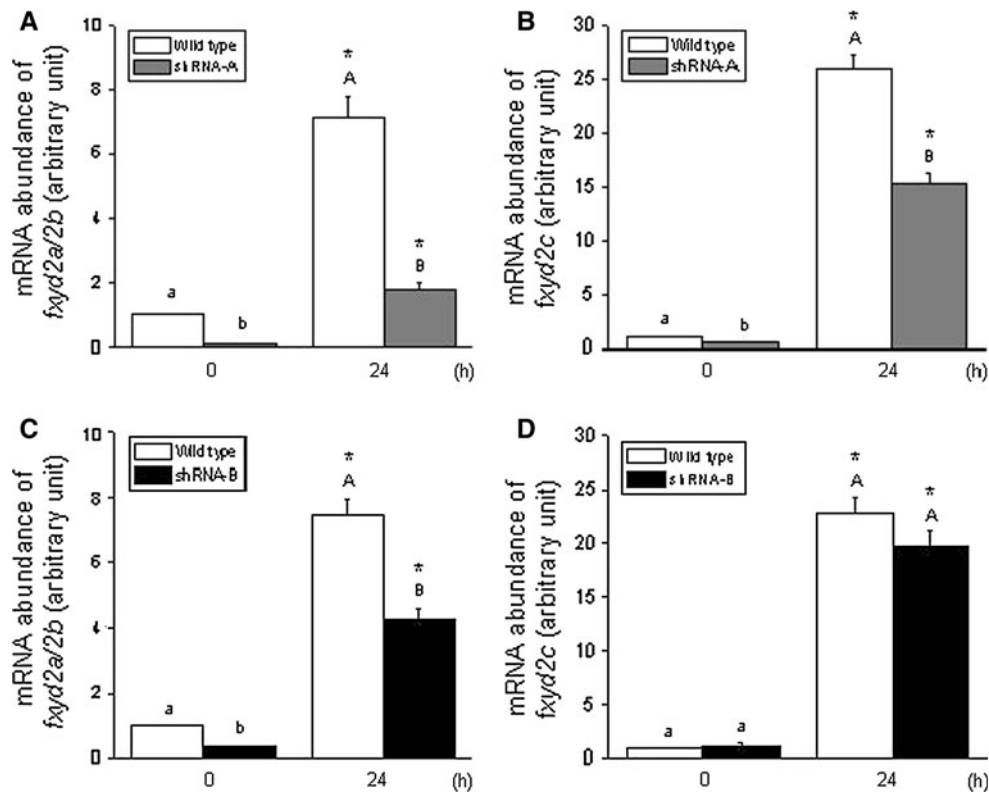


Fig. 6 Functional inhibition of FXYP2a/2b/2c or FXYP2a/2b with shRNA-A or shRNA-B abrogated the mRNA level of *fxyd2c* under isotonic or hypertonic conditions. The HK-2 cells were transfected with 2 μ g/ml shRNA-A clones for 24 h, and the knockdown efficiency was confirmed via the real-time PCR analysis. The mRNA levels of *fxyd2a/2b* (a, c), and *fxyd2c* (b, d) were determined and compared with that of the wild type group upon hypertonic challenge. Densitometric analysis demonstrated the relative ratios of the

respective mRNA/*gapdh* levels. The data represent the mean \pm SEM ($n = 5$). Statistical analysis was performed using Student's *t* test. Significant differences were labeled with different lowercase letters (a and b) among wild type, shRNA-A, or shRNA-B constructs at 0 h. Different capital letters (A and B) indicated significant differences between different constructs at hypertonic conditions (24 h). The asterisk indicated significant differences between 0 and 24 h in the same group

1.2-, 2.1-, and 3.3-fold after 24 h hypertonic exposure in the wild type, shRNA-A, and shRNA-B constructs, respectively (Fig. 8b). NKA activity varied significantly among the three groups in the isotonic group (0 h) or the hypertonic group (24 h).

Discussion

Human kidney is a major osmoregulatory organ, and the regulatory mechanisms of ionic and water homeostasis are mediated by renal epithelial cells (Bens et al. 2006; Blanco and Mercer 1998; Ho 2006). Renal epithelial cells trigger the transepithelial transporters of ions, e.g., NKA, the primary active pump, to respond to the highly variable extracellular ion concentrations (Beck et al. 1998; Blanco and Mercer 1998). Alteration in NKA activity is an important mechanism for cells to adapt to changes in the osmolality of extracellular environments (Bens et al. 2006; Burg et al. 2007). Changes in NKA kinetics and activity might be caused by different isozymes with various isoforms

composed of NKA α (1–4) and β (1–3) subunits (Blanco and Mercer 1998). To reveal a new candidate gene involved in modulating NKA activity in renal cells, DNA microarray was used primarily to select target genes in HK-2 cells upon hypertonic challenge. In the preliminary data, the hypertonic treatment of HK-2 cells induced the shrinkage of the cells, reduction of cell volume, and the appearance of some floating cells, indicating that the hypertonic challenge was performed successfully (Suppl. Fig. 1).

FXYPs are the major regulatory subunit of NKA, which is named based on the conserved domain, the FXYP domain (Geering 2006). In the kidney, several FXYP proteins (i.e., FXYP1, FXYP2, FXYP4, and FXYP5) have been identified (Arystarkhova et al. 2007; Béguin et al. 2001; Bogaev et al. 2001; Lubarski et al. 2005; Mercer et al. 1993; Pihakaski-Maunsbach et al. 2008). FXYPs were suggested to be obligatory rather than auxiliary components of NKA, and their interchangeability underlies responses of NKA to cellular stress (Arystarkhova et al. 2007). Among them, FXYP2 (γ subunit of NKA) is the most important regulator modulating the properties of NKA in renal cells

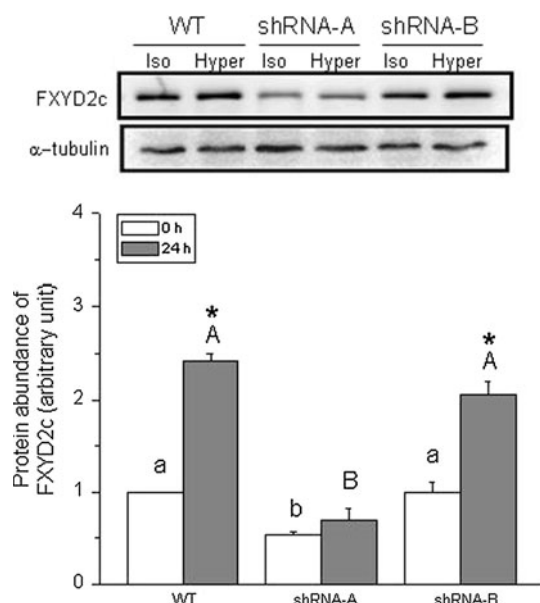


Fig. 7 Functional inhibition of FXYD2a/2b/2c or FXYD2a/2b with shRNA-A or shRNA-B, respectively, diminished the protein levels of FXYD2c at isotonic (0 h) or post-exposure hypertonic (24 h) conditions. FXYD2c protein levels simultaneously increased upon hypertonic challenge when HK-2 cells were treated with shRNA-A or shRNA-B clones. The upper panel showed the immunoblots of FXYD2c, and the lower panel showed the quantification values representing the relative ratios of the respective protein/ α -tubulin levels. The data represented the mean \pm SEM ($n = 5$). When compared with the wild type WT, the statistical analysis for shRNA-A (knockdown *fxyd2a/2b/2c*) and shRNA-B (knockdown *fxyd2a/2b*) constructs under isotonic (0 h) or hypertonic (24 h) conditions was performed using one-way ANOVA with Tukey's pairwise method. Significant differences were labeled with different lowercase letters (*a* and *b*) among wild type, shRNA-A, and shRNA-B constructs at 0 h. Different capital letters (*A* and *B*) indicated significant differences between different constructs at hypertonic conditions (24 h). The asterisk indicated significant differences as assessed by Student's *t* test between 0 and 24 h in the same group

(Arystarkhova et al. 2007; Capasso et al. 2001a, b; Lindzen et al. 2003; Pihakaski-Maunsbach et al. 2008; Therien et al. 1999). Additionally, FXYD2 protein levels are increased upon hypertonic challenge (Capasso et al. 2001b). Microarray analysis revealed that FXYD2 protein expression was upregulated upon hypertonic challenge (Table 2). Previous studies of FXYD2 have focused on its two transcript variants—FXYD2a and FXYD2b (Arystarkhova and Sweadner 2005, Arystarkhova et al. 2007; Therien et al. 1999). In our study, we identified *fxyd2c* (accession number: BAC04693) as a candidate gene. Moreover, the relationship between *fxyd2c* and NKA is unknown. The period for inducing mRNA expression of *fxyd2c* is shorter than that of *fxyd2a* and *fxyd2b* at 12 h after hypertonic exposure. The protein levels of FXYD2c were induced at 24 h, as was observed with the corresponding mRNA expression pattern upon hypertonic challenge. The results suggested that the enhancement of FXYD2c after hypertonic exposure might

be regulated at the transcriptional level in human renal epithelial cells. On the other hand, the present study used the NKA $\alpha 1$ subunit to explore the effects of hypertonic challenge in HK-2 cells, as NKA isoform $\alpha 1\beta 1$ has been shown to be the principal isozyme of the kidney (Blanco and Mercer 1998). The protein and mRNA levels of the NKA $\alpha 1$ subunit and NKA activities were significantly increased in HK-2 cells upon hypertonic challenge, which correlates with previous findings described for the inner medullary collecting duct cells (Bens et al. 2006; Ohtaka et al. 1996). NKA activities were significantly increased at 12 h after hypertonic exposure. The protein levels of the NKA $\alpha 1$ subunit and FXYD2c were, however, elevated at 24 h. The results suggested that the increased NKA activity at 12 h might be regulated by other mechanisms, but not on the transcription level of *fxyd2c*. Dynamic exchange of other members of the FXYD proteins or changes of the interaction level between NKA and FXYD2c might be enough to up-regulate the NKA activity in response to stress (Arystarkhova and Sweadner 2005, Arystarkhova et al. 2007; Béguin et al. 2001; Lindzen et al. 2003; Lubarski et al. 2005).

Upon hypertonic challenge, the induction of FXYD2c correlated with an increase of NKA responses (Figs. 2 and 3). To address the potential function of FXYD2c, it is important to examine its cellular localization and interaction with NKA. To examine the interaction between NKA and FXYD2c, specific antibodies against FXYD2c and the NKA $\alpha 1$ subunit were used in this study. To exclude the possibility of antibody cross-reaction, we applied bioinformatics predictions to ensure that the epitope sequence of the anti-FXYD2c antibody was highly specific. Furthermore, the experiment of antibody pre-absorption with antigen has demonstrated that the anti-FXYD2c antibody specifically recognized the FXYD2c and did not cross-react with FXYD2a and FXYD2b (Fig. 4a). IP analysis showed that the protein levels of FXYD2c and the NKA $\alpha 1$ subunit were both elevated, suggesting that hypertonic challenge increased binding of FXYD2c and NKA $\alpha 1$ subunit to the NKA. In other words, hypertonic stress enhanced the interaction between FXYD2c and the NKA α subunit in HK-2 cells (Fig. 5b, c). The NKA $\alpha 1$ subunit was localized primarily to the plasma membrane in cultured renal cells (Sweadner et al. 2011). The co-localization of several FXYD proteins and NKA at the plasma membrane in different mammalian cells has been reported (Cornelius and Mahmoud 2003; Delprat et al. 2007). In the basolateral membrane of renal epithelial cells, FXYD2a and FXYD2b were found to co-localize with NKA (Cornelius and Mahmoud 2003). IF staining was used to assess the localization of FXYD2c, which showed that FXYD2c is predominantly expressed at the cell membrane and co-localizes with NKA (Figs. 5). These results were in agreement with previous findings of Arystarkhova et al.

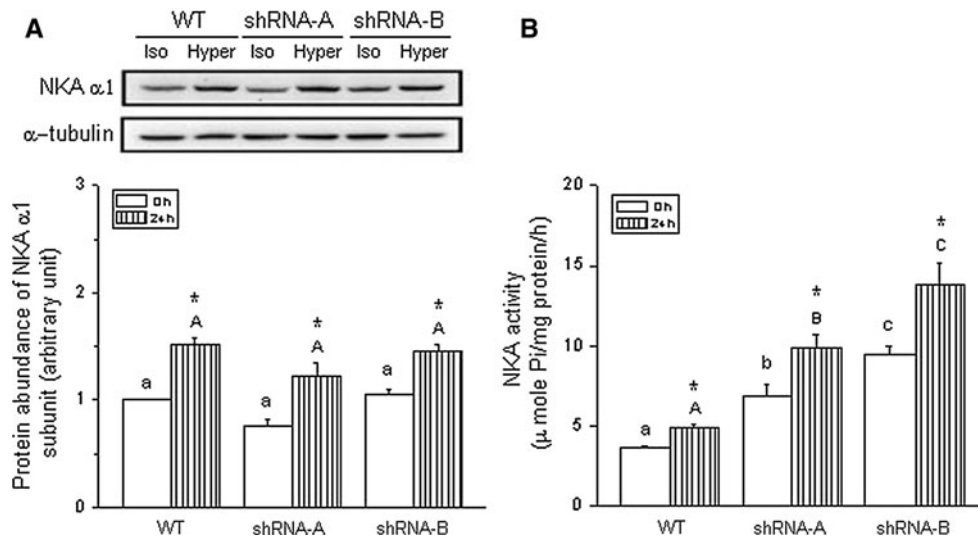


Fig. 8 Functional inhibition of FXYD2a/2b/2c or FXYD2a/2b alleviated the protein levels of NKA α1 subunit, but simultaneously elevated the specific activity levels of NKA upon hypertonic challenge. The *upper panel* showed immunoblots of the NKA α1 subunit, and the *lower panel* showed the quantification values representing the relative ratios of the respective protein/α-tubulin levels (**a**). The data represented the mean ± SEM ($n = 6$). The NKA activity of HK-2 cells in different constructs when exposed to hypertonic environments (**b**). NKA activities were assayed using the

specific inhibitor of NKA (ouabain) and the measurement of Pi concentrations. The data were the mean ± SEM ($n = 4$). Significant differences between the wild type, shRNA-A, and shRNA-B constructs at 0 h were labeled with *different lowercase letters* (a and b). *Different capital letters* (A and B) indicated significant differences between the constructs at 24 h. Both statistics were performed using the Tukey's pairwise method. The *asterisk* indicated significant differences as assessed by Student's *t* test between 0 and 24 h in the same group

(1999) in which the gamma subunit modulated Na⁺ and K⁺ affinity of renal NKA.

Previous studies have reported that FXYD2a and FXYD2b exhibited an inhibitory function in regulation of the NKA activity when mammalian cells were exposed to hypertonic stress (Crambert and Geering 2003; Therien et al. 1999; Wetzel et al. 2004). To clarify the potential function of FXYD2c in NKA regulation to hypertonic shock, shRNAs were used in the FXYD knockdown experiments. Although the shRNA silencing efficiency of FXYD2c was not higher than that of FXYD2a and FXYD2b (the mRNA was silenced approximately 56 % in shRNA-B constructs), the mRNA levels of *fxyd2c* were obviously repressed in the shRNA constructs compared with the wild type cells upon hypertonic challenge at 24 h. Moreover, the protein levels of FXYD2c in the shRNA constructs were lower than the wild type group. These results of the knockdown experiment provided evidence confirming that the expression of FXYD2c was certainly reduced. Relative protein levels of NKA α1 subunit were slightly decreased in the shRNA-A constructs. Meanwhile, there was no significant effect on NKA α1 subunit protein abundance in response to hypertonic stress among the three groups. Accordingly, FXYD2c might regulate NKA properties via mechanisms other than modulation of NKA protein expression. In contrast, because alteration of NKA activity is important for cells to compensate for

hypertonicity-induced disturbances (Therien et al. 1999), the role of FXYD2c in regulation of NKA activity was examined by analyzing NKA activity in FXYD2c-silenced cells (Fig. 8b). NKA activities were increased in hypertonic conditions in all groups. The highest NKA activity was found in shRNA-B constructs (knockdown of FXYD2a and FXYD2b) which coincided with previous findings showing that FXYD2a and FXYD2b exhibited an inhibitory role in modulating of NKA activity upon hypertonic exposure (Wetzel et al. 2004). Compared with the group of shRNA-B constructs, however, shRNA-A (knockdown of all FXYD isoforms) treatment led to an approximate 25 % decrease in NKA activity. By comparing the activity and abundance of mRNA and protein of NKA between the shRNA-A and shRNA-B constructs, the specific function of FXYD2c in regulation of NKA activity was elucidated. According to our data, FXYD2c would act as an enhancer in regulation of NKA activity upon hypertonic challenge, whereas FXYD2a and FXYD2b play a repressor role. Moreover, the other FXYD family proteins might also be involved in modulating NKA kinetic properties. Identifying the signaling pathways involved in modulation of NKA activity via FXYD2c in response to osmotic disturbances requires further investigation. Taken together, our findings suggested that FXYD2c played a role in regulation of NKA activity by enhancing the expression of NKA in HK-2 cells upon hypertonic challenge.

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Conflict of interest The authors declare no conflicts of interest.

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