Tankyrase-mediated β-catenin activity regulates vasopressin-induced AQP2 expression in kidney collecting duct mpkCCDc14 cells

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Aqua2ors (AQP5S) are water channel proteins that transport water molecules across the biomembrane. Aquaporin-2 (AQP2) is the key water channel protein expressed in the kidney connecting tubules and collecting ducts for arginine vasopressin (AVP)-mediated water reabsorption (5, 10, 12, 20, 22). Vasopressin V2-receptor (V2R)-mediated cAMP/PKA signaling has been shown to be a principal pathway for both AQP2 trafficking and protein expression via activation of Gα proteins (36). Recent studies demonstrated that tankyrase also plays a role in the AVP-mediated cAMP-PKA response element binding protein, which increases transcription of the AQP2 gene (41).

The AVP-induced cAMP-PKA signaling pathway interacts with other signaling pathways, such as phosphoinositide pathways (19, 31) and Wnt/β-catenin signaling (34). However, cross talk between AVP and Wnt/β-catenin signaling in the kidney collecting ducts, particularly for the regulation of AVP-induced AQP2 expression, is unknown. Previous studies demonstrated that AQP2 expression is regulated by phosphorylation (Ser 552) and nuclear translocation of β-catenin (34). Moreover, the binding sites of DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF), the main partners for Wnt target genes, were identified at the promoter region of the AQP2 gene (28). It is therefore hypothesized that transcriptional activity of β-catenin could also play a role in AVP-induced AQP2 expression.

Tankyrase-1 and tankyrase-2 are isoforms of the poly(ADP-ribose) polymerase (PARP) family. They are involved in a variety of cellular processes upon their catalytic transfer of ADP-ribose from NAD+ to acceptor proteins, possibly including Gα proteins (36). Recent studies demonstrated that tankyrase also plays a role in the AVP-mediated cAMP-PKA signaling pathway through regulation of stability of the axin/β-catenin complex, which mediates β-catenin activation for the transcription of Wnt target genes (15). In contrast, the tankyrase inhibitor XAV939 stimulates β-catenin degradation by stabilizing axin, and hence it inhibits β-catenin-mediated transcription (15). Previous studies demonstrated that mRNA expression of tankyrase is seen in the kidney, and the expression correlated with canonical Wnt activity (7, 27). Moreover, tankyrase has been demonstrated to play a key role in kidney development (18). In this study, we aimed to study whether tankyrase plays an important role in AVP-induced AQP2 upregulation via ADP-ribosylation of G protein-α (Gα) and/or β-catenin-mediated transcription of AQP2 in the kidney collecting duct cell line mpkCCDc14. The results would provide novel insights into vasopressin-mediated urine concentration and homeostasis of body water metabolism.

MATERIALS AND METHODS

Culture of mouse cortical collecting duct mpkCCDc14 cells. mpkCCDc14 cells (passages 28–36) were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium (DMEM/F12) containing 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM L-glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM HEPES, 0.1% penicillin-streptomycin solution, 20 mM D-glucose, and 2% heat-inactivated FBS at...
37°C, as previously described (23, 30). mpkCCDc14 cells were cultured in a semipermeable filter of the Transwell system (0.4-µm pore size, Transwell Permeable Supports, catalog no. 3460, Corning) for 7 days and starved in serum- and hormone-free medium for another 24 h before the experiment. To inhibit tankyrase activity, the apical and basolateral side of the polarized cells were treated with 10 µM tankyrase inhibitor 3,5,7,8-tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-1 (XAV939; Cayman Chemical).

Primary culture of inner medullary collecting duct cells of the rat kidney. The animal protocols were approved by the Animal Care and Use Committee of the Kyungpook National University, Korea (KNU 2012-10). Primary cultures enriched in inner medullary collecting duct (IMCD) cells were prepared from pathogen-free male Sprague-Dawley rats (200–250 g, Charles River, Seongnam, Korea), as previously described in detail (24). Briefly, rats were anesthetized under enflurane inhalation, and kidneys were rapidly removed. After isolation of an IMCD cell suspension, cells were seeded on a collagen-coated 35-mm glass-base dish (Asahi Techno Glass, Tokyo, Japan). IMCD cells were fed every 48 h and were grown in hypertonic culture medium (640 mosmol/kg H2O) supplemented with 10% fetal bovine serum at 37°C in a 5% CO2-95% air atmosphere for 3 days, and then in fetal bovine serum-free culture medium for additional 1 day before the experiment at day 5. The culture medium was Dulbecco’s modified Eagle’s medium/F12 without phenol red, containing 80 mM urea, 130 mM NaCl, 10 mM HEPES, 2 mM t-glutaminate, 10,000 U/ml penicillin-streptomycin 50 mM hydrocortisone, 5 µM 3,3,5-triiodothyronine, 1 nM sodium selenate, 5 µg/ml transferrin, and 10% fetal bovine serum (pH 7.4, 640 mosmol/kg H2O).

RT-PCR analysis. Total RNA from homogenates of mouse whole kidney (male C57BL/6 mice) and mpkCCDc14 cells was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions (19, 23). Total RNA from homogenates of mouse whole kidney (male C57BL/6 mice) and mpkCCDc14 cells was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RT-PCR was performed using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RT-PCR was performed using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RT-PCR was performed in total RNA using the Access RT-PCR system (Promega, Madison, WI) according to the manufacturer’s instructions (19, 23). Amplification of the mRNA for the housekeeping gene was low in the basal state, whereas it is increased when a PKA substrate is phosphorylated. RT-PCR analysis was performed to determine the expression level of the housekeeping gene β-actin was used as an internal control. The primer sequences are described in Table 1. RT-PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide.

Quantitative immunoblot analysis. The cell lysates were obtained in RIPA buffer (10 mM Tris HCl, 0.15 M NaCl, 1% NP-40, 1% Na-deoxycholate, 0.5% SDS, 0.02% sodium azide, 1 mM EDTA, pH 7.4) including proteinase and phosphatase inhibitors (0.4 µg/ml leupeptin, 0.1 mg/ml pefabloc, 1 mM Na2VO4, 25 mM NaF, and 0.1 µM okadaic acid). Semi-quantitative immunoblotting was performed, as previously described (19, 30). Densitometric values were corrected by the densitometry value of β-actin. Primary antibodies used were anti-AQP2 (1:2,000, H7661AP) (23, 29), anti-tankyrase [Abcam ab13587 for semi-quantitative immunoblotting (1:1,000) and Abcam ab39378 for immunohistochemistry (1:120)], anti-poly(ADP-ribose) (1:1,000, Millipore), anti-Gx (1:1,000, Abcam), and anti-β-actin (1:100,000, Sigma). The film was scanned (Epson Perfection V700 Scanner, Long Beach, CA), and band density was quantitated by ImageJ (National Institutes of Health (NIH) Image, NIMH, NIH, Bethesda, MD). The densitometric values for each protein were corrected by the densitometry of β-actin and were normalized to facilitate comparisons (19, 23, 30).

Immunohistochemistry. Left kidneys of male C57BL/6 mice were fixed by retrograde perfusion via the aorta with 3% paraformaldehyde in PBS, pH 7.4. Immunolabeling was performed on sections from a paraffin-embedded preparation (2-µm thickness) using methods described previously in detail (23, 29).

Pull-down of biotin-ADP ribose-conjugated proteins. Biotin-NAD (Trevigen) was used as a substrate for pull-down assay of ADP-ribosylated proteins. mpkCCDc14 cells were cultured on a semipermeable filter of the six-well Transwell system for 7 days and incubated in serum-free and hormone-deprived medium for another 24 h. After starvation, mpkCCDc14 cells were incubated in the serum-free and hormone-deprived medium containing 6 µg of biotin-NAD in the presence or the absence of 10−9 M dDAVP for 24 or 48 h. The cells were lysed in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4). Cell lysates were incubated with streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, Carlsbad, CA) for 30 min at room temperature. The beads were washed with PBS containing 0.1% BSA four times and incubated in Laemmli sample buffer at 65°C for 15 min. After removal of beads, samples were stored at 4°C.

Fluorescence resonance energy transfer analysis of PKA activation using AKAR3EV indicator. LLC-PK1 cells expressing AKAR3EV were seeded on a collagen-coated 35-mm glass-base dish (3911-035, Iwaki) as an indicator of PKA activation (21), and PKA activation in response to dDAVP or forskolin was analyzed for 20 min. In this probe design, the fluorescence resonance energy transfer (FRET) level is low in the basal state, whereas it is increased when a PKA substrate peptide (LLRATLVD) is phosphorylated by PKA, and hence demonstrates PKA activation. After incubation in serum- and hormone-free medium for 16–20 h, the solution was loaded to the dish fixed in a microscope stage incubator (37°C, 5% CO2). In XAV939-treated cells, XAV939 (10 µM) was pretreated in the XAV939-treated group for 30 min before perfusion of forskolin- or dDAVP-containing medium and cotreated with forskolin or dDAVP during analysis. PKA activation was time-lapse obtained by a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with Cascade 512B (EMCCD) camera (Roper Scientific, Trenton, NJ), using excitation and emission filter wheels (MAC5000, Ludl Electronic Products, Hawthorne, NY). All systems were controlled by MetaMorph software (Universal Imaging, Downingtown, PA). Fluorescent images were acquired sequentially through cyan fluorescent protein (CFP) and FRET filter channels. Images were acquired by using the 2×2 binning mode and 100-ms exposure time. The FRET/CFP ratio was created with MetaMorph software, and the values were normalized to 1 at the starting time point in both groups.

Table 1. Primer sequences of RT-PCR

<table>
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<th>Gene (Accession No.)</th>
<th>Sequences</th>
<th>Expected Product Size, bp</th>
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<tr>
<td>Mouse Tnk-1 (NM_175091.3)</td>
<td>Sense: 5'-ATTGGAGGGAGATTTGAGGCA-3'&lt;br&gt;Antisense: 5'-TATGCCACGCCCATTTGACACT-3'</td>
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<tr>
<td>Mouse Tnk-2 (NM_001000101.2)</td>
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<td>213</td>
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<tr>
<td>Mouse β-actin (NM_007393)</td>
<td>Sense: 5'-GATGCCCTGAGGCTTTTTC-3'&lt;br&gt;Antisense: 5'-TGACGAAATGCCGGTGCTACTA-3'</td>
<td>172</td>
</tr>
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Tnk, tankyrase.
incubated in serum-free and hormone-free medium for 24 h before dDAVP treatment. The basolateral side of the polarized cells was treated with dDAVP (10^{-9} M) for 48 h. Protein expression of AQP2 and β-catenin was analyzed by semiquantitative immunoblot analysis.

Computer-based silico analysis of transcription factor binding sites. To identify the putative transcriptional factor binding site of AQP2, the 5'-flanking region of the AQP2 (3,000 bp) gene was analyzed using MatInspector (Genomatix Software, Ann Arbor, MI).

Luciferase reporter assay. The pGL3-basic vector (Promega) was used to examine the promoter activity in mpkCCDc14 cells. The 1.7-kb 5'-flanking region of the AQP2 gene was inserted into the pGL3-basic vector. The dual luciferase assays were performed according to the manufacturer’s instructions, as described previously (30). The reporter constructs (0.5 μg) and the pRL-SV40 vector (10 ng, Promega) per well were cotransfected using 0.5 μl of Lipofectamine 2000 in mpkCCDc14 cells seeded on a 24-well plate (SPL Life Sciences, Seoul, Korea) for 24 h. Then, to inhibit tankyrase activity the cells were treated with forskolin (10^{-5} M) for 6 or 12 h and XAV939 (10 μM). The dual luciferase assays (Promega) were performed according to the manufacturer’s instruction. Briefly, cells were lysed with passive lysis buffer. Luciferase assay buffer II containing a luciferase substrate was added to the lysate in the 96-well plate (Victor X2, Perkin Elmer 2030, Waltham, MA). After the same volume of dual and stop reagent was added to each well, Renilla luminescence was measured in the same plate order as the firefly luminescence.

Statistical analysis. Quantitative data are presented as means ± SE. Comparisons were made by unpaired t-test (between 2 groups) or one-way ANOVA followed by Tukey’s multiple comparisons post hoc test (more than 2 groups). Multiple comparison tests were only applied when a significant difference was determined in the ANOVA (P < 0.05). P values <0.05 were considered statistically significant.

RESULTS

Tankyrase expression was seen in mouse cortical collecting duct mpkCCDc14 cells and mouse kidney tissues. Tankyrase expression in mouse kidney cortical collecting duct mpkCCDc14 cells and mouse kidney tissues was examined. RT-PCR showed mRNA expression of tankyrase-1 and -2 in mpkCCDc14 cells and kidney tissues from male C57BL/6 mice (Fig. 1A). In parallel, immunoblots of tankyrase revealed the protein expression of tankyrase in mpkCCDc14 cells and mouse kidneys (Fig. 1B). Specifically, protein expression of tankyrase was seen in whole kidney homogenates, intracellular vesicle (200,000-g pellet)-, or cytosol (200,000-g supernatant)-enriched fractions from mouse kidneys, but not in the plasma membrane (17,000-g pellet)-enriched fraction (Fig. 1C). The expression was the most abundant in the cytosol-enriched fraction (Fig. 1C).

Immunohistochemistry of tankyrase in mouse kidneys. Immunoperoxidase microscopy of tankyrase in mouse kidneys (male C57BL/6 mice) demonstrated that tankyrase labeling was mainly seen in the proximal tubules (indicated as P in Fig. 2, A and B), thick ascending limb (indicated as T in Fig. 2, A and B), and collecting duct cells (indicated as C in Fig. 2, A–D). The labeling of tankyrase was exclusively cytosolic, and labeling at the plasma membrane was not seen, consistent with the data demonstrated in Fig. 1C. In particular, in the proximal tubules, the labeling was not seen in the brush border membranes (Fig. 2, A and B). In the collecting ducts, some cells were weakly labeled or unlabeled (indicated by asterisks in Fig. 2, A, C, and D). Immunoperoxidase labeling without primary antibodies against tankyrase was negative (not shown).

![Fig. 1](http://ajprenal.physiology.org/) Tankyrase expression in mouse cortical collecting duct mpkCCDc14 cells and mouse kidney tissues. A: RT-PCR showing the mRNA expression of tankyrase in mouse cortical collecting duct mpkCCDc14 cells and kidney tissues from male C57BL/6 mice. RT-PCR showed tankyrase-1 (273-bp), tankyrase-2 (213-bp), and β-actin (172-bp) products in mpkCCDc14 cells and mouse kidney tissues. Negative controls were shown by omission of reverse transcriptase (−RT). B: immunoblotting of tankyrase in mouse cortical collecting duct mpkCCDc14 cells and kidneys from male C57BL/6 mice. The immunoblots were reacted with anti-tankyrase antibody and revealed ~130- and 170-kDa bands in mpkCCDc14 cells and ~130- and 140-kDa bands in mouse kidneys. Mouse kidneys were fractionated by differential centrifugation: 4,000-g supernatant for whole kidney homogenates, 17,000-g pellet for plasma membrane fraction, and 200,000 g for intracellular vesicles (pellet) and cytosol (supernatant). Cor: cortex; ICV, intracellular vesicle fraction; IM, inner medulla; OM, outer medulla; PM, plasma membrane fraction; Tnk-1, tankyrase-1; Tnk-2, tankyrase-2; WK, whole kidney fraction; −, RT negative; +, RT positive.
Inhibition of tankyrase attenuated dDAVP-induced AQP2 upregulation in mpkCCDc14 cells. To examine the effect of tankyrase inhibition on dDAVP-induced AQP2 expression, mpkCCDc14 cells were treated with dDAVP (10^{-9} M) in the absence or the presence of tankyrase inhibitor XAV939 (10^{-9} M) for 48 h. dDAVP stimulation markedly upregulated AQP2 expression (162 ± 14% of vehicle-treated cells, P < 0.05) (Fig. 3A), whereas XAV939 cotreatment significantly attenuated dDAVP-induced AQP2 upregulation [101 ± 8% of vehicle-treated cells, not significant (n.s.)] (Fig. 3A). Notably, XAV939 treatment induced accumulation of tankyrase protein (Fig. 3A), since inhibition of PARP activity of tankyrase results in the decrease in autoPARsylation, leading to a significant decrease in proteasomal degradation of tankyrase (42). Moreover, mpkCCDc14 cells transiently transfected with siRNAs against both tankyrase-1 and -2 (70 ± 4% of nontarget siRNA-treated cells, P < 0.05) (Fig. 3B) demonstrated that dDAVP-induced AQP2 upregulation observed in nontarget siRNA-treated cells (130 ± 7% of vehicle-treated cells, P < 0.05) (Fig. 3B) was not present in the cells with tankyrase knockdown (92 ± 3% of vehicle-treated cells, n.s.) (Fig. 3B).

Additionally, the effect of tankyrase inhibition on cAMP-responsive AQP2 induction was examined. Treatment with the adenyl cyclase activator forskolin (10^{-6} M, for 24 h) induced AQP2 expression (144 ± 9% of vehicle-treated cells, P < 0.05) (Fig. 4A). In contrast, in Fig. 4B, AQP2 upregulation induced by forskolin treatment (157 ± 3% of vehicle-treated cells, P < 0.05) was not seen when mpkCCDc14 cells were cotreated with XAV939 (106 ± 2% of vehicle-treated cells, n.s.).

Inhibition of tankyrase attenuated dDAVP-induced AQP2 upregulation in primary culture of IMCD cells from rat kidneys. To further examine the effect of tankyrase inhibition on dDAVP-induced AQP2 expression, primary cultured IMCD cells from rat kidneys were treated with dDAVP (10^{-10} M) for 24 h. dDAVP stimulation markedly upregulated AQP2 expression (156 ± 16% of vehicle-treated cells, P < 0.05) (Fig. 5, A and B). In contrast, XAV939 cotreatment significantly attenuated dDAVP-induced AQP2 upregulation (99 ± 6% of vehicle-treated cells, n.s.) (Fig. 5A and B). As seen in mpkCCDc14 cells (Fig. 3A), XAV939 treatment also induced accumulation of tankyrase protein in primary cultured IMCD cells (Fig. 5A).

dDAVP-induced cAMP/PKA activation was intact despite decreased ADP-ribosylation of Gα protein under inhibition of tankyrase. Tankyrase-mediated ADP-ribosylation and PARsylation of Gα have not been reported. To examine whether Gα is the target protein of the ADP-ribosylating activity of tankyrase, whole ADP-ribosylated proteins in mpkCCDc14 cells were isolated. Cells were incubated with biotinylated-
NAD⁺ (6 µg/well) in the absence or presence of dDAVP stimulation (10⁻⁹ M for 48 h). Then, biotin-conjugated ADP-ribosylated proteins were pulled down by streptavidin-conjugated magnetic beads and subjected to Gα immunoblotting (Figs. 6A and 7A). dDAVP treatment increased ADP-ribosylation of Gα (Fig. 6, A and B), whereas XAV939 cotreatment (10 µM for 48 h) markedly reduced the dDAVP-induced ADP-ribosylation of Gα (Fig. 7A). Moreover, PARylation of ~45-kDa-sized proteins, including Gα protein, was decreased by XAV939 treatment (Fig. 7B).

Next, to examine whether tankyrase-mediated ADP-ribosylation of Gα is critical in dDAVP-induced cAMP/PKA signaling pathways, changes in PKA activity was examined by FRET image analysis using AKAR3EV as an indicator of PKA activation (21). FRET responses were analyzed in LLC-PK1 cells expressing AKAR3EV after dDAVP or forskolin stimulation in the absence or the presence of XAV939 cotreatment. dDAVP (10⁻⁹ M) increased PKA activity to the maximum level within 2–3 min. FRET responses were similar despite XAV939 cotreatment (Fig. 7C). Similarly, forskolin (10⁻⁶ M) increased intracellular PKA activity, which was not affected by tankyrase inhibition. Therefore, the observed absence or attenuation of dDAVP-induced AQP2 upregulation under tankyrase inhibition was not likely due to the decreased ADP-ribosylation and PARylation of Gα.

Inhibition of tankyrase attenuated dDAVP-induced phosphorylation of β-catenin (S552) and nuclear translocation. Phosphorylation of β-catenin at S552 was examined in
mpkCCDc14 cells and LLC-PK1 cells. Tankyrase inhibition by XAV939 treatment without dDAVP cotreatment did not affect the expression of &beta;1-catenin in both cell lines (Figs. 8A and 9A). The polarized mpkCCDc14 cells were treated with dDAVP (10^{-9}M) in the absence or the presence of cotreatment with XAV939 (10 &mu;M, 24 h). The immunoblots were reacted with anti-AQP2 (28- to 33-kDa deglycosylated bands and 33- to 50-kDa glycosylated bands), and anti- &beta;-actin antibody (42-kDa bands). B: semiquantitative immunoblotting of AQP2 was carried out from mpkCCDc14 cells treated with vehicle and forskolin (10^{-6} M, 24 h) in the absence or the presence of cotreatment with XAV939 (10 &mu;M, 24 h). The immunoblots were reacted with anti-AQP2 (28- to 33-kDa deglycosylated bands and 33- to 50-kDa glycosylated bands) and anti- &beta;-actin antibody (42-kDa bands). n, No. of cell lysate preparations in each group; FSK, forskolin; V, vehicle; X, XAV939. *P < 0.05.

Immunofluorescence microscopy revealed that dDAVP treatment increased nuclear translocation of p- &beta;1-catenin (S552) in LLC-PK1 cells (Fig. 9C). In contrast, less nuclear translocation was observed in the cells cotreated by both dDAVP and XAV939, and immunolabeling was also seen at the plasma membrane (Fig. 9C). The results indicated that inhibition of tankyrase significantly decreased the dDAVP-induced phosphorylation of &beta;1-catenin and its nuclear translocation, possibly affecting the &beta;1-catenin-mediated transcriptional regulation of target genes.

Fig. 4. Tankyrase inhibition attenuated forskolin-induced AQP2 upregulation in mpkCCDc14 cells. A: semiquantitative immunoblotting of AQP2 was carried out from mpkCCDc14 cells treated with vehicle and forskolin (10^{-6} M, 6 or 24 h). The immunoblots were reacted with anti-AQP2 (28- to 33-kDa deglycosylated bands and 33- to 50-kDa glycosylated bands), and anti- &beta;-actin antibody (42-kDa bands). B: semiquantitative immunoblotting of AQP2 was carried out from mpkCCDc14 cells treated with vehicle and forskolin (10^{-6} M, 24 h) in the absence or the presence of cotreatment with XAV939 (10 &mu;M, 24 h). The immunoblots were reacted with anti-AQP2 (28- to 33-kDa deglycosylated bands and 33- to 50-kDa glycosylated bands) and anti- &beta;-actin antibody (42-kDa bands). n, No. of cell lysate preparations in each group; FSK, forskolin; V, vehicle; X, XAV939. *P < 0.05.

Fig. 5. Tankyrase inhibition attenuated dDAVP-induced AQP2 upregulation in primary cultured inner medullary collecting duct cells from rat kidney. A: semiquantitative immunoblotting of AQP2 and tankyrase (Tnk) was carried out from primary cultured inner medullary collecting duct cells from rat kidneys treated with vehicle, dDAVP (10^{-10} M), XAV939 (10 &mu;M), or both (dDAVP and XAV939) for 24 h. The immunoblots were reacted with anti-AQP2 (28- to 33-kDa deglycosylated bands and 33- to 50-kDa glycosylated bands), anti-tankyrase (130- and 170-kDa bands), and anti- &beta;-actin antibody (42-kDa bands). Abbreviations are defined as in the legend for Fig. 3. *P < 0.05.

Immunofluorescence microscopy revealed that dDAVP treatment increased nuclear translocation of p- &beta;1-catenin (S552) in LLC-PK1 cells (Fig. 9C). In contrast, less nuclear translocation was observed in the cells cotreated by both dDAVP and XAV939, and immunolabeling was also seen at the plasma membrane (Fig. 9C). The results indicated that inhibition of tankyrase significantly decreased the dDAVP-induced phosphorylation of &beta;1-catenin and its nuclear translocation, possibly affecting the &beta;1-catenin-mediated transcriptional regulation of target genes.
Inhibition of Akt/GSK3β/β-catenin signaling also decreased nuclear translocation of β-catenin and diminished dDAVP-induced AQP2 expression. Previous studies demonstrated that the transcriptional activity of β-catenin induced by its nuclear translocation is also regulated by other signaling pathways, e.g., phosphoinositide 3-kinase (PI3K)/Akt signaling (9, 35). To further examine the critical role of β-catenin in AQP2 transcription as a transcriptional regulator, changes in dDAVP-induced AQP2 expression were examined in mpkCCDC14 cells subjected to inhibition of PI3K/Akt signaling.

LY294002 (25 µM) inhibited AQP2 induction by dDAVP (10^{-9} M) treatment for 6 or 48 h (Fig. 10, A and B). Expression of GSK3β, one of the Akt substrates, was decreased by LY294002 treatment. Phosphorylated GSK3β (S9) was significantly decreased by LY294002, and the ratio of p-GSK3β (S9) to total GSK3β was also diminished. In contrast, phosphorylation of β-catenin (S552) was increased in response to dDAVP, and the phosphorylation was more intensified by LY294002 cotreatment for 48 h. Cotreatment of dDAVP and LY294002 also induced a remarkable increase in the ratio of β-catenin (S552) to total β-catenin (S552) at 48 h (Fig. 10, A and B).

To examine the nuclear translocation of p-β-catenin (S552), immunolabeling of p-β-catenin (S552) was observed in LLC-PK₁ cells treated with dDAVP or dDAVP with LY294002 for 6 and 12 h (Fig. 10C). dDAVP treatment induced a remarkable nuclear translocation of p-β-catenin (S552). In contrast, LY294002 cotreatment decreased dDAVP-induced nuclear translocation of p-β-catenin (S552), and the labeling of the cytosol and plasma membrane became more prominent (Fig. 10C).

siRNA-mediated knockdown of β-catenin abolished dDAVP-induced upregulation of AQP2. To examine whether β-catenin, the transcriptional regulator in the downstream signaling of tankyrase, affects AQP2 expression, changes in AQP2 protein expression were examined in the cells with siRNA-mediated knockdown of β-catenin. siRNA-transfected mpkCCDC14 cells were cultured for 7 days on semipermeable filters without dDAVP stimulation, and at day 8 cells were treated with dDAVP (10^{-9} M) at the basolateral side in serum- and hormone-free media for another 48 h. In control siRNA-treated cells, dDAVP treatment markedly increased AQP2 protein expression (1,032 ± 201% of vehicle-treated cells, P < 0.05) (Fig. 11, A and C), whereas AQP2 expression was not induced by dDAVP treatment (104 ± 11% of vehicle-treated cells, n.s.) (Fig. 11, A and C) in the cells with β-catenin knockdown (31 ± 2% of control siRNA-treated cells, P > 0.05) (Fig. 11B).

siRNA-mediated knockdown of β-catenin decreased forskolin-induced AQP2 transcription. In silico prediction analysis of the transcription factor binding site of AQP2 revealed the binding sites of the TCF/LEF family transcription factor in the 3,000 bp of the 5’-flanking region of AQP2, which has been shown to interact with β-catenin during the transcriptional process (2). To examine whether β-catenin affects AQP2 transcription, mpkCCDC14 cells transfected by a construct of 1.7 kb of the 5’-flanking region of the rat AQP2 gene was
stimulated with forskolin (10⁻⁵ M) for 6 and 12 h. β-Catenin expression was remarkably reduced by treatment of siRNAs specific for β-catenin (Fig. 12, A and B). Luciferase activity in the cells transfected with the AQP2 promoter was significantly increased by forskolin treatment at 6 and 12 h compared with vehicle (DMSO)-treated cells (Fig. 12C). In the cells with β-catenin knockdown, however, luciferase activity was not induced in response to forskolin (Fig. 12C), indicating that β-catenin plays a role in AQP2 transcription.

**DISCUSSION**

Tankyrase, belonging to a PARP family with PARsylating activity, participates in diverse cellular processes mainly through the functional regulation of target proteins via ADP-ribosylation (32, 36). Moreover, tankyrase also plays a role in the Wnt signaling pathway through mediating β-catenin activation for the transcription of Wnt target genes (15). The present study demonstrated mRNA and protein expression of tankyrase in mouse kidney and mouse cortical collecting duct mpkCCDc14 cells. Immunohistochemistry revealed that tankyrase labeling in mouse kidneys was widely observed in the tubular epithelial cells, and the expression was exclusively cytosolic. In particular, tankyrase labeling was seen in the collecting duct cells, but some cells were weakly labeled or unlabeled, presumably intercalated cells. The specific colocalization of tankyrase and AQP2 in the principal cells was not directly seen in this study, since these two antibodies were unlabeled, presumably intercalated cells. The specific colocalization of tankyrase and AQP2 in the principal cells was not directly seen in this study, since these two antibodies were unlabeled, presumably intercalated cells.
though XAV939 showed in vitro an IC50 value with high activity of tankyrase-1 and tankyrase-2, respectively (15). Significantly attenuated by the inhibition of tankyrase with 10 μM XAV939 treatment or siRNA-mediated knockdown, indicating that tankyrase and/or tankyrase-mediated signaling is importantly involved in AQP2 regulation. This finding was further demonstrated in primary cultured IMCD cells from rat kidneys, as shown in Fig. 5.

To examine the effects of tankyrase-mediated signaling on vasopressin-induced AQP2 regulation in collecting duct cells, mpkCCDc14 cells were cotreated with the tankyrase-selective inhibitor XAV939. In previous studies, XAV939 has been used as a selective inhibitor of tankyrase activity and tankyrase-mediated Wnt signaling (1, 8, 15). Although XAV939 showed in vitro an IC50 value with high selectivity, higher concentrations (5–10 μM) of XAV939 were commonly chosen for cell-based experiments to examine the inhibitory effects of XAV939 on tankyrase activity and tankyrase-mediated Wnt signaling (1, 8, 14). Importantly, dDAVP- or forskolin-induced AQP2 upregulation was significantly attenuated by the inhibition of tankyrase with 10 μM XAV939 treatment or siRNA-mediated knockdown, indicating that tankyrase and/or tankyrase-mediated signaling is importantly involved in AQP2 regulation. This finding was further demonstrated in primary cultured IMCD cells from rat kidneys, as shown in Fig. 5.

To investigate the underlying mechanisms for the effects of tankyrase inhibition on dDAVP-induced AQP2 expression, the effects induced by altered ADP-ribosylation of Goα on cAMP/PKA activation were examined, as the first step. Goα, a subunit of the ubiquitously expressed heterotrimeric G protein that couples the receptor to the effector enzyme adenylyl cyclase, is required for receptor-stimulated generation of intracellular cAMP (3). In the kidney collecting duct cells, V2R-coupled Goα activation is initiated via replacement of GDP to GTP and Goα is dissociated from β- and γ-subunits of the G protein complex. With the translocation of Goα into the cytosol, ADP-ribosylation has been suggested as an important posttranslational protein modification that regulates the activity of Goα (17). Actually, cholera toxin with ADP-ribosylating activity activates Goα by ADP-ribosylation and leads to an increase in intracellular cAMP via adenyl cyclase activation (13, 16). In contrast, ADP-ribosylation of Goα induced by pertussis toxin inhibits cAMP induction and cAMP-stimulated AQP2 trafficking (39).

In the present study, a pull-down assay against ADP-ribosylated proteins demonstrated that tankyrase mediates...
PKA activity was monitored by time-lapse FRET imaging analysis to assess the affects dDAVP-induced cAMP/PKA signaling, intracellular ribosylation of Gαi/αo. dDAVP stimulation catalyzes ADP-ribosylation of Gαi/αo, which binds to the DNA and increases transcription of the AQP2 gene (37). Activation of PKA causes phosphorylation of a cAMP-response element binding protein (CREB protein), which binds to the DNA and increases transcription of the AQP2 gene. To investigate whether decreased ADP-ribosylation of Gαi resulting from tankyrase inhibition affects dDAVP-induced cAMP/PKA signaling, intracellular PKA activity was monitored by time-lapse FRET imaging analysis in live LLC-PK1 cells. Interestingly, PKA activation in response to dDAVP or forskolin stimulation was not affected by cotreatment with the tankyrase inhibitor XAV939, suggesting that the observed decrease in dDAVP-induced AQP2 upregulation under tankyrase inhibition was not likely due to the decreased PARsylation of Gαi protein per se.

Previous proteomic studies on vasopressin signaling in kidney collecting duct cells have suggested that the Wnt signaling pathway could also be involved in vasopressin-mediated AQP2 regulation (4, 33, 34). Recently, Schenk et al. (34) demonstrated that vasopressin induced a change in the abundance of the transcriptional regulator β-catenin in the nuclear proteome of cortical collecting duct mpkCCD (clone 11) cells. This finding potentially suggested that transcriptional regulator β-catenin could be involved in the transcriptional regulation in response to vasopressin stimulation. In the present study, we demonstrated that β-catenin regulates AQP2 expression, where tankyrase was importantly involved. dDAVP induced phosphorylation of β-catenin (S552) and nuclear translocation of phosphorlated β-catenin, both of which were significantly
AQP2 transcription (28). The findings, therefore, suggested that 

**TANKYRESE/β-CATENIN IN AQP2 REGULATION**

- Tankyrase inhibition decreased dDAVP-induced phosphorylation of β-catenin (S552) and nuclear translocation of phosphorylated β-catenin, associated with decreased dDAVP-induced AQP2 upregulation. Moreover, a luciferase reporter assay demonstrated that forskolin-induced AQP2 transcription was significantly decreased in the cells with siRNA-mediated knockdown of β-catenin. In addition, several consensus sites for TCF/LEF gene 5'-flanking regions, suggesting that β-catenin could serve as an activator of TCF-dependent AQP2 transcription (28). The findings, therefore, suggested that transcriptional regulation by β-catenin is likely to be a critical mechanism for AQP2 regulation.

Inhibition of PI3K/Akt, which was also associated with significantly decreased nuclear translocation of p-β-catenin (S552), diminished dDAVP-induced AQP2 expression, further indicating that β-catenin plays a role in AQP2 regulation. Recent studies have suggested that the PI3K/Akt/GSK3β signaling pathway interacts with Wnt/β-catenin signaling. Wnt-1 decreased by tankyrase inhibition. Moreover, siRNA-mediated knockdown of β-catenin expression significantly decreased forskolin-induced AQP2 transcription and dDAVP-induced AQP2 protein abundance. The results therefore indicated that β-catenin is likely to play a critical role in vasopressin-mediated AQP2 regulation, and tankyrase is importantly involved.

β-Catenin has been known to regulate cell-to-cell adhesion mediated by cadherin (26, 40), and hence a decreased β-catenin level might affect the cell adhesion, possibly leading to redistribution of V2R localization along the plasma membrane. Basolateral expression of V2R is a critical factor in the vasopressin-induced response in kidney collecting duct cells. Therefore, the observed decrease in dDAVP-induced AQP2 expression in mpkCCD cells with siRNA-mediated β-catenin knockdown might suggest that dysregulation of the cadherin cell adhesion complex could be involved in the attenuated AQP2 response to dDAVP stimulation. Nevertheless, tankyrase inhibition decreased dDAVP-induced phosphorylation of β-catenin (S552) and nuclear translocation of phosphorylated β-catenin, associated with decreased dDAVP-induced AQP2 upregulation. Moreover, a luciferase reporter assay demonstrated that forskolin-induced AQP2 transcription was significantly decreased in the cells with siRNA-mediated knockdown of β-catenin. In addition, several consensus sites for TCF/LEF gene 5'-flanking regions, suggesting that β-catenin could serve as an activator of TCF-dependent AQP2 transcription (28). The findings, therefore, suggested that transcriptional regulation by β-catenin is likely to be a critical mechanism for AQP2 regulation.

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induced phosphorylation of Akt (T308 and S473) and GSK3β (S9), and these phosphorylations were inhibited by Wnt antagonist-secreted Frizzled-related protein-1 (sFRP-1) (11). Moreover, activation of Akt induces phosphorylation of GSK3β via interacting with activated Dvl, which consequently leads to inhibition of degradation of β-catenin and maintenance of cytosolic free β-catenin levels regulating transcription of Wnt target genes (11, 25, 38). We and other researchers have previously reported that PI3K/Akt activation was induced by vasopressin in the collecting duct cells (19, 31). Short-term dDAVP treatment induced phosphorylation of Akt at T308 and S473, whereas a PI3K inhibitor (LY294002) abolished Akt phosphorylation and resulted in attenuated vasopressin signaling. Moreover, PI3K/Akt activation in response to insulin was associated with dDAVP-responsive AQP2 mRNA and protein expression in the mpkCCDc14 cells (6).

In summary, tankyrase is likely to play an important role in vasopressin-induced AQP2 upregulation via β-catenin-mediated transcription in the kidney collecting duct cells. This study provides a novel insight into vasopressin-mediated urine concentration and body water homeostasis.

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