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

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AQP2 is the key water channel protein expressed in the kidney connecting tubules and collecting ducts for arginine vasopressin (AVP)-induced water reabsorption in the kidney collecting duct. AVP regulates AQP2 expression primarily via Gs-cAMP/PKA signaling. Tankyrerase, a member of the poly(ADP-ribose) polymerase family, is known to mediate Wnt/β-catenin signaling-induced gene expression. We examined whether tankyrerase plays a role in AVP-induced AQP2 regulation via ADP-ribosylation of G protein-α (Gα) and/or β-catenin-mediated transcription of AQP2. RT-PCR and immunoblotting analysis revealed the mRNA and protein expression of tankyrerase in mouse kidney and mouse collecting duct mpkCCDc14 cells. dDAVP-induced AQP2 upregulation was attenuated in mpkCCDc14 cells under the tankyrerase inhibition by XAV939 treatment or small interfering (si) RNA knockdown. Fluorescence resonance energy transfer image analysis, however, revealed that XAV939 treatment did not affect dDAVP- or forskolin-induced PKA activation. Inhibition of tankyrerase decreased dDAVP-induced phosphorylation of β-catenin (S552) and nuclear translocation of phospho-β-catenin. siRNA-mediated knockdown of β-catenin decreased forskolin-induced AQP2 transcription and dDAVP-induced AQP2 expression. Moreover, inhibition of phosphoinositide 3-kinase/Akt, which was associated with decreased nuclear translocation of β-catenin, diminished dDAVP-induced AQP2 upregulation, further indicating that β-catenin mediates AQP2 expression. Taken together, tankyrerase plays a role in AVP-induced AQP2 regulation, which is likely via β-catenin-mediated transcription of AQP2, but not ADP-ribosylation of Gα. The results provide novel insights into vasopressin-mediated urine concentration and homeostasis of body water metabolism.

AQUAPORINS (AQPs) are water channel proteins that transport water molecules across the biomembrane. 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 Gsα-mediated adenyl cyclase activity. Increased intracellular cAMP concentration and activation of PKA phosphorylate cAMP-response element binding protein, which increases transcription of the AQP2 gene (41).

The AVP-induced cAMP/PKA signaling pathway interacts with other signals, 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 AVP-mediated signaling induces 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 partner for β-catenin in transcriptional regulation of 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.

Tankyrerase-1 and tankyrerase-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 protein (36). Recent studies demonstrated that tankyrase also plays a role in the Wnt 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 tankyrerase 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 was 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 in the kidney collecting duct cells 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 mM 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-thiopyranopyrano[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/kgH2O) 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 L-glutamine, 10,000 U/ml penicillin-streptomycin 50 nM hydrocortisone, 5 pM 3,3,5-triiodothyronine, 1 mM sodium selenate, 5 ng/ml transferrin, and 10% fetal bovine serum (pH 7.4, 640 mosmol/kgH2O).

**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). Amplification of the mRNA for the housekeeping gene WI) according to the manufacturer’s instructions (19, 23). Amplification of the mRNA for the housekeeping gene 

**Table 1. Primer sequences for RT-PCR**

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Sequences</th>
<th>Expected Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Tnk-1 (NM_175901.3)</td>
<td><strong>Sense:</strong> 5'-ATGGAGGGATCTTGGAGGC-3'</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td><strong>Antisense:</strong> 5'-ATTGGAGGGATCTTGGAGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Mouse Tnk-2 (NM_001000101.2)</td>
<td><strong>Sense:</strong> 5'-ATTGGAGGGATCTTGGAGGC-3'</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td><strong>Antisense:</strong> 5'-ATTGGAGGGATCTTGGAGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Mouse β-actin (NM_007393)</td>
<td><strong>Sense:</strong> 5'-ATTGGAGGGATCTTGGAGGC-3'</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td><strong>Antisense:</strong> 5'-ATTGGAGGGATCTTGGAGGC-3'</td>
<td></td>
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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-luminometer (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).

A

![AQP2 mRNA expression](image1)

B

![Tankyrase immunoblotting](image2)

C

![Tankyrase localization](image3)

Fig. 1. 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. C: immunoblotting of tankyrase 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. 3 A), whereas XAV939 cotreatment significantly attenuated dDAVP-induced AQP2 upregulation (101 ± 8% of vehicle-treated cells, not significant (n.s.)) (Fig. 3 A). Notably, XAV939 treatment induced accumulation of tankyrase protein (Fig. 3 A), 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. 3 B) demonstrated that dDAVP-induced AQP2 upregulation observed in nontarget siRNA-treated cells (130 ± 7% of vehicle-treated cells, P < 0.05) (Fig. 3 B) was not present in the cells with tankyrase knockdown (92 ± 3% of vehicle-treated cells, n.s.) (Fig. 3 B).

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. 4 A). In contrast, in Fig. 4 B, 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. 5 A and B). As seen in mpkCCDc14 cells (Fig. 3 A), XAV939 treatment also induced accumulation of tankyrase protein in primary cultured IMCD cells (Fig. 5 A).

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 \(\mu\)g/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 G\(_\alpha\) immunoblotting (Figs. 6A and 7A). dDAVP treatment increased ADP-ribosylation of G\(_\alpha\) (Fig. 6A and B), whereas XAV939 cotreatment (10 \(\mu\)M for 48 h) markedly reduced the dDAVP-induced ADP-ribosylation of G\(_\alpha\) (Fig. 7A). Moreover, PARylation of \(~45-\)kDa-sized proteins, including G\(_\alpha\) protein, was decreased by XAV939 treatment (Fig. 7B).

Next, to examine whether tankyrase-mediated ADP-ribosylation of G\(_\alpha\) 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\(^{-8}\) 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 G\(_\alpha\).

**Inhibition of tankyrase attenuated dDAVP-induced phosphorylation of β-catenin (S552) and nuclear translocation.** Phosphorylation of β-catenin at S552 was examined in
mpkCCD14 cells and LLC-PK1 cells. Tankyrase inhibition by XAV939 treatment without dDAVP cotreatment did not affect the expression of β-catenin in both cell lines (Figs. 8A and 9A). The polarized mpkCCD14 cells were treated with dDAVP (10^{-9} M) in the absence or the presence of cotreatment with XAV939 (10 μM, 24 h). The immunoblots were reacted with anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands), and anti-β-actin antibody (~42-kDa bands). B: semiquantitative immunoblotting of AQP2 was carried out from mpkCCD14 cells treated with vehicle and dDAVP (10^{-6} M, 24 h) in the absence or the presence of tankyrase inhibition for 12 h, whereas this was not seen at 12 h under tankyrase inhibition (XAV939, 10 μM) (Fig. 9B).

Immunofluorescence microscopy revealed that dDAVP treatment increased nuclear translocation of p-β-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 β-catenin and its nuclear translocation, possibly affecting the β-catenin-mediated transcriptional regulation of target genes.

Fig. 4. Tankyrase inhibition attenuated forskolin-induced AQP2 upregulation in mpkCCD14 cells. A: semiquantitative immunoblotting of AQP2 was carried out from mpkCCD14 cells treated with vehicle and forskolin (10^{-6} M, 6 or 24 h). The immunoblots were reacted with anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands), and anti-β-actin antibody (~42-kDa bands). B: semiquantitative immunoblotting of AQP2 was carried out from mpkCCD14 cells treated with vehicle and forskolin (10^{-6} M, 24 h) in the absence or the presence of cotreatment with XAV939 (10 μM, 24 h). The immunoblots were reacted with anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands) and anti-β-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 μM), or both (dDAVP and XAV939) for 24 h. The immunoblots were reacted with anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands), anti-tankyrase (~130- and 170-kDa bands), and anti-β-actin antibody (~42-kDa bands). B: semiquantitative immunoblotting of AQP2 was carried out from primary cultured inner medullary collecting duct cells treated with vehicle, dDAVP (10^{-10} M), XAV939 (10 μM), or both (dDAVP and XAV939) for 24 h. The immunoblots were reacted with anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands), anti-tankyrase (~130- and 170-kDa bands), and anti-β-actin antibody (~42-kDa bands). Abbreviations are defined as in the legend for Fig. 3. *P < 0.05.
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 6 or 48 h. Cotreatment of dDAVP and LY294002 also induced a remarkable increase in the ratio of p-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

![Fig. 6](http://ajprenal.physiology.org/)

**Fig. 6.** dDAVP treatment increased ADP-ribosylation of Ga. Semiquantitative immunoblotting of AQP2, tankyrase, Ga, and ADP-ribosylated Ga in mpkCCDc14 cells treated with dDAVP (10−9 M, 24 or 48 h). A: immunoblots were reacted with anti-AQP2 (−28-kDa deglycosylated bands and −33- to 50-kDa glycosylated bands), anti-tankyrase (−130- and 170-kDa bands), anti-Ga (−43-kDa bands), and anti-β-actin antibody (−42-kDa bands). B: ADP-ribosylation of Ga was significantly increased in dDAVP-treated cells, as demonstrated by the increased ratio of ADP-ribosylated Ga to total Ga by dDAVP treatment (24 h: 180 ± 30 and 48 h: 210 ± 30% of vehicle-treated cells, P < 0.05). AQP2 expression was increased by dDAVP treatment at 24 h (246 ± 25% of vehicle-treated cells, P < 0.05) in contrast, expressions of tankyrase and Ga were unchanged by dDAVP treatment for 24 and 48 h. Abbreviations are defined as in the legend for Fig. 3. *P < 0.05.
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 raised in the same host rabbit.
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). An in vitro IC_{50} test revealed that the IC_{50} of XAV939 was 4 and 11 nM against activity of tankyrase-1 and tankyrase-2, respectively (15). Although XAV939 showed in vitro an IC_{50} 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 G_{α} on cAMP/PKA activation were examined, as the first step. G_{α}, 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 G_{α} activation is initiated via replacement of GDP to GTP and G_{α} is dissociated from β- and γ-subunits of the G protein complex. With the translocation of G_{α} into the cytosol, ADP-ribosylation has been suggested as an important posttranslational protein modification that regulates the activity of G_{α} (17). Actually, cholera toxin with ADP-ribosylating activity activates G_{α} by ADP-ribosylation and leads to an increase in intracellular cAMP via adenylyl cyclase activation (13, 16). In contrast, ADP-ribosylation of G_{α} 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 of a cAMP-response element binding protein (CREB) probe (37). Activation of PKA causes phosphorylation of a cAMP-responsive element in the 5′-flanking region of the AQP2 gene (37). Activation of PKA causes phosphorylation of β-catenin (37). Consistent with this, there is known to be a β-catenin regulates AQP2 expression, where the observed decrease in dDAVP-induced AQP2 upregulation under tankyrase inhibition was not likely due to the decreased PARylation of Gα 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 phosphorylated β-catenin, both of which were significantly

ADP-ribosylation of Gα. Additionally, abundance of ADP-ribosylated Gα was increased by dDAVP, indicating that dDAVP stimulation catalyzes ADP-ribosylation of Gα. Transcriptional mechanisms for vasopressin-induced AQP2 upregulation has been well defined, e.g., cAMP/PKA activation (41). Consistent with this, there is known to be a cAMP-responsive element in the 5′-flanking region 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 Gα 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...
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.

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

Fig. 10. dDAVP-induced AQP2 upregulation and nuclear translocation of β-catenin were decreased by inhibition of phosphoinositide 3-kinase (PI3K)/Akt signaling. A and B: semiquantitative immunoblotting of AQP2, Akt, phosphorylated Akt (p-Akt, S473), GSK3β, phosphorylated GSK3β (p-GSK3β, S9), β-catenin, and phosphorylated β-catenin (p-β-catenin, S552) in mpkCCDc14 cells treated with dDAVP (10−8 M) in the absence or the presence of LY294002 (25 μM) for 6 h or 48 h. The immunoblots were reacted with anti-Akt (p-Akt, S473, ~60-kDa bands), anti-phosphorylated Akt (p-Akt, S473, ~60-kDa bands), GSK3β (~47-kDa bands), anti-phosphorylated GSK3β (p-GSK3β, S9, ~47-kDa bands), anti-β-catenin (~94-kDa bands), anti-phosphorylated β-catenin (p-β-catenin, S552, ~94-kDa bands), and anti-AQP2 antibody (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands). C: immunofluorescence microscopy of p-β-catenin (S552) in LLC-PK1 cells treated with dDAVP in the absence or presence of LY294002 for 6 h. The nuclei were stained by DAPI. n, No. of cell lysate preparation; V, vehicle; D, dDAVP; and LY, LY294002. *P < 0.05 compared with vehicle-treated cells. #P < 0.05 compared with dDAVP-treated cells.
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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Fig. 11. siRNA-mediated knockdown of β-catenin inhibited the dDAVP-induced AQP2 upregulation in mpkCCDc14 cells. A–C: dDAVP-induced upregulation of AQP2 was significantly attenuated by siRNA-mediated knockdown of β-catenin for 48 h. Semiquantitative immunoblotting of β-catenin and AQP2 in mpkCCDc14 cells treated by either nontarget siRNA or siRNA against β-catenin. The immunoblots were reacted with anti-β-catenin (~94-kDa bands), anti-AQP2 (~28-kDa deglycosylated bands and ~33- to 50-kDa glycosylated bands), and β-actin antibody (~42-kDa bands). Abbreviations are defined as in the legend for Fig. 3. *P < 0.05 compared with nontarget siRNA-treated cells.

Fig. 12. siRNA-mediated knockdown of β-catenin decreased forskolin-induced AQP2 transcription. A and B: semiquantitative immunoblotting of β-catenin in mpkCCDc14 cells treated by either nontarget siRNA or siRNA against β-catenin. The immunoblots were reacted with anti-β-catenin (~94-kDa bands) and β-actin antibody (~42-kDa bands). C: luciferase assay of AQP2 promoter activity in the absence or presence of β-catenin knockdown. Closed bars, vehicle-treated cells; open bars, forskolin (10 μM)-treated cells. The promoter activity of the 1.7 kb of the 5′-flanking region of the rat AQP2 gene in response to forskolin (FSK; 10−5 M) was analyzed in mpkCCDc14 cells. Abbreviations are defined as in the legend for Fig. 3. *P < 0.05 compared with nontarget siRNA-treated cells.
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


