

# Different effects of CsA and FK506 on aquaporin-2 abundance in rat primary cultured collecting duct cells

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**Abstract** Calcineurin (Cn) inhibitors (CnI) such as cyclosporine A (CsA) and FK506 are nephrotoxic immunosuppressant drugs, which decrease tubular function. Here, we examined the direct effect of CnI on aquaporin-2 (AQP2) expression in rat primary cultured inner medullary collecting duct cells. CsA (0.5–5  $\mu$ M) but not FK 506 (0.01–1  $\mu$ M) decreased expression of AQP2 protein and messenger RNA (mRNA) in a concentration and time dependent manner, without affecting mRNA stability. This effect was observed despite similar inhibition of Cn activity by both CnI, thereby suggesting that the CsA-dependent decrease in AQP2 expression was Cn independent. Another inhibitor of cyclophilin A, the primary intracellular target of CsA, had no effect on AQP2 expression. In order to investigate the mechanism of decreased AQP2 transcription, we studied activation status of two suggested transcriptional regulators of AQP2, cAMP-responsive element binding protein (CREB), and tonicity enhancer binding protein (TonEBP). Localization of TonEBP, as well as TonEBP-mediated gene transcription, was not affected by CsA. Phosphorylation of CREB at an activating phosphorylation site (S133) was decreased by CsA, but not by FK506. However, both CnI did not affect cellular cAMP levels. We show that CsA decreases transcription of AQP2, a process that is in part independent of Cn or cyclophilin A and suggests dependence on decreased activity of CREB.

**Keywords** Aquaporin-2 · Calcineurin · Cyclosporin-A · FK506 · Cyclic adenosine monophosphate (cyclic AMP) · Osmolarity

## Introduction

In the kidney collecting duct, aquaporin-2 (AQP2) is responsible for regulated water reabsorption from the tubular lumen, whereas AQP3 and AQP4 mediate water flow across the basolateral membrane [33]. AQP2 gene expression is partially regulated by the neuropeptide arginine-vasopressin (AVP) via the vasopressin-2-receptor. Binding of AVP leads to activation of adenylate cyclase isoform 6 (AC) and a cAMP-dependent activation of protein kinase A (PKA) and further basophilic protein kinases [22, 38]. In long-term, this process results in phosphorylation of the cAMP-responsive element binding protein (CREB) at S133, a process believed to be crucial for regulation of long-term AQP2 abundance [17, 19, 43]. In addition, recent evidence from cell culture and knockout mouse models show a significant involvement of NF $\kappa$ B, the glycogen synthase kinase 3 beta (Gsk3b) pathway and transcription factors of the nuclear factor of activated T cell (NFAT) family (Nfatc1–4 and Nfat5/TonEBP) in the regulation of AQP2 abundance [18, 20, 25, 36]. The tonicity-dependent transcription factor TonEBP has been characterized as a major regulating element of AQP2, Slc14a2 urea channels (UT-A1 and UT-A3) and several other osmoprotective genes, among these aldose reductase (AR), heat shock protein 70 (HSP70), and betaine  $\gamma$ -amino-butyrate transporter 1 (BGT-1) [5, 14, 16, 18, 47]. Bioinformatic analysis of the AQP2 promotor revealed the presence of two major conserved transcription factor binding site elements, which may be crucial for the regulation of AQP2 transcription [50].

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Calcineurin inhibitors (CnIs), such as cyclosporine A (CsA) and tacrolimus (FK506), are well-established immunosuppressant drugs. The nephrotoxic side effects of this substance class include a decrease of glomerular and tubular function; however, the molecular etiology of these processes is not completely understood [6]. Long-term CsA treatment of Brattleboro rats (3 weeks) was found to decrease AQP2 gene and protein expression in the kidney [26]. In a similar model, decrease in medullary Na<sup>+</sup> transporters and a subsequent decrease in medullary tonicity were followed by inactivation of TonEBP and a decrease in AQP2 protein expression; these data hint at an indirect effect of CsA on AQP2 protein abundance [27]. However, the knockout mouse for calcineurin A alpha has no difference in expression of AQP2 protein in the kidney when compared to her wild-type littermates [15].

The aim of this study was to investigate the effects of CsA on primary cultured inner medullary collecting duct cells, which endogenously express the water channel AQP2. Here, we show that CsA, but not FK506, directly reduces the expression of AQP2 on the messenger RNA (mRNA) and protein level. Analyzing signaling pathways involved in the regulation of AQP2 abundance, we show that a cAMP-independent decrease in CREB phosphorylation may be a crucial element in a complex signaling network, which contributes to a decrease in AQP2 transcription under CsA treatment.

## Materials and methods

**Cell culture** Primary cultured inner medullary collecting duct (IMCD) cells were prepared as previously described [37]. Female Wistar rats (age 2–3 months) were killed by decapitation, and kidneys were removed. Kidney inner medulla, including papilla, was isolated, chopped into small pieces, and digested in phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany) containing 0.2% hyaluronidase (Sigma, Deisenhofen, Germany) and 0.2% collagenase type CLS-II (Sigma) at 37°C for 90 min. Cells were seeded in 24-well plates (Falcon, Germany) or on glass cover slips, coated with collagen type IV (Becton-Dickinson, Heidelberg, Germany) at a density of approximately 10<sup>5</sup> cells/cm<sup>2</sup> and cultivated in Dulbecco's modified Eagle's medium containing penicillin 100 IU/ml and streptomycin 100 µg/ml, 0.2% glutamine, 1% non essential amino acids, and 1% ultrosor (BioSeptra Inc., Marlborough, MA, USA). The osmolality was adjusted to 600 mOsm/kg by addition of 100 mM NaCl and 100 mM urea. A medium without NaCl and urea supplements was determined to be 300 mOsm/kg. Osmolality was checked using an osmometer (Knauer, Berlin, Germany) with an appropriate calibration solution of 400 mOsm/kg (Braun, Berlin, Germany). Initially, cells

were seeded at 600 mOsm/kg, the medium was changed, and defined media (600 or 300 mOsm/kg) were added after 24 h. Medium was changed every 48 h. Cells were cultured for 5 days and were incubated with CsA (Novartis, Basel, Switzerland), FK506 (Fujisawa, Osaka, Japan), Forskolin or dbcAMP (Sigma), or the cyclophilin A inhibitor 239836 (Merck Biosciences, Darmstadt, Germany) for different time periods and concentrations as indicated. For time course studies, an appropriate vehicle control treatment (24 h) was conducted, and all cells had the same age at the time point of harvest (day 6). An effect of different duration of vehicle treatment was excluded because of parallel vehicle pretreatment of the shorter treatment groups (<12 h). All experiments were conducted with at least three independent cell cultures from different rats. All animal experiments were conducted according to German animal protection guidelines and were approved by a governmental committee on animal welfare.

**Immunofluorescence** The localization of TonEBP was determined via immunofluorescence using a commercially available polyclonal TonEBP/NEAT5 antibody (Abcam, Cambridge, UK). IMCD cells were fixed in PBS containing 4% paraformaldehyde for 20 min. Cells were washed with PBS (three times, 10 min), permeabilized in PBS containing 0.1% Triton X100 for 5 min, and washed three times for 10 min with PBS. To block unspecific binding sites, cells were incubated for 20 min in blocking solution (0.35% fish skin gelatine in PBS) at 37°C. Cells on cover slips were incubated at 37°C in a humidified chamber for 90 min with specific antibodies against TonEBP, washed three times (PBS, 10 min), and incubated for 90 min with an Alexa 488 labeled anti-rabbit IgG antibody (Molecular Probes, Leiden, The Netherlands). Cells were washed three times for 10 min in PBS and mounted with Crystallmount (Biomedica, Foster City, CA, USA). Images were taken by a fluorescence microscope with an apotome function (Axiovert, Zeiss, Oberkochen, Germany).

**RNA-Isolation, qPCR, and mRNA stability assay** Total RNA was isolated using RNeasy-Kit (Qiagen, Hilden, Germany). The complementary DNA synthesis of freshly isolated RNA was performed using SuperScript II Kit (Invitrogen, Karlsruhe, Germany). All procedures were done following the manufacturer's instructions. The quantitative PCR (qPCR) was performed using the SYBR Green PCR Master Mix with the ABI PRISM 7900 Sequence Detection System. All instruments and reagents were purchased from Applied Biosystems (Darmstadt, Germany). Specific primer pairs were used as listed in Table 1, and specificity was confirmed using melting point analysis and gel electrophoresis. Relative gene expression values were

**Table 1** Accession number, gene names, primer sequences, and gene symbols of genes used for qPCR

Ref_Seq	Gene name	Primer sequence	Gene symbol
NM_012909	Aquaporin-2	Sense: TGCATCTTTGCCTCCACCGACGAG Antisense: CATGGAGCAACCGGTGAAATAG	Aqp2
NM_031703	Aquaporin-3	Sense: TTCACTGCCCTGGCTGGCTG Antisense: AAGGCGGGGGCTGCTCCAGG	Aqp3
NM_012825	Aquaporin-4	Sense: CATGGGAAACTGGGAAAACCCAC Antisense: GCGACGTTTGAGCTCCACGTC	Aqp4
NM_012498	Aldose reductase	Sense: GTGCTGATCCGGTTCCTCCATC Antisense: CTCATCAAGGCGCACACCTC	Akr1b1
NM_017335.1	Betaine/Gaba Transporter 1	Sense: CCTCCATGGCCTGTGTACCGC Antisense: GAGTCTTGCTTGACTGGAGAG	Slc6a12
NM_153629.1	Heat shock protein 70	Sense: GCACCAATGAGGCGATGGAGTG Antisense: GGACTGCAAATATTGTGTCAGCTC	Hspa4
NM_017008	GAPDH	Sense: CATCAACGACCCCTTCATT Antisense: ACTCCACGACATACTCAGCAC	Gapdh
NM_019347.1	UTA 1+3	Sense: TCTACGTCATCACCTGGCAGAC Antisense: TGCCAGGGTTGTTGGTTGTGAG	Slc14a2
NM_001107425	TonEBP	Sense: GTCAGCTGTTAACTTCTGGGCC Antisense: GCCAGTGGGGCAGATTCTGG	Nfat5

evaluated with the  $2^{-\Delta\Delta Ct}$  method using GAPDH as reference gene [28]. The mRNA stability assay was performed as previously described [7]. Briefly, cells were preincubated with 50 ng/mL actinomycin-D, an inhibitor of transcription for 15 min. Subsequently, cells were incubated as indicated, and mRNA expression was determined using qPCR.

**Immunoblotting** Semiquantitative immunoblotting was performed as previously described [42]. Briefly, same protein amounts were separated by sodium dodecyl sulfate (SDS) polyacrylamide (4–20%) electrophoresis and transferred to a polyvinylidene fluoride membrane (Amersham, Freiburg, Germany). Membranes were subsequently incubated with primary antibodies in a 1:1,000 dilution. Antibodies raised against C-terminal AQP2 were obtained from Dr. Enno Klussmann (Berlin, Germany), Dr. Mark Knepper (Bethesda, MD, USA K5007 antibody), and Alomone Labs (Jerusalem, Israel). All other antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). A secondary, horseradish peroxidase-linked antirabbit antibody was used. Membranes were stripped using SDS buffer and reincubated with an antibody raised against GAPDH to ensure equal loading. Semiquantification of specific signals was performed using the Lumianalyst software (Roche, Basel, Switzerland).

**cAMP assay** Total cAMP levels in treated IMCD cells were determined using a cAMP specific ELISA (KGE#002, RnD Systems, Minneapolis, USA), following the instructions of the manufacturer. Absorbance at 450 nm was analyzed employing a microplate reader (Tecan Spectra, Crailsheim,

Germany), and cAMP concentrations were calculated using the WinFitting Software (Tecan).

**Calcineurin assay** Cells were treated as indicated with various CnI concentrations. Measurement of cellular calcineurin activity in cellular lysates was performed using a calcineurin activity assay kit (Enzo life sciences, Ann Arbor, USA) according to the manufacturer's instruction [10]. This assay is based on in vitro dephosphorylation of a peptide substrate (RII-peptide, sequence Asp–Leu–Asp–Val–Pro–Ile–Pro–Gly–Arg–Phe–Asp–Arg–Arg–Val–pSer–Val–Ala–Ala–Glu) by purified cellular calcineurin in the presence of calmodulin.

**Statistics** Data were analyzed with one-way ANOVA using GraphPad-Prism 5.0 (San Diego, CA, USA) with a Tukey's post-test. All values are presented as mean values  $\pm$  SEM.  $P \leq 0.05$  was regarded as significant. Asterisks indicate significant changes.

## Results

Cyclosporin A decreases AQP2 protein and mRNA expression in primary cultured IMCD cells

Primary cultured IMCD cells (cultured in medium of 600 mOsm/kg) were incubated with CsA (5  $\mu$ M) for 48 h. Proteins were extracted and immunoblotting for AQP2 expression was performed. CsA led to a decrease in AQP2 expression (twofold, data presented as  $\log_2$  of

densitometric values) compared to vehicle treatment. As a control, dbcAMP (500  $\mu$ M, 48 h) led to an increase in AQP2 protein abundance (greater than twofold, Fig. 1a). Time course experiments revealed that AQP2 was already significantly decreased after 24 h of CsA incubation (Fig. 1b).

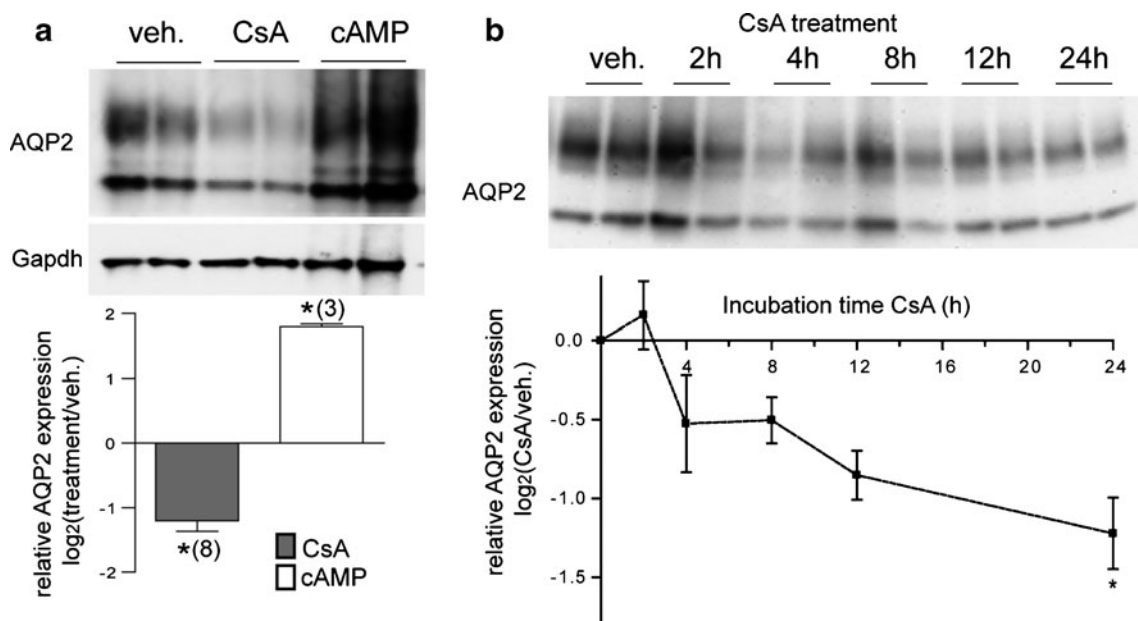
In order to investigate whether the changes in AQP2 protein were dependent on regulation of transcription, mRNA levels of AQP2 were determined at various time points. Incubation with CsA for 0.5, 24, and 48 h led to a significant decrease in AQP2 mRNA abundance (Fig. 2a). The effect after 0.5 h was concentration dependent (Fig. 2b). In order to investigate whether AQP2 mRNA was downregulated by mRNA degradation or alteration of transcription, we performed analysis of mRNA stability. For this, we pretreated cells with actinomycin-D (ActD, 50 ng/mL, 15 min), an irreversible inhibitor of RNA polymerase. There was no significant difference between cells that were treated with ActD alone and those treated with ActD and subsequent CsA (Fig. 2c).

Decrease of AQP2 by CsA is independent of calcineurin

Calcineurin-mediated transcriptional changes may be responsible for downregulation of AQP2 by CsA. This is

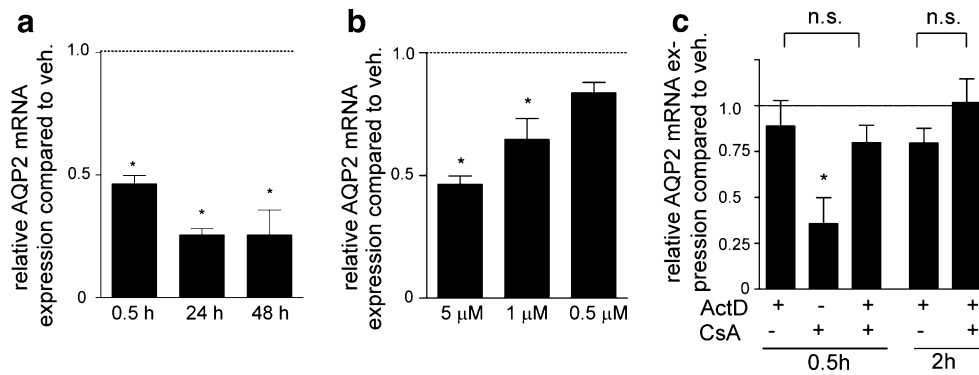
suggested by cell culture studies in immortalized mouse cortical collecting duct cells (mpkCCD) [25], whereas the calcineurin A alpha knockout mouse has no difference in renal AQP2 expression [15]. To clarify this difference at least for this particular rat cell culture model, we performed immunoblot analysis of cells treated with FK506 (tacrolimus), an alternative inhibitor with a higher affinity to calcineurin [12]. FK506 (0.1  $\mu$ M) had no effect on AQP2 protein expression (Fig. 3a). For confirmation, we performed independent experiments using an antibody that targets a region of the AQP2 protein, which is not believed to be modified by posttranslational modification such as ubiquitinylation or phosphorylation (K5007 antibody). Similar results were obtained (Fig. 3b). Even higher concentrations of FK506 (1  $\mu$ M) were not able to suppress AQP2 gene expression (Fig. 3c). To verify FK506-mediated calcineurin inhibition, an *in vitro* peptide dephosphorylation assay was performed [10]. Both CnIs (5  $\mu$ M CsA and 0.1  $\mu$ M FK506) reduced calcineurin activity by approximately 90% after 24 h in IMCD cells. This effect was time and concentration dependent (Fig. 3d). These findings suggest a calcineurin-independent effect for the observed decrease in AQP2 expression.

The CsA specific decrease in AQP2 mRNA and protein may be induced by a mechanism depending on cyclophilin



**Fig. 1** Immunoblot analysis of CsA-induced effects on AQP2 protein expression in cultured rat collecting duct cells. **a** IMCD cells were treated with CsA (5  $\mu$ M, 48 h) or vehicle (DMSO, 48 h) or dbcAMP (500  $\mu$ M, 48 h). In immunoblots, AQP2 was detected using an anti-AQP2 antibody, and the signal intensities were densitometrically analyzed. The number of replicates is given as a small number close to the column. **b** Time course of CsA effect on AQP2 expression in primary cultured collecting duct cells compared to vehicle treatment.

Cells were treated for the indicated time periods, and lysates were harvested and analyzed as described above ( $n=8$ ). All data are presented as logarithmic ratio compared to the vehicle control ( $\log_2$ [treatment/vehicle]). Negative values indicate decreases, whereas positive indicate increases in protein expression. Significant changes in the densitometric analysis are indicated by an asterisk ( $p<0.05$ , ANOVA)



**Fig. 2** Time and concentration dependent effect of CsA on transcription of AQP2. **a** IMCD cells were treated for different time points with CsA, and AQP2 mRNA expression levels were determined using qPCR as indicated in “Materials and methods.” The expression value of the respective control group was normalized to the value of 1. **b** Cells were incubated with different concentrations of CsA for 0.5 h,

and mRNA expression was analyzed using qPCR. **c** mRNA stability assay was carried out using actinomycin-D (ActD, 50 ng/mL) as indicated in “Materials and methods.” There was no significant CsA-dependent decrease in AQP2 mRNA after pretreatment with actinomycin D. In all graphs, significant changes compared to vehicle treatment are indicated by an asterisk ( $p < 0.05$ , ANOVA,  $n = 5$ )

A, the primary binding site of CsA in the cell [46]. Thus, we performed experiments with a commercially available cyclophilin A inhibitor in concentrations known to be sufficient for complete inhibition of cyclophilin A. Cyclophilin A inhibitor treatment, however, had no effect on either AQP2 mRNA (Fig. 3e) or protein expression (Fig. 3f, g). Therefore, we decided to further study signaling pathways, which are known to be disturbed by CsA and which have been suggested to alter AQP2 abundance in various cell culture models.

CsA does not interfere with TonEBP localization or TonEBP mediated gene transcription

It has been established that TonEBP regulates AQP2 expression [18]. However, there are conflicting results concerning the effect of CsA on TonEBP activity and localization [40, 41]. Very similar to these previous studies, we analyzed expression of characteristic target genes and the subcellular distribution of TonEBP to estimate its activation status by different osmolality compositions of the culture media (6 days cultivation, 300 and 600 mOsm/kg, medium differing in supplemental urea and NaCl). At 300 mOsm/kg, TonEBP protein was localized in the cytosol and was found more in the nucleus at 600 mOsm/kg (Fig. 4a). Total expression levels of TonEBP mRNA did not differ between 300 and 600 mOsm/kg culturing conditions. CsA treatment had no effect on TonEBP localization at 600 mOsm/kg (Fig. 4a).

Expression of genes known to be regulated by TonEBP, such as AR, UT-A, BGT-1, and AQP2, was decreased at 300 mOsm/kg (Fig. 4b). HSP-70 expression was not altered. Consistent with the immunofluorescence data, mRNA levels of AR, HSP70, UT-A, and BGT-1

showed no decrease after 0.5, 24 (data not shown), and 48 h of CsA incubation, unlike the downregulation of AQP2 (Fig. 4c).

Studies elevating medium osmolality for shorter periods of time (24 h, elevation of 600–900 mOsm/kg) have been performed in the absence and presence of calcineurin inhibitor treatment. Here, we did not see any difference in TonEBP localization as well as TonEBP mediated transcription [40].

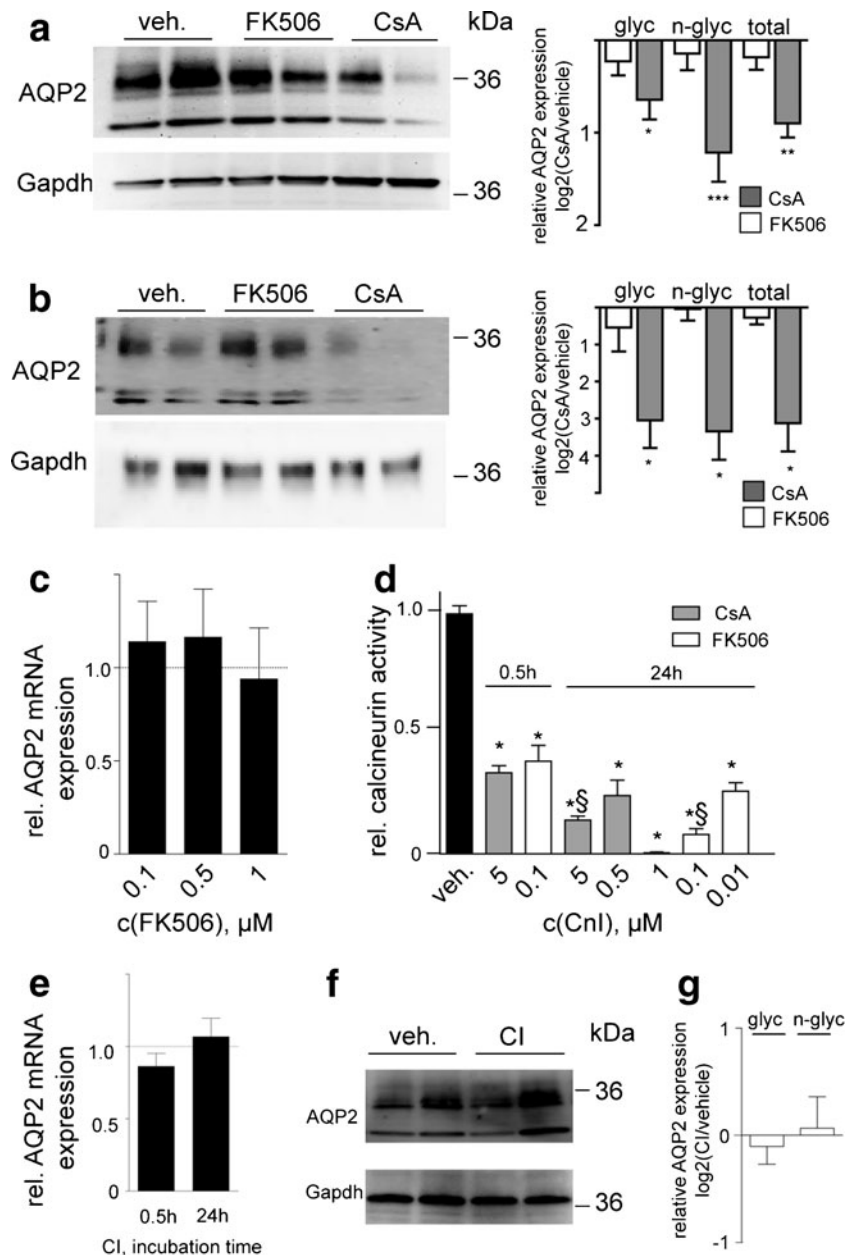
Gsk3b and  $\beta$ -catenin abundance are affected by CsA

Recently, the kinase Gsk3b has been suggested to be involved in regulation of AQP2 abundance [36]. Immunoblot analysis of protein expression showed that there was an increased expression of the kinase Gsk3b in response to both CsA and FK506 (24 h treatment; Fig. 5a). However, phosphorylation of this kinase at a regulatory, inhibitory site (S9) was not altered. Protein expression of  $\beta$ -catenin, a downstream effector of Gsk3b activity [49], was decreased by CsA, but not by FK506 (Fig. 5b). We also observed increases in  $\beta$ -catenin phosphorylation at S675 in response to CsA and FK506, but not at S552 or T41/45 after 24 h (Fig. 5b).

Activity of MAP-kinases ERK and JNK is increased by CsA and FK506

Recently, it has been established that the mitogen-activated kinase (MAP-kinase) p38-kinase (p38) plays a significant role in regulation of AQP2 protein degradation [32]. Immunoblotting was performed in order to investigate the protein abundance and activity of the three classical MAP-kinases extracellular signal regulated kinase (ERK), Jun-



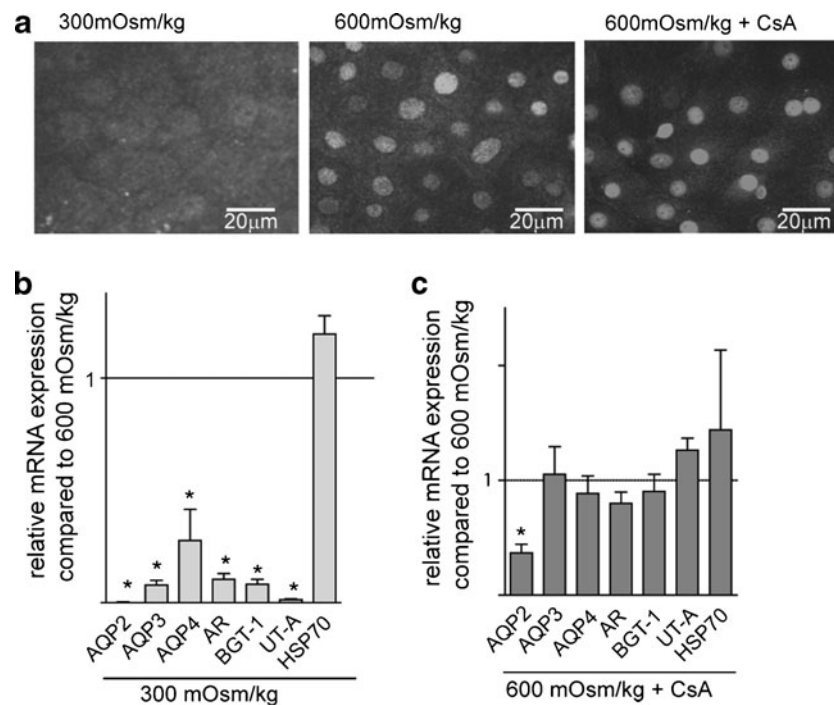


**Fig. 3** Calcineurin dependence of observed effects on AQP2. **a** AQP2 protein expression was analyzed in the presence of the calcineurin inhibitors (CnIs) FK506 (0.1  $\mu$ M, 24 h) and CsA (5  $\mu$ M, 24 h;  $n=6$ ). Data obtained from densitometric analysis are presented as logarithmic ratio ( $\log_2[\text{CnI}/\text{veh.}]$ ). Quantification data are provided for both glycosylated (glyc) and non-glycosylated (n-glyc) AQP2 protein. **b** AQP2 protein expression was analyzed in independent experiments using a different antibody (K5007;  $n=6$ ). In all immunoblot experiments, *asterisks* indicate statistical significance compared to vehicle treatment ( $p<0.05$ , ANOVA). **c** mRNA expression of AQP2 in response to various concentrations (c) of FK506 (24 h treatment). **d** Cells were incubated with calcineurin inhibitors with the indicated

concentrations and for the indicated time periods. An in vitro peptide dephosphorylation assay was used to quantify cellular calcineurin activity (see “Materials and methods” for details). *Asterisks* indicate significance compared to vehicle control. *Section sign* denotes a statistical significance compared to the 0.5 h data of the respective drug ( $p<0.05$ , ANOVA,  $n=5$ ). **e** Cells were incubated with cyclophilin A inhibitor for the indicated time periods (CI; 0.5  $\mu$ M). The mRNA expression level of AQP2 was analyzed ( $n=3$ ). **f** AQP2 protein expression was analyzed in the presence of CI (24 h, 0.5  $\mu$ M). **g** Quantification of densitometric signals of the immunoblot analysis (Fig. 7f) ( $n=6$ )

Kinase (JNK), and p38. Total protein levels did not change in response to CsA (Fig. 6). However, there was an increase in phosphorylation at activating phosphorylation sites of

ERK and JNK, but not of p38. This was observed to a similar extent with FK506 treatment, pointing at a calcineurin-dependent mechanism.



**Fig. 4** CsA does not affect TonEBP localization or TonEBP-dependent transcription. **a** IMCD cells were cultivated in media of 300 mOsm/kg (lower osmolality) and at 600 mOsm/kg in the presence of CsA (5  $\mu$ M, 24 h). Localization of TonEBP protein was detected using a specific polyclonal antibody. **b** Expression of TonEBP-dependent genes and AQP3 and AQP4 in cells cultured at 300 mOsm/kg compared to 600 mOsm/kg. mRNA expression levels were determined using quantitative PCR and compared to cells cultured at 600 mOsm/kg (*AQP2* aquaporin-2, *UT-A* urea channel

*Slc14a2*, *BGT* betaine  $\gamma$ -amino-butyrat transporter, *AR* aldose reductase, *HSP70* heat shock protein 70). **c** Expression of TonEBP-dependent genes in cells treated with CsA (osmolality=600 mOsm/kg). mRNA expression levels were determined using quantitative PCR and compared to cells cultured at 600 mOsm/kg. All results were obtained with c(CsA)=5  $\mu$ M for 48 h. Merely identical results were obtained for 0.5 and 24 h.  $n \geq 5$  replicates, asterisks mark significance ( $p < 0.05$ , in one-way ANOVA)

#### CsA decreases CREB phosphorylation independent of cAMP generation

The PKA/cAMP pathway is a regulator of long-term AQP2 expression. Especially phosphorylation at S133 of CREB has been suggested as a key regulator of AQP2 expression, as described above. CsA, but not FK506, induced a reduction in phosphorylation at S133 (Fig. 7a, b). Immunoblot analysis of total CREB protein showed no difference in response to either immunosuppressant. However, there was no effect on intracellular cAMP levels by CsA or FK506 (0.5 h, 24 h, Fig. 7c). Forskolin (10  $\mu$ M, 0.5 h) increased cellular cAMP levels, which were not altered by additional treatment with either CsA or FK506.

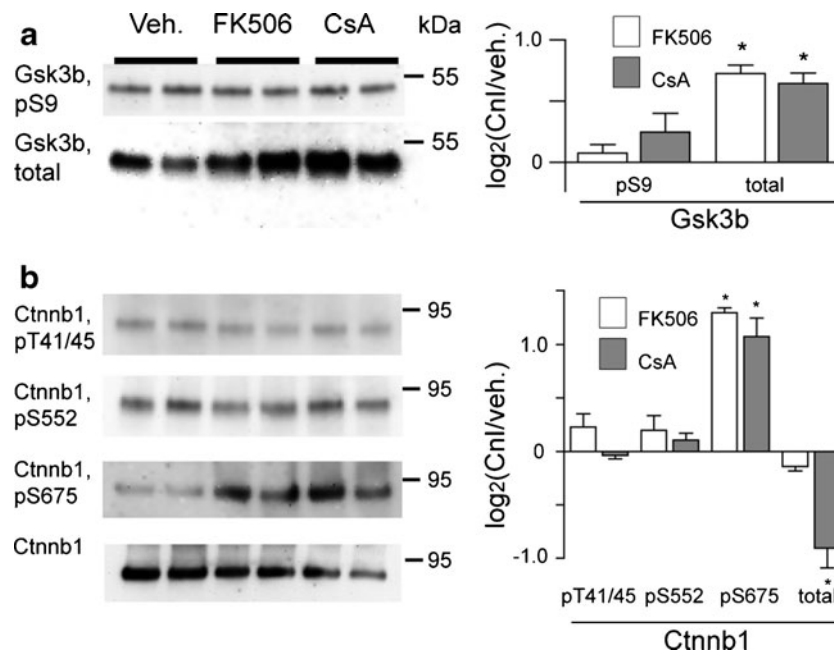
To investigate the mechanism of this event, we performed analysis of p-CREB abundance in cells treated with Forskolin or with Forskolin and simultaneous CsA stimulation. The results showed that Forskolin treatment (10  $\mu$ M, 1 h) significantly induced p-CREB phosphorylation at S133 (Fig. 7d, e). However, co-treatment with CsA had no additional effect. Simultaneously, we performed analysis of mRNA levels induced by long-term AVP treatment of the cells. Treatment with AVP ( $10^{-8}$  or

$10^{-9}$  M, 48 h) significantly induced AQP2 mRNA levels. Again, this effect was not altered by additional treatment with CsA (Fig. 7f).

#### Discussion

Investigations of the transcriptional regulation of AQP2 have been performed in a variety of cell culture models [21]. Cultured rat IMCD cells have been used for investigation of AQP2 mRNA and protein regulation [30], and are grown at an elevated medium osmolality. Endogenous signaling pathways leading to AVP-dependent AQP2 translocation are conserved in these cells [23]. In addition, IMCD cells endogenously express *Slc14a2* urea channels, AQP3, and AQP4. In our view, these features qualify them as a cell model for the analysis of collecting duct effects of CsA on AQP2 protein and gene expression.

Our results demonstrate that AQP2 abundance in IMCD cells is significantly affected by CsA. Data obtained from CsA-treated Brattleboro rats showed that decreased AQP2 abundance is preceded by decreased expression of NKCC2 in the thick ascending limb of Henle, leading to diminished



**Fig. 5** Analysis of the Gsk3b and  $\beta$ -catenin on protein level. **a** Cells were treated with CsA (5  $\mu$ M) or FK506 (0.1  $\mu$ M) for 24 h. Immunoblot analysis with specific antibodies was performed to determine protein expression of Gsk3b and phosphorylation at S9, an inhibitory site of the kinase. **b** Immunoblot analysis of protein expression and phosphorylation status of  $\beta$ -catenin (Ctnnb1) in

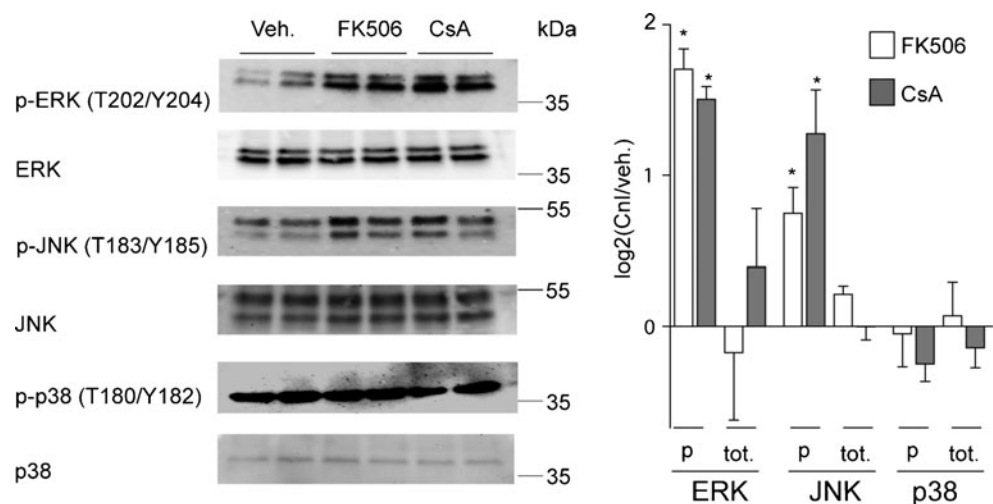
response to CnI treatment (CsA, 24 h, 5  $\mu$ M; FK506, 24 h, 0.1  $\mu$ M). Phosphorylation sites analyzed using phosphospecific antibodies included T41/45, S552, and S675. All specific bands were densitometrically analyzed, and quantification data are presented as logarithmic ratio (CnI treatment over vehicle treatment) ( $n \geq 5$ ,  $p < 0.05$  was considered significant)

interstitial tonicity and subsequent decrease in TonEBP activity [27]. Here, we demonstrate a direct effect of CsA on AQP2 mRNA and protein expression in IMCD cells, which is independent of systemic interference. The time course of AQP2 downregulation is rapid on mRNA level and—as expected for a process involving transcriptional regulation—occurs later on the protein level. CsA does not acutely affect endogenously expressed UT-A, AQP3, or AQP4. These proteins have been shown to be decreased by long-term CsA treatment (up to 3 weeks) in vivo [26]. In

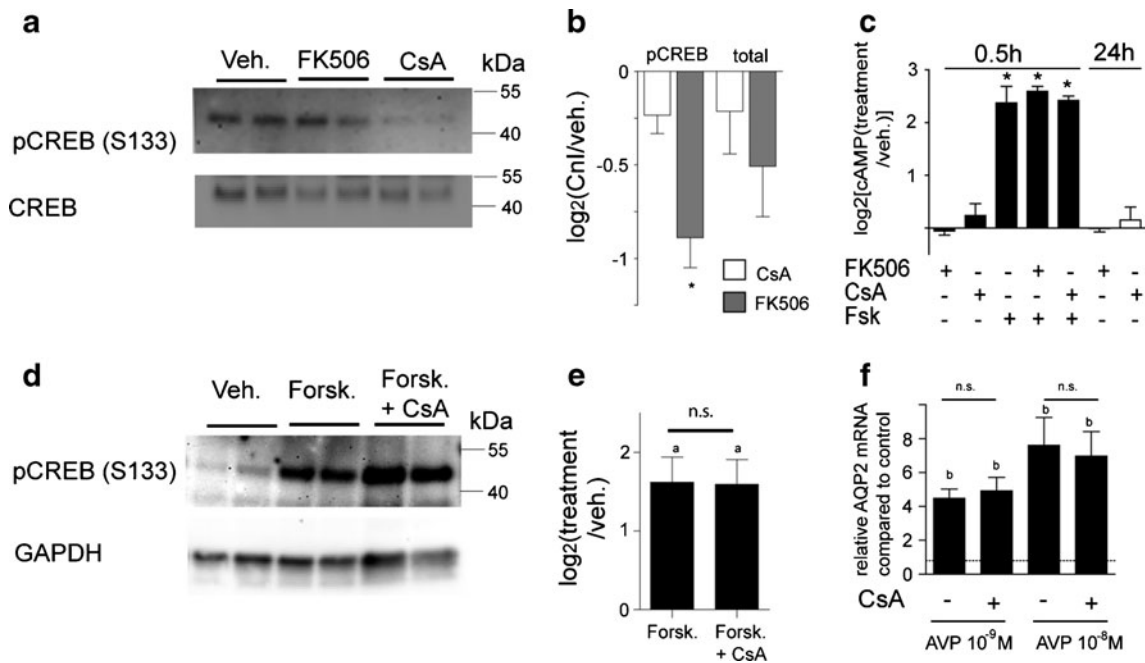
conclusion, there are two ways how CsA may influence AQP2 expression in the collecting duct in vivo. One involves AQP2 decrease as a secondary response to the decreased papillary osmolality gradient; the second may be a direct effect on the collecting duct cells, as demonstrated here. We believe that further in vivo studies, such as additional blocking of NKCC2 with diuretics during CsA treatment, will be necessary to address this question.

The calcineurin inhibitor FK506 in various concentrations did not decrease AQP2 expression. FK506 in a slightly lower

**Fig. 6** Analysis of expression and phosphorylation of MAP kinases on protein level. Immunoblot analysis was performed for protein expression and phosphorylation levels at activating sites for the three MAP-kinases ERK, JNK, and p38. Densitometric values are presented as logarithmic ratio (CnI treatment over veh. treatment). “p” indicates the phosphorylated form of the respective MAP kinase whereas “tot” indicates the total abundance of the respective protein ( $n \geq 5$ , treatment: CsA, 24 h, 5  $\mu$ M; FK506, 24 h, 0.1  $\mu$ M;  $p < 0.05$  considered significant)







**Fig. 7** **a** Immunoblot analysis of protein expression and phosphorylation of CREB (S133) in cells treated with CsA or FK506 ( $n \geq 6$ , treatment: CsA, 24 h, 5  $\mu\text{M}$ ; FK506, 24 h, 0.1  $\mu\text{M}$ ;  $p < 0.05$  was considered significant). Specific antibodies were used. **b** Quantification of densitometric signals of the immunoblot analysis (**a**). **c** cAMP levels were measured in cells treated with FK506 and CsA. Furthermore, cAMP generated by treatment with forskolin (10  $\mu\text{M}$ , 0.5 h) was analyzed in the presence of both CnIs ( $n \geq 5$ , treatment: CsA, 5  $\mu\text{M}$ ; FK506, 0.1  $\mu\text{M}$ ;  $p < 0.05$  was considered significant). In

the forskolin experiments, pretreatment with CsA or FK506 was performed (15 min). **d** Immunoblot analysis of phosphorylation of CREB in cells treated with forskolin (10  $\mu\text{M}$ , 1 h) alone or with CsA (5  $\mu\text{M}$ , 1 h). **e** Quantification of densitometric signals of the immunoblot analysis was performed (**d**) ( $n = 6$ ,  $p > 0.05$ ). The letter *a* denotes a statistical significance compared to vehicle control. **f** mRNA expression of AQP2 induced by AVP in the absence or presence of CsA ( $n = 6$ ,  $p > 0.05$ ). The letter *b* denotes a statistical significance compared to vehicle control

concentration than CsA inhibited calcineurin to a similar extent compared to CsA (Fig. 3c). Consequently, the observed effects seem to be independent of calcineurin. Reasons for this may be found in the drug-specific intracellular primary binding sites (cyclophilin vs. FK506 binding protein 12), with potential different downstream effects [46]; however, this idea appears to be less likely with regard to the missing effect of a cyclophilin inhibitor on AQP2 mRNA or protein expression (Fig. 3e–g). An alternative explanation may be the different calcineurin inhibitor specificities, a common finding in several cell models [1, 48]. In this study, calcineurin inhibition alone was not sufficient to reduce AQP2 expression, in contrast to a report by Li et al. [25]. The latter study carefully scrutinizes the AQP2 promoter and mutates certain NFAT binding sites necessary for AQP2 transcription in mpkCCD cells. Still, there are marked differences in the experimental setup compared to the data reported here, which may not allow a direct comparison of the results. These include the use of not only different cell lines (immortalized mouse cortical collecting duct in [25]) and culturing conditions (cultured at approximately 300 mOsm/kg with dDAVP added [4]) but also different treatment schemes [25]. Further experiments will be necessary to decipher the differences between the two

studies and cell culture models. One of these may be the use of calcium-releasing and NFATc-activating agents (PMA and/or Ionomycin), which were used in the study above. However, in our particular cell model, even short incubation times  $< 4$  h with comparable drug concentrations induced massive morphological changes and detachment in the cultured cells, probably indicating apoptosis.

Data obtained from in vivo studies appear to support the findings reported here: Long-term treatment of rats with FK506 did not have any effect on AQP2 protein abundance and urinary concentration, but changed expression patterns of transporters involved in acid–base balance [31]. Furthermore, mice lacking calcineurin A alpha, which is the catalytic subunit of the enzyme, do not show a decreased abundance but a decreased apical localization of AQP2 [15].

One study suggested that TonEBP nuclear localization was significantly inhibited by CsA in MDCK cells [41]. Other studies do not report an inhibition of TonEBP by CsA [2, 29]. In the cell model analyzed here, there were no differences in TonEBP localization between CsA-treated and control cells. This absence of an effect on localization correlated with gene expression of TonEBP-dependent genes. Still, there are other mechanisms of TonEBP

regulation such as phosphorylation [13], and, additionally, the expression of the analyzed genes may be regulated by other factors. From a functional view, however, we suggest that TonEBP is not the main factor responsible for CsA mediated decrease in AQP2 expression in this cell culture model.

During CsA treatment, Gsk3b phosphorylation was not changed, whereas its total protein amount was slightly induced. Gsk3b phosphorylation was analyzed for two specific reasons: First, a mouse knockout model of Gsk3b showed a significantly reduced AQP2 protein expression [36]. Second, CsA was shown to specifically inhibit Gsk3b in other tissues by blocking its dephosphorylation at the inhibiting site S9 [24]. A rather surprising finding was a CsA-specific decreased expression of  $\beta$ -catenin, which was analyzed as a downstream effector of Gsk3b [49], but whose expression of course can also be modified by other mechanisms. Still, there is little known about the role of  $\beta$ -catenin in the collecting duct with regard to regulation of gene expression and cell-specific differentiation [3]. The AQP2 promoter shows several binding sites for TCF, the transcription factor depending on direct  $\beta$ -catenin interaction [34]. Increased abundance of both Gsk3b and  $\beta$ -catenin is observed in lithium-induced diabetes insipidus in rats, a condition characterized by AQP2 downregulation [34].

The family of MAP kinases has been recently linked to regulation of protein expression of AQP2 [32, 38]. Inhibition of p38 was shown to be involved in degradation of AQP2 via decreased phosphorylation of AQP2 (S261) in primary cultured IMCD cells [32]. We observed increased phosphorylation at activating sites of ERK and JNK, but not p38, and no change in total protein abundance. Thus, calcineurin inhibitor treatment significantly induced ERK and JNK activation, a finding shown in multiple cell culture and animal models (for examples, see [11, 39, 45]). Phosphorylation of both kinases at the described sites is increased in rats with lithium-induced diabetes insipidus [34]. However, since both CnIs had a similar effect on the activation of these kinases, we believe that alteration of these signaling pathways does not sufficiently explain the CsA-specific decrease in AQP2 mRNA and protein abundance.

Finally we demonstrated that phosphorylation of CREB is significantly decreased in response to CsA, but not FK506 under basal conditions (Fig. 7). This finding is not mediated by inhibition of adenylate cyclase activity, since cellular cAMP levels were not altered by CsA. In addition, forskolin-stimulated cAMP levels were not differentially altered. Knockout mice lacking Gsk3b exhibited decreased cAMP generation rates [36]; however, in this cell culture model, cAMP generation as well as Gsk3b activity as indicated by phosphorylation (Fig. 6a) was not changed. The cAMP levels and the CREB phosphorylation were increased by

short-term Forskolin treatment. However, under these cAMP-stimulating conditions, CREB phosphorylation was not affected by CsA, and the repressive effect of CsA is most likely bypassed or overridden (Fig. 7c–f). Thus, the mechanism responsible for decreased phosphorylation of CREB under basal conditions remains to be identified. A decreased transcriptional activity of CREB in response to CsA has previously been shown in cultured fibroblasts [35] and might partially account for the decreased AQP2 abundance in this experimental setting. Further signaling events may involve not only inhibition of another basophilic kinase but also activation of a phosphatase.

Apart from a transcriptional regulation by CREB, the data obtained here may also hint at a mechanism involving mRNA stability. ActD treatment abolished the CsA-induced decrease in AQP2 mRNA; however, ActD itself had no effect on AQP2 mRNA as previously shown in the same cell culture model [32] (Fig. 2c). A mechanism for this could involve CsA-altered gene expression of factors, which are directly involved in regulation of AQP2 mRNA stability. An example may be the CsA-induced expression of a destabilizing microRNA (this expression would also be blocked by ActD).

We would like to stress that effects of CsA on AQP2 may be completely different under pathophysiological in vivo conditions such as renal transplantation and graft rejection. Studies in this field show that AQP2 abundance—along with several other key transporters in the distal nephron—is decreased in animal models of acute graft rejection [44]. These effects are consistent with tubular damage induced by the stimulated immune system and are known to be ameliorated or even reversed by calcineurin inhibitor treatment [8, 9].

To conclude, we show that several mechanisms coincide in this cell culture model of decreased AQP2 expression upon CnI treatment: Calcineurin inhibition alone and cyclophilin A inhibition are not sufficient. However, additional loss of phosphorylation of CREB is observed. Further roles may be played by altered MAP kinase and  $\beta$ -catenin signaling. We suggest that these complex events are part of a comprehensive signaling network, which results in decreased AQP2 transcription. In addition, this study supports the view that effects of calcineurin inhibitors in physiological systems need to be carefully reviewed with regard to complex side effects, especially in long-term administration.

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