Osmolalities and solute composition are strong regulators of AQP2 expression in renal principal cells

R. Storm, E. Klussmann, A. Geelhaar, W. Rosenthal, and K. Maric. Osmolality and solute composition are strong regulators of AQP2 expression in renal principal cells. Am J Physiol Renal Physiol 284: F189–F198, 2003. First published August 13, 2002; 10.1152/ajprenal.00245.2002.—The water permeability of the renal collecting duct is regulated by the insertion of aquaporin-2 (AQP2) into the apical plasma membrane of epithelial (principal) cells. Using primary cultured epithelial cells from the inner medulla of rat kidney (IMCD cells), we show that osmolality and solute composition are potent regulators of AQP2 mRNA and protein synthesis, as well as the classical cAMP-dependent pathway, but do not affect the arginine vasopressin-induced AQP2 shuttle. In the presence of the cAMP analog dibutyryl cAMP (DBcAMP, 500 μM), NaCl and sorbitol, but not urea, evoked a robust increase of AQP2 expression in IMCD cells, with NaCl being far more potent than sorbitol. cAMP-responsive element-binding protein phosphorylation increased with DBcAMP concentrations but was not altered by changes in osmolality. In the rat and human AQP2 promoter, we identified a putative tonicity-responsive element. We conclude that, in addition to the arginine vasopressin/cAMP-signaling cascade, a further pathway activated by elevated effective osmolality (tonicity) is crucial for the expression of AQP2 in IMCD cells, and we suggest that the effect is mediated via the toxicity-responsive element.

osmolality; aquaporin-2; kidney; gene regulation; toxicity responsive enhancer

THE WATER CHANNEL aquaporin-2 (AQP2), expressed in epithelial (principal) cells of renal collecting ducts, is required for the vasopressin-dependent concentration of urine (7). AQP2 abundance increases from the kidney cortex to the inner region of the kidney medulla (29), as does osmolality. The water permeability of the inner medullary collecting duct (IMCD) is rapidly regulated (within minutes) by the antidiuretic hormone arginine vasopressin (AVP), which binds to heptahelical vasopressin V2 receptors (V2R), located mainly in the basolateral plasma membrane of principal cells. Activation of the V2R causes stimulation of adenyl cyclase via the G protein Gs, leading to elevation of cAMP. The subsequent activation of protein kinase A (PKA) initiates the translocation of AQP2-bearing vesicles from the cytosol to the plasma membrane, in which AQP2 is inserted by an exocytosis-like process (short-term regulation) (D. Lorenz, A. Krylov, V. Hagen, J. Zipper, W. Rosenthal, P. Pohl, and K. Maric, unpublished observations; 30). In addition, the signaling cascade described above governs the expression of AQP2 (long-term regulation) by PKA-mediated phosphorylation of the transcription factor cAMP-responsive element (CRE)-binding protein (CREB) (13, 16). Given the fact that AQP2 biosynthesis is usually shut off shortly after IMCD cells are established in primary culture (10), most studies on AQP2 long-term regulation have been performed using animal models (29, 37). We recently established primary cultured IMCD cells as a model system (17, 18, 21, 22). These cells exhibit sustained AQP2 expression when grown in hypertonic medium (600 mosmol/l) in the presence of 500 μM dibutyryl cAMP (DBcAMP). Thus this cell model allows the investigation of isolated aspects of kidney function: the short- and/or long-term regulation of AVP-mediated water reabsorption in IMCD cells endogenously expressing AQP2.

Several studies have provided evidence for a V2R/cAMP-independent regulation of AQP2 expression. In rats the downregulation of AQP2 expression by V2R antagonist treatment was reversed by subjecting the animals to thirst (24). In addition, senescent (30-mo-old) rats exhibited a significantly decreased AQP2 expression and papillary osmolality compared with younger (10-mo-old) animals, while papillary cAMP remained unaffected (32).

Taking advantage of our cell culture system, we investigated the role of extracellular osmolality and solute composition in the long-term regulation of AQP2. We examined whether these effects are mediated by the transcription factor CREB or whether other, possibly cAMP-independent, pathways are involved.

MATERIALS AND METHODS

Cell culture. IMCD primary cultures were prepared as described previously (21). Renal inner medullae of Wistar rats (2–3 mo old; both sexes) were the source of primary
cultured IMCD cells. All media were based on Dulbecco’s modified Eagle’s medium (containing 110 mmol/l NaCl and 300 mosmol/l) routinely supplemented with 500 μM DBcAMP, 4.5% glucose, and 1% serum substitute Ultroser (Life Technologies, Karlsruhe, Germany) instead of 10% FCS. This basic medium, termed 300N, contained 500 μM DBcAMP (if not indicated otherwise) for the maintenance of AQP2 expression. Media of elevated osmolalities (600 and 900 mosmol/kgH2O after equimolar addition of NaCl and urea) were termed 600N and 900N, respectively. 

At 1 day after seeding, this routine culture cells were seeded at a density of 300N and the osmolality of the medium are indicated. The process of elevating medium osmolality, the amounts of NaCl added to NaCl and/or urea were termed 600U 0/300, 600NaCl/U 0/300, 600NaCl/U 0/600, 600NaCl/U 0/900, 600NaCl/U 0/1200, 600NaCl/U 0/1800, 600NaCl/U 0/2400, 600NaCl/U 300/900, 600NaCl/U 600/1200, 600NaCl/U 900/1800, 600NaCl/U 1200/2400, 600NaCl/U 1800/3000, 600NaCl/U 2400/3600, 600NaCl/U 3000/4500, 600NaCl/U 3600/4800, 600NaCl/U 4500/6000, 600NaCl/U 6000/9000, and 600NaCl/U 9000/12000. When only NaCl was used to elevate medium osmolality, the amounts of NaCl added to 300N and the osmolality of the medium are indicated. The cells were seeded at a density of ~10^5/cm² in 600N to select for IMCD cells. At 1 day after seeding, this routine culture medium was replaced by the desired culture medium. Cultures were split 1:10 and seeded on culture dishes in 300N or 600N.

WT-10 cells [Madin-Darby canine kidney (MDCK) cells that were stably transfected with AQP2 construct, driven by cytomegalovirus (CMV) promoter (6)] were cultured without DBcAMP in 300N with 5% FCS and passed every 4–5 days. At 6 days before membrane preparation, WT-10 cells were split 1:10 and seeded on culture dishes in 300N or 600N.

**Protein preparations and Western blot analysis.** All procedures were performed at 4°C. Cells cultured in 60-mm-diameter culture dishes were rinsed twice with ice-cold phosphate-buffered saline (PBS), scraped off the culture dishes, and homogenized in 1.5 ml of ice-cold PBS with a glass-Teflon homogenizer (10 strokes, 750 rpm). For membrane preparations, the homogenates were centrifuged at 800 g for 5 min to remove nuclei and debris; the supernatant was centrifuged at 200,000 g for 1 h, and the resulting pellet (membrane fraction) was resuspended in ice-cold PBS. Samples (15 μg) were subjected to SDS-PAGE (12% acrylamide in separating gels). Cells grown on 24-well plates (2 cm²/well) were rinsed twice with ice-cold PBS and lysed in modified Laemmli buffer [40 μl/well; 4% (wt/vol), instead of 2%, SDS] by incubation at room temperature. The solubilized material was then sonicated (Sonopuls UW 2070, Bandelin Electronic, Berlin, Germany) for 5 s at 40% power to shear chromosomal DNA and subjected to SDS-PAGE (total homogenate from 1 cm² of confluent cell monolayer was loaded per lane onto 12% SDS-polyacrylamide gels). Size-separated proteins were transferred to nitrocellulose filters (Optitran, Schleicher & Schuell, Dassel, Germany). Protein transfer and equal loading were verified by Ponceau red staining (not shown).

Filters were blocked for 1 h in blocking buffer [Tris-buffered saline + Tween (TBST) containing 5% (wt/vol) low-fat dry milk], incubated with the desired primary antibodies, and subsequently washed in TBST (5 times for 5 min each). As primary antibodies, rabbit polyclonal antisera against AQP2 (20), which, in addition to AQP2, also detects the histone H2A1 (14), was used at a dilution of 1:1,500 in blocking buffer for 1 h at room temperature, and rabbit polyclonal antisera against phosphorylated CREB diluted 1:500 in TBST containing 5% BSA (New England Biolabs, Frankfurt am Main, Germany) was used overnight at 4°C. As secondary antibodies, peroxidase-coupled goat anti-rabbit F(ab)² fragments (Dianova, Hamburg, Germany) were used at a dilution of 1:2,000 in blocking buffer, 1 h at room temperature). Blots were washed five times for 8 min each in TBST and then incubated for 5 min in Lumi-Light solution (Roche Diagnostics, Mannheim, Germany). Chemiluminescence was visualized and band densities were quantified using a Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany). Results (means ± SE) are expressed as percentage of control signal intensity. Statistical evaluation was carried out by ANOVA, using GraphPad Prism software (GraphPad Software, San Diego, CA). GraphPad Prism software was also used to perform nonlinear regression analysis on the data obtained in time-course experiments.

**Northern blot analysis.** Total RNA was prepared from IMCD cells grown in 60-mm culture dishes using TRizol reagent (GIBCO-BRL, Karlsruhe, Germany). Northern blot analysis with 15 μg of total RNA was essentially performed as previously described (12). Hybridization was carried out using 32P-labeled rat AQP2 cDNA (labeled according to the manufacturer’s instructions; Megaprime DNA Labeling Kit, Amersham Pharmacia Biotech, Freiburg, Germany). For standardization of AQP2 mRNA signals, blots were rehybridized with a 32P-labeled (44-bp) cDNA fragment specific for rat 18S rRNA (5’ ACAGAATGGCCCGCGGTCCCTTTAAATCAGGGCTCTAGT RCCG 3’; terminal transferase end-labeling kit from the manufacturer’s instructions; Boehringer, Mannheim, Germany). Signals were visualized using a STORM 830 PhosphorImager and quantified using Image Quant 5.1 software (Amersham Pharmacia Biotech). For semiquantitative analysis, band densities for AQP2 mRNA were standardized to the corresponding 18S rRNA signals. Standardized signals (means ± SE) are expressed as percentage of control signal intensity (IMCD cells cultivated in 600N) for each set of experiments. Statistical evaluation was carried out by ANOVA using GraphPad Prism software.

**Sequence searches and alignments.** DNA sequences for the human and rat AQP2 promoters were obtained by using the National Center for Biotechnology Information nucleotide search. Alignments were performed using GeneTool software (version 1.0, BioTools).

**Immunofluorescence studies.** Immunofluorescence experiments for the detection of AQP2 in IMCD cells was essentially performed as previously described (21). AQP2 was detected by fluorescence microscopy (Leica DMLB microscope with Sensicam 12 Bitled charge-coupled device camera, Bensheim, Germany) using rabbit polyclonal anti-AQP2 antibodies and Cy3-conjugated anti-rabbit secondary antibodies (Dianova).

**RESULTS**

**AQP2 protein expression is stimulated by DBcAMP.**

Figure 1A shows a representative AQP2 immunoblot analysis of total homogenates derived from IMCD cells cultured for 6 days in 24-well plates. The analysis of total homogenates derived from IMCD cells grown in 24-well plates was favored over membrane preparations, because the latter require 10 times more cells and, thus, animals. Another advantage to using total homogenates is that histone H2A1, a nuclear protein detected therein by the anti-AQP2 antisemur (14), can be used as a marker to compare cell numbers and amounts of protein loaded per lane. Cells were grown in 600N supplemented with 5 μM, 50 μM, 200 μM, 500 μM (control), 2 mM, and 5 mM DBcAMP, a membrane-permeable cAMP analog. The results are summarized in Fig. 1B. DBcAMP stimulated AQP2 expression in a dose-dependent manner; its maximum effect was at 200 and 500 μM. Higher concentrations led to a de-
crease in AQP2 expression, while cell morphology, assessed by transmission microscopy, remained unchanged (not shown). These findings suggest that DBcAMP can affect AQP2 expression bidirectionally in IMCD cells grown in 600N. DBcAMP at 500 μM, also used in previous studies (17, 18, 21, 22), yielded maximal AQP2 expression in IMCD cells grown in 600N and was therefore routinely employed in experiments designed to identify pathways other than the cAMP-dependent pathway.

AQP2 protein expression is dependent on elevated osmolality and altered by solute composition. Increased AQP2 expression in rats is induced by water deprivation, despite chronic administration of V2R antagonists (24, 30), possibly as a consequence of increased medullary osmolality derived from urea and NaCl. We therefore tested whether cAMP-stimulated AQP2 expression is altered by increased medium osmolality derived from NaCl, urea, or sorbitol concentration. Media supplemented solely with sorbitol were used to distinguish the effects of elevated osmolality from the effects of NaCl and urea. Figure 2A shows a representative AQP2 immunoblot analysis of total homogenates from IMCD cells cultured in 24-well plates for 6 days in 300N, 600S, 600N (control), 900S, and 900N. The results are summarized in Fig. 2B. For IMCD cells cultivated in 300N, only weak AQP2 signals were detectable. An elevation of medium osmolality to 600 mosmol/kgH2O with the addition of sorbitol (600S) clearly increased AQP2 protein expression. Maximal AQP2 expression, however, was achieved when equimolar NaCl and urea were used to elevate medium osmolality to 600 mosmol/kgH2O (as in 600N). A further elevation of the osmolality to 900 mosmol/kgH2O by sorbitol (900S) drastically reduced AQP2 expression, whereas AQP2 expression was only slightly reduced when NaCl and urea were employed (900N). These results indicate that osmolality and a specific action of NaCl and urea determine the level of AQP2 protein expression in IMCD cells.

Osmolality changes do not influence AQP2 protein expression in WT-10 cells stably transfected with AQP2. MDCK cells stably expressing human AQP2 under the control of a CMV promoter (WT-10 cells) (6) and IMCD cells were exposed to 300N and 600N. Figure 3A shows a representative immunoblot of total membrane preparations derived from WT-10 and IMCD cells. The results are summarized in Fig. 3B. The data indicate that the CMV promoter-governed expression of AQP2

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**Fig. 1.** Effect of dibutyryl cAMP (DBcAMP) on aquaporin-2 (AQP2) protein levels in inner medullary collecting duct (IMCD) cells. IMCD cells were cultured for 6 days in 24-well plates containing medium in which osmolality was elevated to 600 mosmol/kgH2O with NaCl and urea (600N) supplemented with 5 μM, 50 μM, 200 μM, 500 μM (control), 2 mM, and 5 mM DBcAMP. A: AQP2 [glycosylated (g) and nonglycosylated (ng)] and histone H2A1 detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-polyacrylamide gel, total homogenate (protein) from 1-cm² confluent cell monolayer was loaded. B: densitometric analysis of AQP2 protein levels (glycosylated and nonglycosylated). Values are means ± SE (n = 4). *P < 0.05 vs. control.

**Fig. 2.** Dependence of AQP2 expression on elevated osmolality and solute composition. IMCD cells were cultured for 6 days in 24-well plates in the presence of 500 μM DBcAMP in basal medium (300N), medium in which osmolality was elevated to 600 mosmol/kgH2O with sorbitol (600S), 600N (control), medium in which osmolality was elevated to 900 mosmol/kgH2O with sorbitol (900S), and medium in which osmolality was elevated to 900 mosmol/kgH2O with NaCl and urea (900N). A: AQP2 and histone H2A1 detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-polyacrylamide gel, total homogenate (protein) from 1-cm² confluent cell monolayer was loaded. B: densitometric analysis of AQP2 protein levels (glycosylated and nonglycosylated). Values are means ± SE (n = 8). *P < 0.05 vs. control.
in WT-10 cells is not affected by changes in osmolality. In contrast, AQP2 expression in IMCD cells increased with increasing osmolality (Fig. 2). These findings suggest that the AQP2 gene's natural promoter is required for an effect of osmolality on AQP2 expression.

**AQP2 mRNA expression is regulated by osmolality and solute composition.** To investigate whether the above-described changes in AQP2 protein levels result from corresponding changes in AQP2 mRNA levels, Northern blot experiments were performed. We used RNA preparations from IMCD cells cultured in 300N, 600S, 600N (control), or 900N (Fig. 4A) and 600N (control) or 900S (Fig. 4B). Figure 4C summarizes AQP2 mRNA signals. The changes in the mRNA level correspond closely to the changes in the protein level. Thus osmolality and solute composition regulate AQP2 expression, most likely on the transcriptional level.

CREB phosphorylation is stimulated by DBcAMP. The promoter region of the AQP2 gene contains a CRE, known to be involved in the AVP-induced AQP2 expression (13, 25). CREB, once phosphorylated at Ser133 (pCREB) by PKA, binds to the CRE and, thereby, presumably stimulates AQP2 transcription. We performed Western blot experiments to examine whether DBcAMP increases the level of pCREB in IMCD cells.

**DBcAMP-induced CREB phosphorylation is not affected by changes in osmolality and solute composition.** We next assessed whether osmolality and solute composition alter the amount of pCREB in IMCD cells grown for 6 days in the presence of 500 μM DBcAMP, thereby regulating the expression of AQP2. Figure 6A shows a representative Western blot analysis of total homogenates of IMCD cells probed with a specific antiserum against pCREB and the same blot probed for the histone H2A1. The results are summarized in Fig. 5B. The level of pCREB in IMCD cells increased with DBcAMP concentrations in the culture medium.

**Kinetics of AQP2 regulation in response to hyper- and hyposmotic challenge provide evidence for an involvement of a tonicity enhancer element identified in the**

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**Fig. 3. Comparison of the effect of osmolality and solute composition on Madin-Darby canine kidney (MDCK1) cells stably expressing AQP2 (WT-10 cells) and IMCD cells.** WT-10 cells are stably transfected with AQP2; its expression is controlled by a cytomegalovirus (CMV) promoter (6). Cells were cultured for 6 days in 300N or 600N (control). A: AQP2 detected by immunoblotting with anti-AQP2 antiserum in membrane preparations. Per lane of an SDS-polyacrylamide gel, 15 μg of membrane protein were loaded. B: densitometric analysis of AQP2 protein levels of WT-10 (n = 3) and IMCD (n = 6) cells. Values are means ± SE. *P < 0.05 vs. control.

**Fig. 4. Regulation of AQP2 mRNA expression by osmolality and solute composition in IMCD cells.** IMCD cells were cultured for 6 days in the presence of 500 μM DBcAMP and 300N, 600S, 600N (control), or 900N (A) and 600N (control) or 900S (B). Total RNA was isolated, and Northern blots (15 μg of total RNA per lane) were performed. AQP2 mRNA was detected with 32P-labeled AQP2 cDNA. Blots were stripped and reprobed with a 32P-labeled cDNA fragment specific for 18S rat rRNA for detection of 18S rRNA. Results are from 2 independent experiments per culture condition. C: densitometric analysis of AQP2 mRNA levels from 4 independent experiments. Values for AQP2 mRNA signals (means ± SE; n = 4) were standardized to corresponding 18S rRNA signals. *P < 0.05 vs. control.
AQP2 promoter. To show that the effects of hyper- and hyposmotic challenge on AQP2 expression are reversible and to assess the time courses of increase and decrease in AQP2 expression, we performed the experiments described below. To analyze the time course of upregulation in response to a hyperosmotic challenge, IMCD cells were seeded in 600N that was changed 24 h later to 300N (except for controls). Starting 72 h after the shift, cells were exposed to 600N for 72, 48, 24, and 6 h before lysis. Figure 7A shows the densitometric analysis of AQP2 expression levels as measured by Western blot analysis. Nonlinear regression analysis suggests that the AQP2 expression started to increase with a lag time of 15 h after exposure to hyperosmolality. The AQP2 expression level of IMCD cells kept for 72 h in 300N (see also Fig. 7B) increased in 600N within 72 h to 75% of that of controls (cells continuously cultured in 600N, 168-h time point). To analyze the time course of AQP2 downregulation in response to hyposmotic challenge, cells were seeded in 600N that was changed to 300N (except for controls) for 144, 72, 48, 24, 6, and 3 h before lysis. Figure 7B shows the densitometric analysis of AQP2 expression levels as detected by Western blot analysis. A downregulation of AQP2 protein expression in response to hyposmolality was detectable after 6 h. Thereafter, AQP2 expression continued to decrease and dropped to baseline levels 72 h after exposure to 300N (Fig. 7B).

Within the promoter of the AQP2 gene we located a regulatory element that could potentially mediate the effects of osmolality changes on AQP2 expression. Figure 7C shows that the rat and human AQP2 promoter [National Center for Biotechnology Information accession nos. D87128 (33) and U30469 (13)] contain elements matching the consensus sequence for the tonicity responsive enhancer (TonE) (26, 34). The TonE consensus sequence is recognized by the recently identified transcription factor TonE-binding protein (TonEBP) (27, 36). In agreement with the present data, activation of TonEBP in response to a hypertonic challenge requires >10 h (27), whereas a hypotonic challenge rapidly decreases TonEBP activity (27, 38).

Hypertonic challenge and not urea, promotes AQP2 protein expression. To discriminate further between the effects of NaCl and urea on AQP2 protein expression in IMCD cells, a set of experiments was performed using media elevated to 600 mosmol/kgH2O with different concentrations of either solute. Figure 8A shows two representative AQP2 immunoblots obtained with total homogenates from IMCD cells cultured for 6 days in the indicated media. The results are summarized in Fig. 8B. Culture media with a high effective osmolality...
AQP2 expression depends on elevated extracellular NaCl. The effect of different NaCl concentrations on DBcAMP-dependent AQP2 expression was further analyzed (Fig. 9). For this purpose, IMCD cells were seeded in 600N and grown for 6 days in 300N with 10, 20, 35, 50, 100, 150, 200, 250, and 300 mM NaCl. AQP2 protein expression of the cells was analyzed by Western blot analysis. A representative set of experiments is shown in Fig. 9B. The expression of AQP2 was increased when up to 150 mM NaCl was added and appeared to decrease when higher concentrations of NaCl were used. These findings underline the assumption that NaCl concentration is a key component in mediating hypertonic induction of AQP2 expression.

Osmolality and solute composition do not interfere with the short-term regulation of AQP2. Immunofluorescence microscopy studies were performed to investigate whether medium osmolality and solute composition alter the AVP-dependent AQP2 translocation from intracellular stores to the plasma membrane, i.e., the short-term regulation of AQP2. As shown in Fig. 7, kinetics of AQP2 regulation in response to hyper- and hypotonic challenge provide evidence for involvement of a tonicity enhancer element identified in the AQP2 promoter. IMCD cells were cultured for a total of 168 h (6 days) in 24-well plates in the presence of 500 μM DBcAMP. A: cells were seeded in 600N, which was changed 24 h later to 300N to induce a downregulation of AQP2. Medium osmolality was not changed in the case of controls (cells continuously grown in 600N; 168-h time point). Upregulation of AQP2 was induced by changing medium to 600N for 72, 48, 24, and 6 h before cell lysis and Western blot analysis (not shown). AQP2 was detected and quantified as described in Figs. 1, 2, and 8. Values from densitometric analysis of AQP2 protein (glycosylated and nonglycosylated) are means ± SE; n = 8. Graph was obtained by performing nonlinear regression analysis (GraphPad Prism; R² (unweighted) = 0.9999). B: cells were seeded in 600N. Downregulation of AQP2 was induced by changing culture medium to 300N (except controls; 0-h time point) for 144, 72, 48, 24, 6, and 3 h before cell lysis (168 h after seeding) and Western blot analysis (not shown). AQP2 was detected and quantified as described in Figs. 1, 2, and 8. Values from densitometric analysis of AQP2 protein (glycosylated and nonglycosylated) are means ± SE; n = 4. Graph was obtained by performing nonlinear regression analysis (GraphPad Prism; R² (unweighted) = 0.9990).
AQP2 expression is regulated by tonicity

10. AQP2 was localized intracellularly in unstimulated IMCD cells and translocated to the plasma membrane in response to AVP stimulation (21). The AQP2 shuttle was observed under all conditions tested. These findings indicate that osmolality and solute composition do not influence the AVP-induced translocation of AQP2. In addition, they show that IMCD cells are viable under the different conditions tested.

**DISCUSSION**

In the present study we show that, in the continuous presence of DBcAMP (500 μM), the levels of AQP2 mRNA (Fig. 4C) and protein (Fig. 2B) in primary cultured IMCD cells are strongly increased by an increase in osmolality and influenced by solute concentration (NaCl and urea vs. sorbitol). The CMV promoter-governed AQP2 expression in stably transfected MDCK cells (WT-10 cells) (6) was not influenced, suggesting that, in IMCD cells, osmolality and solute composition act on AQP2 transcription, rather than affect AQP2 mRNA or protein stability (Fig. 3). Addition of DBcAMP to the cell culture medium increased the phosphorylation of the transcription factor CREB dose dependently, whereas osmolality and solute composition did not influence CREB phosphorylation (Figs. 5 and 6). The effects of hypo- and hyperosmotic challenge on AQP2 expression were reversible (Fig. 7). Hyperosmolality increased AQP2 expression by one-half within 2 days (Fig. 7A), whereas hyposmotic challenge reduced expression by one-half within ~18 h (Fig. 7B). The promoters of the rat and human AQP2 gene each contain a binding site for the transcription factor TonEBP (Fig. 7C), which may mediate the effects of osmolality and solute composition on AQP2 expression. A further analysis of the influence of NaCl and/or urea on AQP2 expression revealed that NaCl is a key component in promotion of AQP2 expression, acting in a dose-dependent manner (Figs. 8 and 9). Elevated osmolality by urea alone had no promoting effect on AQP2 expression (Fig. 8), but the effect of NaCl appeared to be enhanced when urea was present.

In vivo, the high interstitial inner medullary osmolality is mainly derived from equiosmolar urea and NaCl concentrations. The concentrations of NaCl and urea gradually increase from the kidney cortex to the tip of the medulla, thus creating an osmotic gradient required for water reabsorption. The osmotic gradient is maintained even during diuresis, and its magnitude is increased during antidiuresis. The concentration of NaCl in the rat renal medulla varies between 140 mM in hydrated animals and 400 mM in dehydrated animals (3). Whereas urea concentrations equilibrate between the interstitium and the interior of the cell, extracellular NaCl is balanced by intracellular accumulation of organic osmolytes such as sorbitol, betaine, glycerophosphocholine, inositol, and taurine (2). These nonperturbing osmolytes have stabilizing effects on protein function and, thus, counteract the denaturing effect of urea (39). In the renal medulla, the transcription of several genes encoding for proteins involved in the intracellular accumulation of organic osmolytes is induced by hypertonicity (11). We show that elevated NaCl concentrations are also required for a sustained expression of AQP2 in IMCD cells. The increase in AQP2 expression from kidney cortex to inner medulla (29) suggests that NaCl-derived tonicity also controls AQP2 expression in vivo.

It is widely accepted that cAMP is an important factor in the expression (25) and trafficking of AQP2 in principal cells (30). There is also multiple, but mechanistically unexplained, evidence for an AVP-independent regulation of the water permeability of principal cells in the intact animal (30). Decreased AQP2 expression without changes in AVP or cAMP levels was observed in fasting and protein-deprived rats and humans (1, 35). Water deprivation increased AQP2 expression in rats chronically given V2R antagonists (24), indicating that the effect of water deprivation on AQP2 expression is not solely due to AVP-elicited cAMP accumulation. Water loading decreased AQP2 expression, despite chronic administration of the V2R-specific agonist desmopressin (8).

Studies have been conducted to investigate the effect of hypertonicity on AQP2 expression, but the results...
are unclear. Furuno et al. (10) reported a small increase in AQP2 mRNA in cultured mouse outer medullary collecting duct cells bathed for 24 h in hypertonic medium. The finding that a 5-day AVP infusion in rats subjected to thirst increased AQP2 expression to a similar extent in renal cortex (low osmolality) and medulla (high osmolality) favored the assumption that only cAMP, and not tissue osmolality or ionic strength, regulates AQP2 expression (37). No decrease in AQP2 expression was observed after medullary osmolyte washout in rats treated with furosemide for 4–5 days (23, 37). A stimulatory effect of osmolality on AQP2 protein levels in vivo has been suggested by Preisser et al. (32), who found a reduced AQP2 expression in senescent rats, which also exhibited a reduced medullary osmolality in the kidney, while papillary cAMP levels remained unchanged.

We observed that NaCl stimulated the expression of AQP2 in a dose-dependent manner when the cAMP-dependent pathway was stimulated (Fig. 9). This raises the question as to whether 4–5 days of furosemide treatment (see above) lowers the interstitial medullary NaCl concentration below a threshold presumably required for AQP2 expression. It is also possible that elevation of AVP, or other factors, compensates for the effect of decreased tonicity. Further evidence for a promoting influence of medullary osmolality is provided by a recent study (5a) in which it was found that AQP2 protein expression increases in senescent rats when medullary osmolality is restored. We suggest that NaCl-derived hypertonicity, which stimulated AQP2 expression in a dose-dependent manner, is a key factor leading to increased AQP2 expression (Fig. 9).

Fig. 9. AQP2 protein expression correlates with extracellular NaCl concentrations. IMCD cells were cultured for 6 days in 24-well plates containing 600N (control) and 300N elevated to indicated osmolalities with 0 mM (300N), 10 mM, 20 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 300 mM NaCl. A: AQP2 and histone H2A1 detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-polyacrylamide gel, total homogenate (protein) from 1-cm² confluent cell monolayer was loaded. B: densitometric analysis of AQP2 protein levels. Values are means ± SE (7 > n > 12). *P < 0.05 vs. control (CTRL).

Fig. 10. Osmolality and solute composition have no effect on hormone-stimulated trafficking of AQP2 in IMCD cells. Cells were cultured for 6 days in 300N, 600S, 600N (control), 900S, and 900N and deprived of DBcAMP 18 h before experiments. Top: unstimulated IMCD cells. Bottom: IMCD cells stimulated with 100 nM AVP for 30 min before fixation. AQP2 was detected with a specific anti-AQP2 antiserum. Cy3-conjugated goat anti-rabbit antibodies were used as a secondary antibody.
In the present study, elevation of medium osmolality from 300 to 600 mosmol/kgH2O by urea alone had no stimulatory effect on AQP2 expression (Fig. 8). Nevertheless, AQP2 expression was increased when IMCD cells were grown in medium containing 50 mM NaCl and 200 mM urea (Fig. 8, 600 NaCl/U 50/200) compared with cells grown without urea in the presence of 50 mM NaCl (Fig. 9). The importance of urea in the urine-concentrating mechanism, however, was first reported decades ago. Protein deprivation leads to a decreased urine-concentrating ability in animals and humans, which can be restored by urea infusion (5, 19, 31). More recently, the protein deprivation-induced decrease in urine-concentrating ability has been linked to a decrease in AQP2 protein expression in the tip of the inner medulla (30). Our findings indicate that urea itself has no effect on AQP2 expression but, instead, enhances the stimulatory effect of elevated NaCl concentrations.

Considering that osmolality and solute composition did not affect CREB phosphorylation, we assume that the effect of media osmolality and solute composition on AQP2 expression is not mediated by cAMP but is achieved via an alternative pathway. The observation that >500 μM DBcAMP reduces AQP2 protein levels but increases CREB phosphorylation suggests that higher DBcAMP levels activate factors repressing AQP2 transcription, AQP2 translation, or protein stability. Taken together, our data indicate that, in addition to elevated cAMP, NaCl-derived hypertonicity is required for sustained expression of AQP2.

A number of genes coding for proteins involved in the accumulation of osmoprotective solutes in the kidney, such as aldose reductase, sodium-myoinositol cotransporter, and glycine-betaine transporter, have recently been shown to be regulated by tonicity. The regulatory element involved is the toxicity (osmolality)-responsive enhancer element TonE/ORE (9, 26, 28). On increases in tonicity, TonEBP abundance is upregulated and TonEBP translocates to the nucleus, where it activates toxicity-responsive genes. Miyakawa et al. (27) reported that full activation of TonEBP in response to hypertonicity required >10 h, which is consistent with the time required for a detectable increase in AQP2 expression elicited by hypertonicity (Fig. 7). Nuclear abundance and overall expression of TonEBP are rapidly decreased by hypotonic challenge (27, 38). As reported for AQP2 (24, 37), dehydration increases the sodium-myoinositol cotransporter mRNA expression in rat renal medulla, whereas water loading decreases it. This response is presumably due to alterations in the amount of TonEBP present in the nucleus (4). The conservation of the TonE element within the rat and human AQP2 promoter (Fig. 7C) supports the idea that this element is most likely relevant to the genes’ transcriptional regulation.

Altered TonEBP activity, due to altered medullary toxicity, might be responsible for AVP/cAMP-indepen-dent changes in AQP2 expression observed in water loading and thirsting (8, 24). The increase in AQP2 expression from kidney cortex to inner medulla (29) is also well explained by an increasing activity of TonEBP from kidney cortex to medulla. Thus the tonicity and the solute composition within a particular region of the renal collecting duct could limit the range of the effect of AVP/cAMP on the expression of AQP2 in vivo.

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