3. Results

3.1 Elevated osmolality is required for the DBcAMP-elicited expression of robust AQP2 protein levels in IMCD cells

Primary cultured IMCD cells, derived from rat inner medullae, were used as a model system for the investigations on the expression of AQP2. These cells were routinely cultured in medium with an osmolality of 600 mosmol/l in the presence of 500 μM DBcAMP (600N). This standard medium was based on Dulbecco´s modified Eagles medium (DMEM, 300 mosmol/l, termed 300N when supplemented with 500 μM DBcAMP) that was elevated to 600 mosmol/l by the addition of NaCl (100 mmol/l) and urea (100 mmol/l, for details see „Experimental procedures"). To investigate whether extracellular osmolality is a determinant of AQP2 expression, IMCD cells were seeded in collagen type IV coated 60 mm diameter culture dishes containing 600N medium that was either refreshed one day later or, for the second group, exchanged by 300N medium. Media were refreshed every other day and after 7 days in culture cells were checked by light microscopy for confluence. Subsequently, confluent cell layers were scraped off the culture dishes and separately processed for membrane preparations (for details see „Experimental procedures"). Equal amounts of membrane proteins were subjected to SDS-PAGE and Western blotting using PVDF-membranes and semi-dry blotting chambers. Equal loading of lanes was confirmed by ponceau red-staining of membranes (not shown). AQP2 was detected with rabbit anti-AQP2 antiserum as primary antibodies and POD-coupled goat anti-rabbit secondary antibodies. After incubating the blots in Lumi-light substrate, chemiluminescence was visualized with a Lumi-Imager. Signals for AQP2 (glycosylated [gAQP2] and non-glycosylated AQP2 [ng AQP2]) were quantified using Lumi-analyst software. A representative experiment is shown in Fig. 1.

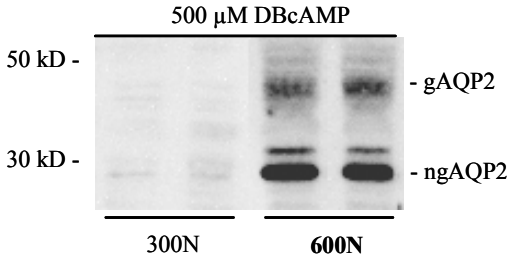

Fig. 1. Hypoosmolality drastically reduces AQP2 protein expression in IMCD cells. Cells were seeded and grown in 600N medium, or were transferred the next day to 300N medium (left lanes). Media supplemented with 500 μM DbcAMP were refreshed every other day. Preparation of membrane fractions was performed 7 days past seeding. Per lane of an SDS-polyacrylamide gel, 20 μg membrane protein was loaded. Glycosylated AQP2 (gAQP2, running at 40-45 kDa) and nonglycosylated AQP2

(ngAQP2, running at 29 kDa) was detected by immunoblotting using rabbit anti-AQP2 antiserum and POD-coupled goat anti-rabbit antibodies. The band appearing above the ngAQP2 band represents core-glycosylated AQP2 (Hasler et al., 2002).

The result shown in Fig. 1 indicate that extracellular osmolality (i.e. medium osmolality) has a strong effect on the expression of AQP2 protein in IMCD cells. Despite the presence of DBcAMP [500 μM] in the culture medium, AQP2 expression was drastically reduced in IMCD cells exposed for 6 days to medium with 300 mosmol/l (300N) compared to cells continuously cultured in medium with 600 mosmol/l (600N medium). This raised the question whether the elevated sodium concentration or the presence of urea is responsible for the robust expression of AQP2. Moreover, it was of interest whether the robust AQP2 expression in 600N medium was due to a compound specific effect of sodium, a specific effect of urea, an increased effective osmolality (tonicity) exerted by the relatively membrane-impermeable solute sodium, or merely the result of increased solute concentration in the medium.

3.1.1 AQP2 protein levels are regulated by extracellular osmolality and solute composition

To investigate the influence of altered osmolality and solute composition on the expression of AQP2 protein, IMCD cells were cultured in the following media. Cultivation of IMCD cells in 300N medium elevated to 600 mosmol/l solely by addition of sorbitol (termed 600S) was intended to give insight whether a high AQP2 expression relies on a specific effect of extracellularly increased NaCl and urea or whether elevation of medium osmolality by sorbitol yields a comparable high AQP2 expression. Medium elevated to 900 mosmol/l by addition of sorbitol (termed 900S) or by equimolar addition of NaCl and urea (termed 900N) was used to elucidate whether the AQP2 expression is to be further increased with osmolality. A representative immunoblot is shown in Fig. 2 (turn page).

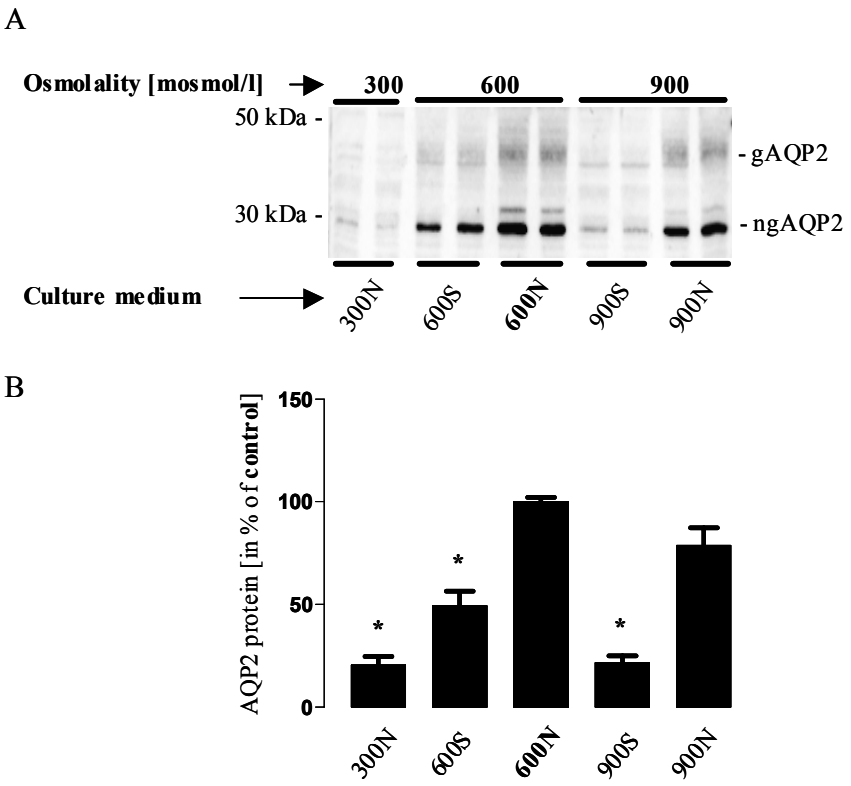

Fig. 2. A combination of hyperosmotic urea and sodium is more potent in increasing the expression of AQP2 than hyperosmotic sorbitol. IMCD cells were cultured for 6 days in 60 mm diameter culture dishes in the continuous presence of 500 μM DBcAMP in A: basal medium (300N), medium elevated to 600 mosmol/l with sorbitol (600S), 600N (control), medium in which osmolality was elevated to 900 mosmol/l with sorbitol (900S), and medium that was elevated to 900 mosmol/l by NaCl and urea (900N). AQP2 was detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-polyacrylamide gel, 20 μg membrane protein was loaded. B: The results of a series of experiments were evaluated by semi-quantitative densitometry using a Lumi-imager and Lumi-analyst software. Signals for AQP2 (g- and ngAQP2) were quantified and raw data was analyzed with Microsoft Exel. Statistical evaluation was performed with Graph Pad Prism (for details see „Experimental procedures"). AQP2 protein expression (glycosylated and nonglycosylated) is expressed in % of control (600N). Values are means +/- SE (6>n>8). *P<0.01 vs. control.

Fig. 2 shows that IMCD cells cultured in 300N medium exhibit only a faint AQP2 expression which is increased by the addition of sorbitol (600S). The expression level of IMCD cells cultured in 600S is significantly lower than that of controls (600N). Cells cultured in 600N medium exhibited the highest expression of AQP2. When sorbitol was used to increase medium osmolality to 900 mosmol/l, the expression of AQP2 was reduced to the low levels of AQP2 detected in cells cultured in 300N medium. In contrast, elevation of medium osmolality by equimolar addition of sodium and urea to 900 mosmol/l yielded only a slight reduction in

AQP2 expression compared to controls (600N). These results indicate that an increase of extracellular osmolality up to 600 mosmol/l by sorbitol, a molecule for which the plasma membrane constitutes a relatively tight barrier, increases the expression of AQP2. Nevertheless, elevating osmolality with equimolar addition of NaCl and urea further increases AQP2 expression, proposing a specific action of NaCl and/or urea in the regulation of AQP2 expression. The solute(s) used seem to determine the threshold up to which hyperosmolality promotes AQP2 expression in IMCD cells. This threshold is higher for sodium and urea then for sorbitol, which has no effect at all when used to elevate medium osmolality to 900 mosmol/l.

3.1.2 The effects of osmolality and solute composition on AQP2 protein levels are due to altered AQP2 mRNA expression

To assess whether the changes in AQP2 protein expression elicited by changed osmolality and solute composition are due to alterations in AQP2 mRNA abundance, altered AQP2 mRNA translation or AQP2 protein stability, Northern blots were performed. For this reason, IMCD cells were seeded in 600N medium and cultured 24 h later in either 300N, 600S, 600N (control), 900S or 900N medium for 6 days in the continuous presence of 500 μM DBcAMP. Total RNA was isolated from IMCD cells cultured in 60 mm diameter culture dishes using TRIzol-reagent (Gibco BRL) according to the manufacturers instructions. Equal amounts of total RNA were separated by agarose gel electrophoresis. RNA was transferred to nylon membranes by Northern blotting and crosslinked AQP2 mRNA was subsequently hybridized to ^{32}P-labelled AQP2 cDNA. After stringent washing, radioactivity was quantified using a PhosphorImager (for details see „Experimental procedures“). A set of representative experiments is shown in Fig. 3.

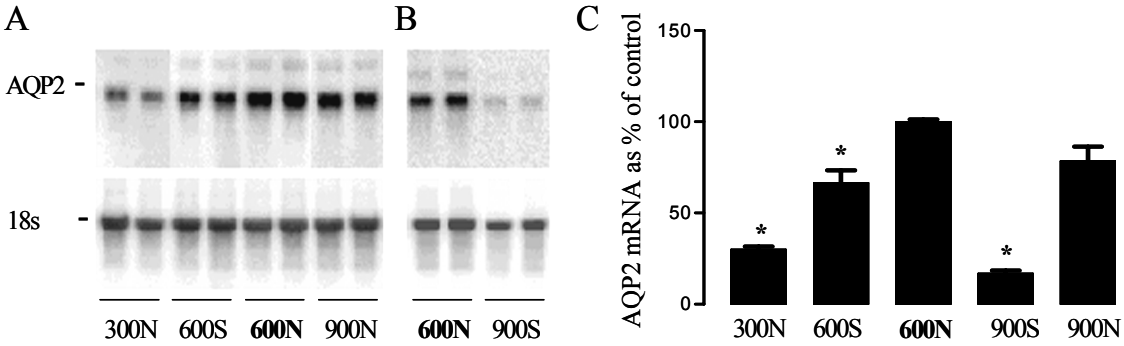

Fig. 3. The effects of osmolality and solute composition on IMCD cell´ AQP2 expression are based on altered AQP2 mRNA expression. IMCD cells were cultured for 6 days in the presence of 500 μM DBcAMP in A: 300N, 600S, 600N (control, bold letters) or 900N medium and B: 600N (control,

bold letters) or 900S medium as indicated (for details see „Experimental procedures"). Total RNA was isolated and Northern blots (15 µg of total RNA per lane) were performed. AQP2 mRNA (upper panels, as indicated) was detected with ^{32}P-labelled AQP2 cDNA. Blots were stripped and reprobed with a ^{32}P-labelled cDNA fragment specific for 18S rat rRNA for the detection of 18S rRNA (lower panels, as indicated). C: Densitometric analysis of AQP2 mRNA levels. Values for AQP2 mRNA signals (means +/- SE; n=4) were standardized to corresponding 18S rRNA signals and are expressed in % AQP2 mRNA expression controls. *P < 0.05 vs. control values.

The changes in the mRNA level closely resemble the findings in AQP2 protein level (Fig. 2). This indicates that either the transcription of AQP2 mRNA, or its stability is affected by osmolality and solute composition.

3.1.3 Altered osmolality has no effect on the cytomegalovirus-governed expression of AQP2 in MDCK1 cells stably transfected with AQP2

To assess whether the effect of osmolality and solute composition on the AQP2 expression in IMCD cells is dependent on the endogenous promoter of the AQP2 gene, with human AQP2 stably transfected MDCK1 cells (WT-10 cells, kindly provided by P.M. Deen) were used. The AQP2 expression of WT-10 cells is governed by a cytomegalovirus (CMV) promoter, leading to constitutive transcription of the AQP2 gene (Deen et al., 1997). WT-10 cells were cultured for 6 days in 300N or 600N medium without DBcAMP and with 5 % FCS instead of 1 % Ultroser (for details see "Experimental procedures"). AQP2 was detected in membrane preparations of confluent cell monolayers by immonoblotting.

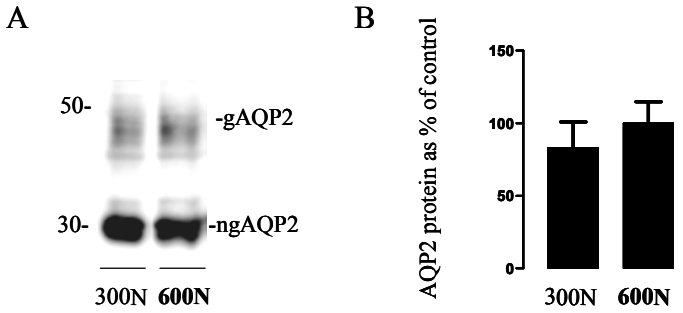

Fig. 4. The viral promotor-governed AQP2 expression of WT-10 cells is not affected by osmolality. WT-10 cells are MDCK1 cells stably transfected with AQP2; its expression is controlled by a CMV promoter (Deen et al., 1997). Cells were cultured for 6 days in 300N or 600N medium (control, bold letters) as indicated. A: AQP2 (g, glycosylated and ng, non-glycosylated) was detected by immunoblotting with anti-AQP2 antiserum in membrane preparations. Per lane of an SDS-PAGE gel, 15 µg of membrane protein was loaded. B: Densitometrical analysis of AQP2 protein (glycosylated and non-glycosylated) levels of WT-10 cells. Values are means +/- SE (n = 3) vs. control.

The results shown in Fig. 4 strongly suggest, that altered osmolality and solute composition do not affect AQP2 mRNA stability *per se*, because AQP2 protein levels were unaltered in WT-10 cells with a constitutiv, cytomegalovirus controlled AQP2 transcription. Therefore, the observed changes in IMCD cell AQP2 mRNA levels elicited by different medium osmolalities and solute compositions (Fig. 3) rely on the endogenous regulatory region of the AQP2 gene and are most likely due to altered transcription.

WT-10 cells, as an immortalized cell line, continue to proliferate in contrast to primary cultured IMCD cells which hardly divide in culture. Therefore it was investigated by immunofluorescence whether WT-10 cells form comparable cell monolayers when cultured for 6 days in 300 mosmol/l or 600 mosmol/l medium. In addition it was investigated whether the number of AQP2 expressing cells is altered by medium osmolality. WT-10 cells were seeded in medium with either 300 or 600 mosmol/l without DBcAMP and were grown for 6 days. Cells were stimulated with 100 nM AVP (for 30 minutes) prior to preparation for immunofluorescence. AQP2 was visualized using rabbit anti-AQP2 antiserum as primary antibodies and Cy3-conjugated goat anti-rabbit secondary antibodies (see Fig. 5). Total cell numbers and AQP2 expressing cells in randomly selected portions of different cell preparations were counted under a fluorescence microscope. Raw data was evaluated using Microsoft Exel software.

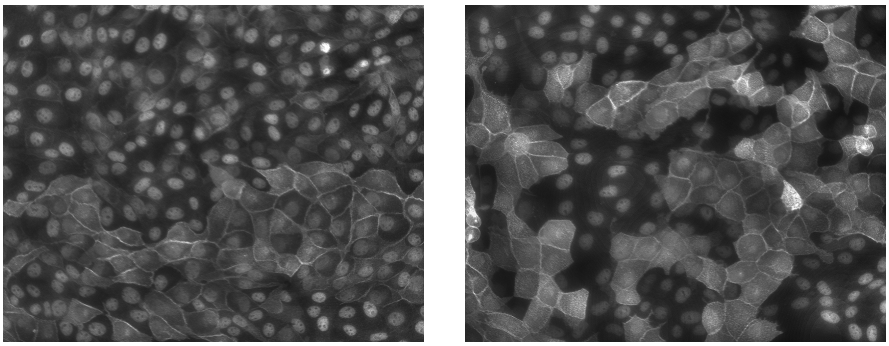

Fig. 5. The AQP2 expression of WT-10 cells is not altered by osmolality. WT-10 cells are stably transfected with AQP2; its expression is controlled by a CMV promoter (Deen et al., 1997). Cells were cultured for 6 days in 300N or 600N medium (without DBcAMP) and were stimulated with 100 nM AVP prior to preparation. AQP2 was detected by immunofluorescence with anti-AQP2 antiserum. AQP2 expressing cells exhibit a clear membrane staining. The nuclei are also stained by the anti-AQP2 antiserum which cross-reacts with the histone H2A1 (Jo et al., 1997). Cy-3-conjugated goat anti-rabbit antibodies were used as a secondary antibody. Cy-3 signals were visualized by fluorescence microscopy using a Leica DMBL microscope with Sensicam 12 Bitled charge-coupled device camera.

The analysis of total cell numbers and percentage of AQP2 expressing cells in randomly selected sectors of WT-10 cell preparations (Fig. 5; n = 7) revealed an average cell number of 257 cells (+/- 53) per section for cells grown at 300 mosmol/l and 207 cells (+/-53) per section for cells grown at 600 mosmol/l. The resulting percentage of AQP2 expressing cells in cultures grown at 300 mosmol/l was 43 +/- 13 % and 47 +/- 11 % (means +/- SE) for cultures grown at 600 mosmol/l and thus not significantly different concerning the two media osmolalities.

3.1.4 Evaluation of an alternative to the quantification of AQP2 protein expression in membrane preparations of IMCD cells: reducing animal consumption

To understand the influence of osmolality and solute composition on the regulation of AQP2 in IMCD cells in which the AQP2 gene is controlled by its endogenous promoter, further experiments were required that relied the analysis of a large number of cell batches. The analysis of AQP2 in membrane preparations requires a high amount of starting material for a single experiment. For a sustained AQP2 expression, the ideal number of IMCD cells seeded per cm^2 was found to be 5 x 10^4 cells / cm^2 (Storm, 1999). For a single experiment, 1.5 x 10^6 cells were seeded into a 60 mm dish.In a first attempt to reduce the starting material and thus the number of animals needed, IMCD cells were seeded in 35 mm diameter culture dishes instead of 60 mm diameter dishes. The amount of crude membrane protein yielded per 35 mm dish was too low for a precise determination of protein concentration by the method of Bradford. As a consequence, results from Western blot analysis were not reliable.

Considering that the anti-AQP2 antiserum crossreacts with the nuclear protein histone H2A1 (Jo et al., 1997), it was intriguing to make use of this feature and use the H2A1 signals as a loading control. As a first step total homogenates of IMCD cells cultured in 300N and 600N in 60 mm dishes were prepared (see "Experimental procedures"), protein concentration was determined by the method of Bradford and equal protein amounts were subjected to SDS-Page (Fig. 6).

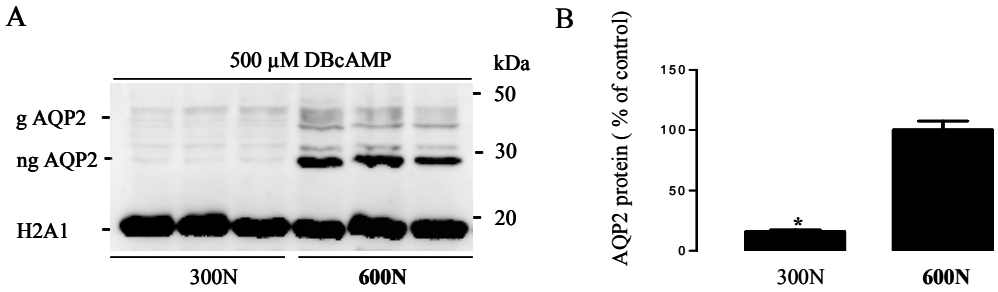

Fig. 6. Analysis of AQP2 protein expression in total homogenates. IMCD cells seeded in 60 mm dishes, were cultured for 6 days in 300N or 600N medium (control, bold letters) as indicated and total homogenates were prepared (see "Experimental procedures"). A: AQP2 (g, glycosylated and ng, non-glycosylated) was detected by immunoblotting with anti-AQP2 antiserum in membrane preparations. Per lane of an SDS-PAGE gel, 40 µg of protein was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated). Values means +/- SE (n = 3) vs. control.

Fig. 6 shows that the levels of AQP2 protein detected in 40 µg of total homogenate protein of IMCD cells grown in either 300N or 600N medium are well comparable to the results obtained analyzing membrane preparations (see Fig. 1 and 2). Moreover, the detected levels of histone H2A1 were not affected by medium osmolality, making the crossreaction of the AQP2 antiserum a suitable intrinsic control for equal protein loading of SDS-PAGE gels and the regularity of protein transfer by Western blotting.

Next, IMCD cells were seeded at a density of 5 x 10^4 cells / cm^2 in 24-well plates with an area of 2 cm^2/well. Cells were grown for 6 days and were subsequently checked for confluency under a light microscope. Confluent cell layers were then lyzed directly in the wells. Chromosomal DNA was sheared by sonication to reduce the viscosity of lysates (for details see "Experimental procedures"). Half the volume of lysate yielded per well (the equivalent to one square cm of confluent cell monolayer) was subjected to SDS-PAGE. AQP2 expression of IMCD cells was analyzed by immunoblotting. Fig. 7 shows a comparison of the results obtained with membrane preparations of IMCD cells (20 µg membrane protein loaded per lane) and with directly lysed IMCD cells (protein amount equal to 1 cm2 of confluent cell monolayer loaded per lane).

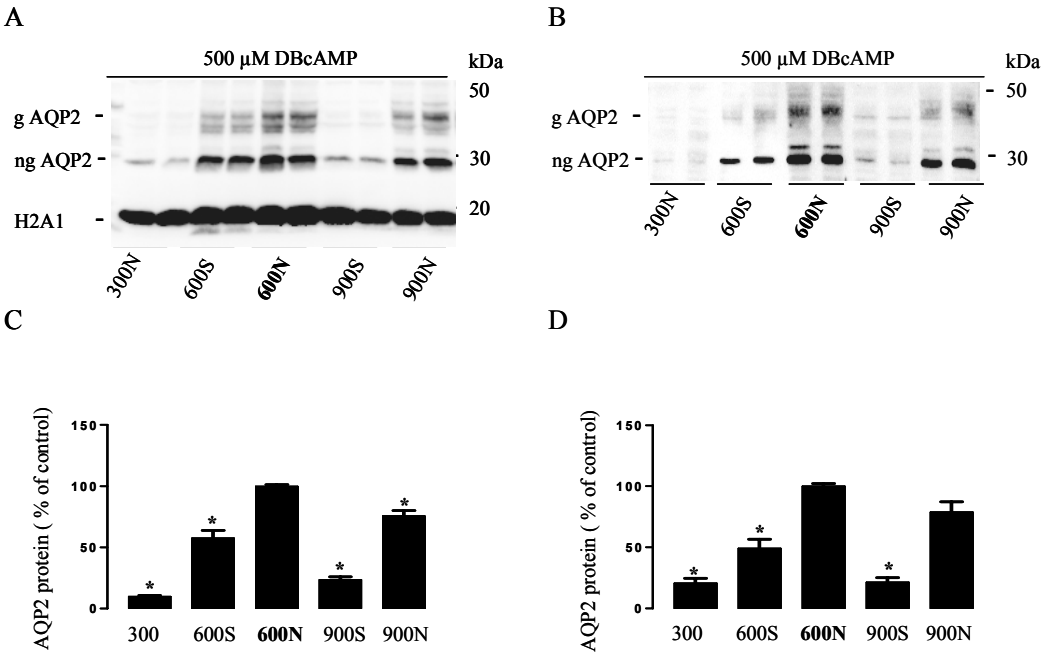

Fig. 7. Analysis of AQP2 in total homogenates and in membrane preparations: two methods of equal quality. IMCD cells were cultured for 6 days in (A) 24-well plates for subsequent total homogenate preparation) or (B) in 60 mm culture dishes for membrane preparations. Cells were cultured in the presence of 500 µM DBcAMP in 300N, 600S, 600N (bold letters, control), 900S and 900N medium (see "Experimental Procedures" for details). A and B: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer (A) or 20 µg membrane protein (B) was loaded. The nuclear protein histone H2A1 is not shown in B because nuclei were largely removed prior to membrane preparation (see "Experimental Procedures" for details). C: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n = 8). *P < 0.05 vs. control. D: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in membrane preparations. Values are means +/- SE (6<n<8). *P < 0.05 vs. control.

As evident from Fig. 7, the analysis of protein expression in IMCD cell total homogenates was found to be qualitatively comparable to the analysis of protein expression in IMCD cell membrane preparations. This approach required 15 times less cell-and animal material and was thus used for all subsequent experiments with primary cultured IMCD cells.

3.2 Osmolality and solute composition have no effect on the hormone-stimulated trafficking of AQP2 in IMCD cells

Immunofluorescence studies were performed to investigate whether medium osmolality and solute composition alter the AVP-dependent translocation of AQP2 from intracellular stores to the plasma membrane (short-term regulation of AQP2). IMCD cells were kept in 600N medium for 24 h past seeding and were thereafter cultured for 6 days in 300N, 600S, 600N, 900S, and 900N medium. Cells were deprived of DBcAMP 18 h before experiments, i.e. stimulation with AVP to induce AQP2 translocation.

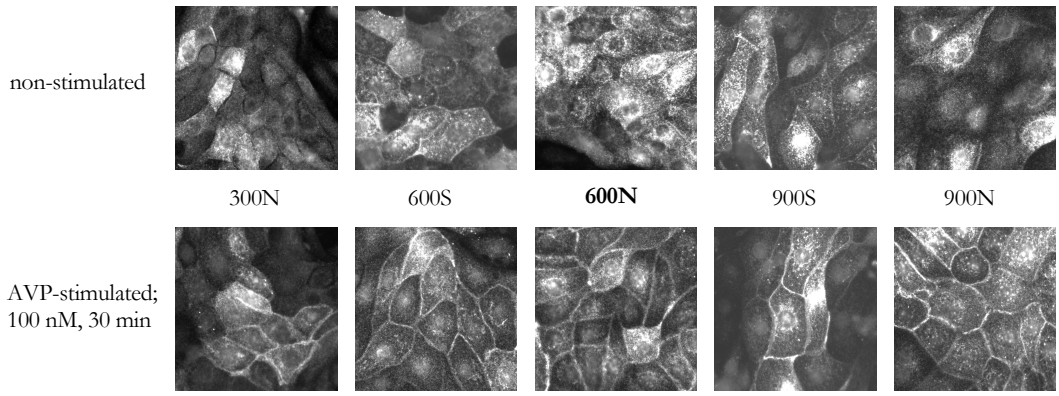

Fig. 8. Osmolality and solute composition have no effect on hormone-stimulated trafficking of AQP2 in IMCD cells. Cells were cultured for 6 days in 300N, 600S, 600N (control), 900S, and 900N medium and deprived of DBcAMP 18 h before experiments. Top: unstimulated IMCD cells. Bottom: IMCD cells stimulated with 100 nM AMP for 30 min prior to fixation. After permeabilization cells were blocked with fish-skin gelatine to prevent binding of the anti-AQP2 antiserum to histone H2A1 in order to reduce the staining of nuclei. Cy-3-conjugated goat anti-rabbit antibodies were used as a secondary antibody. Cy-3 signals were visualized by fluorescence microscopy using a Leica DMBL microscope with Sensicam 12 Bitled charge-coupled device camera. For comparison, exposure times were adjusted to level the differences in AQP2 expression (see Fig. 2).

As shown in Fig. 8, AQP2 was mainly localized intracellularly in unstimulated cells, regardless of the culture medium used. Upon stimulation with 100 nM AVP for 30 min, AQP2 translocated to the plasma membrane. Altered medium osmolality and solute composition had no detectable influence on AQP2 trafficking.

3.3 The role of DBcAMP in the expression of AQP2 in IMCD cells

When the primary cultured IMCD cell model was developed, the supplementation of culture media with 500 μM of the cAMP analogon DBcAMP was found to be a prerequisite for the sustained expression of AQP2 (Maric, et al. 1998). As explained in detail before (see "Introduction"), the binding of AVP to the V_2-receptor elevates intracellular cAMP levels which activate protein kinase A (PKA). PKA-mediated Ser-133 phosphorylation activates CREB, which, bound to the CRE-motif located in the promoter region of the AQP2 gene, is thought to be required for AQP2 transcription (Hozawa et al. 1996).

To evaluate the effect of DBcAMP on the expression of AQP2, IMCD cells were grown in 600N medium supplemented with 5 μM, 50 μM, 200 μM, 500 μM (control), 2 mM and 5 mM DBcAMP. As shown in Fig. 9, DBcAMP stimulated AQP2 expression in a dose-dependent manner; its maximum effect was at 200 and 500 μM. Higher concentrations led to a decrease in AQP2 expression, while cell morphology, as assessed by light microscopy, remained unchanged (not shown).

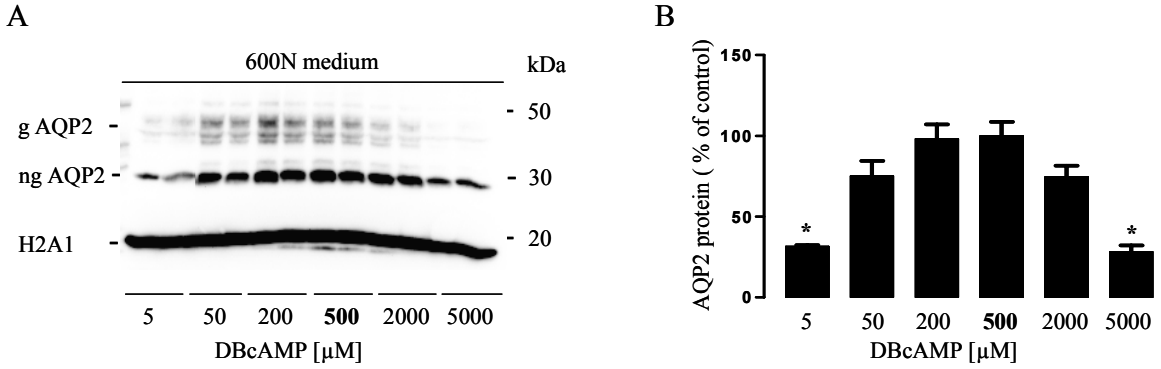

Fig. 9. Effects of DBcAMP on AQP2 protein levels in IMCD cells. IMCD cells were cultured for 6 days in 24-well plates in the presence of 5 μM, 50 μM, 200 μM, 500 μM (control), 2 mM and 5 mM DBcAMP. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n = 4). *P < 0.05 vs. control.

In previous works, DBcAMP had been routinely used to keep up AQP2 expression in IMCD cells at a concentration of 500 μM (Maric et al., 1998, Maric et al., 2001). The results shown above support that this concentration is well suited to maximally stimulate cAMP-dependent

AQP2 expression in primary cultured IMCD cells kept in media elevated to 600 mosmol/l by equimolar addition of sodium chloride and urea (600N, control).

3.3.1 Ser-133 phosphorylation of CREB is stimulated by DBcAMP

As already mentioned, the 5´regulatory region of the AQP2 gene contains a cAMP-responsive element. This element with the sequence GACGTCA is the binding site for the phosphorylated transcription factor cAMP-responsive element binding protein (CREB). The phosphorylation of CREB at residue serine 133 (Ser-133) is required for initiation of transcription of CRE-regulated genes (Gonzales et al., 1989). Using specific antibodies that bind to CREB only when the protein is phosphorylated at Ser-133 (pCREB), the influence of DBcAMP on CREB phosphorylation was tested. IMCD cells were seeded in 600N medium that was supplemented with 5, 50, 200, 500, 2000 and 5000 μM of DBcAMP. As shown in Fig. 10, the detected amounts of pCREB increased with the concentration of the second messenger analogon.

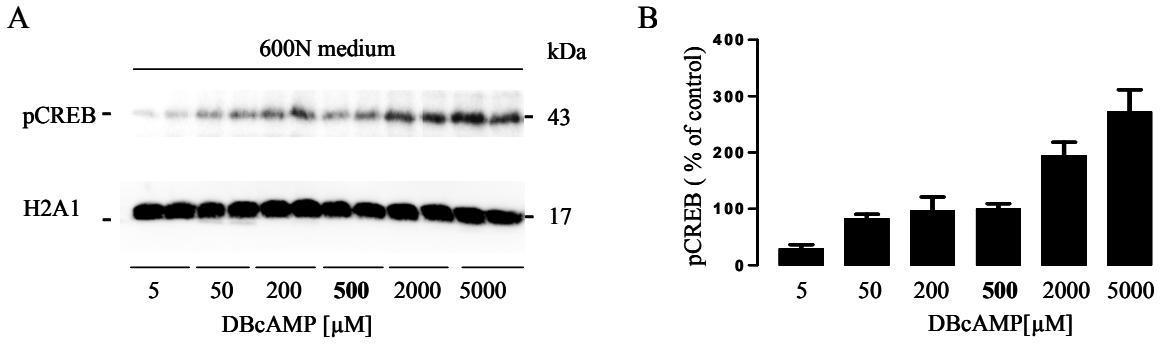

Fig. 10. DBcAMP elicits phosphorylation of the cAMP-responsive-element-binding protein (CREB) in IMCD cells. IMCD cells were cultured for 6 days in 24-well plates in the presence of 5 μM, 50 μM, 200 μM, 500 μM (control), 2 mM and 5 mM DBcAMP (see "Experimental Procedures" for details). A, *top*: Ser-133 phosphorylated CREB (pCREB) detected by immunoblotting with anti-pCREB antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. A, *bottom:* The lower part of the blot was incubated with anti-AQP2 antiserum for the detection of histone H2A1 (as indicated). B: Densitometrical analysis of pCREB protein levels detected in total homogenates. Values are means +/- SE (n = 4).

3.3.2 The DBcAMP-induced CREB phosphorylation is not affected by changes in extracellular osmolality and solute composition

To assess whether alterations in extracellular osmolality and solute composition have the potential to affect CREB phosphorylation and thereby influence AQP2 expression, IMCD cells were exposed to different media in the presence of 500 μM DBcAMP for 6 days. Cell lysates were subjected to SDS-PAGE and immunoblotting. Phosphorylated CREB and the histone H2A1 were detected with pCREB specific antibodies and anti-AQP2 antiserum, respectively. A representative Western blot is shown in Fig. 11 A. Results are summarized in Fig. 11 B.

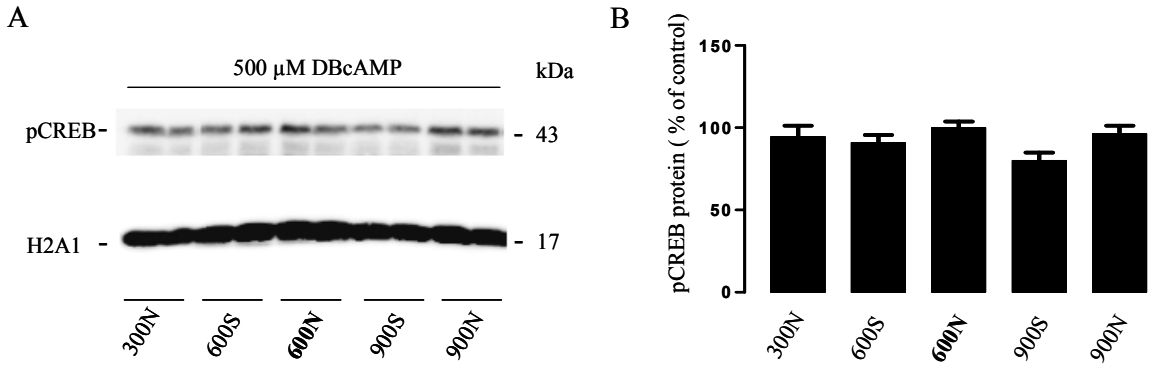

Fig. 11. DBcAMP-induced CREB phosphorylation is not affected by alterations in osmolality and solute composition. IMCD cells were cultured for 6 days in 24-well plates containing 300N, 600S, 600N (control), 900S and 900N in the presence of 500 μM DBcAMP (see "Experimental Procedures" for details). A, *top*: Ser-133 phosphorylated CREB (pCREB) detected by immunoblotting with anti-pCREB antiserum. A, *bottom:* histone H2A1 detected by incubation with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of pCREB protein levels detected in total homogenates. Values are means +/- SE (n = 8). Values were not statistically different from controls.

Fig. 11 shows that changes in the osmolality and solute composition of the culture medium did not affect Ser-133 phosphorylation of CREB in IMCD cells continuously stimulated with 500 μM of the membrane permeable cAMP analogon DBcAMP. This suggests that the regulative effect of osmolality and solute composition on AQP2 mRNA and protein levels is exerted *via* a pathway distinct from CREB phosphorylation.

3.4 Hypertonic challenge, not simple elevation of osmolality by the membrane permeating compound urea, promotes AQP2 protein expression

The results presented beforehand (Figs. 2, 3 and 8) indicated that elevation of media osmolality by sodium and urea as well as sorbitol increased the expression of AQP2. Increasing medium osmolality with sodium or sorbitol increases effective osmolality, i.e. tonicity, because the cell membrane is relatively impermeable to these solutes. Urea on the other hand readily passes the lipid bilayer and is therefore considered an ineffective osmolyte. To discriminate between the individual effects of these solutes and between urea-derived osmolality and sodium or sorbitol-derived tonicity, further experiments with modified media compositions were performed. All media contained 500 μM DBcAMP. IMCD cells were challenged for 6 days with media elevated to 600 mosmol/l by addition of sorbitol, urea or sodium (used in the concentrations indicated). Additionally, the influence of a medium elevated to 900 mosmol/l by 250 mmol/l sodium and 100 mmol/l urea was tested. Two representative Western blots are shown in Fig. 12 A. The results of a set of experiments are summarized in Fig 12 B.

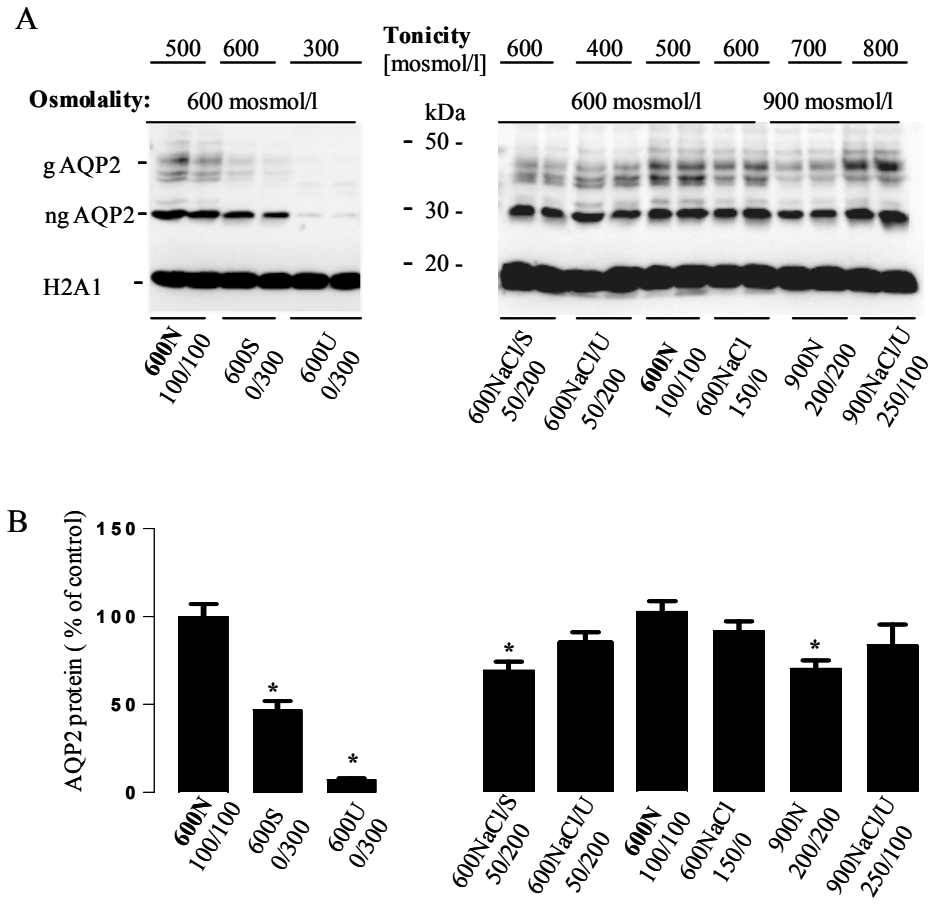

Fig. 12. Hypertonic challenge, not urea, stimulates AQP2 expression. IMCD cells were cultured for 6 days in 24-well plates in the presence of 500 μM DBcAMP in 600N 100/100 medium (control), 600S 0/300, or 600U 0/300 (*left*) and 600NaCl/S 50/200, 600 NaCl/U 50/200, 600N 100/100 (control), 600

NaCl 150/0, 900N 200/200 or 900 NaCl/U 250/100 (*right*). Numbers in front of and behind slash indicate added concentrations (to 300 N medium) of sodium (NaCl), urea (U) or sorbitol (S), (in mM), respectively (for details see "Experimental Procedures"). Both, osmolality and effective osmolality (tonicity) of the media used are indicated. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE ($4<n<12$). *$P < 0.05$ vs. control.

The results presented in Fig. 12 indicate that elevated effective osmolality (tonicity) derived from weakly membrane-permeating solutes as sodium or sorbitol promotes AQP2 expression (as in 600 NaCl/S 50/200, 600 NaCl 150/0 and 600S 0/300), whereas simple elevation of osmolality by urea which readily equilibrates between the cell´s exterior and interior, failed to stimulate the expression of AQP2 (as in 600 U 0/300). Hypertonicity derived from sodium yields a higher expression of AQP2 than sorbitol (compare 600S 0/300 and 600NaCl 150/0). In combination with elevated sodium-derived tonicity, urea appears to enhance the expression of AQP2 (compare 600 NaCl/U 50/200 to 600 NaCl/S 50/200). The expression of AQP2 is not significantly different from controls (600N 100/100) when IMCD cells were challenged with 600 NaCl/U 50/200 or 600 NaCl 150/0. Compared to 900N medium, a slight increase in AQP2 expression was achieved by elevation of the sodium concentration from 200 mmol/l (as in 900N) to 250 mmol/l with concurrent reduction of urea to 100 mmol/l (as in 900 250/100). These results indicate that an elevated tonicity promotes AQP2 expression. A simple elevation of osmolality by urea failed to promote AQP2 expression.