4. Discussion

Previous investigations on the effect of osmolality on AQP2 expression yielded no clear results. Furuno et al. reported a small increase in AQP2 mRNA in cultured mouse outer medullary collecting duct cells bathed for 24 h in hypertonic medium (Furuno et al. 1996), but the effect was not analyzed further in that study. Research on this topic ceased when it was reported that no decrease in AQP2 expression was observed following kidney medullary osmolyte washout after a 4 to 5-day furosemide treatment of rats (Marples et al. 1996, Terris et al. 1996). The finding that a 5-day AVP infusion in thirsted rats increased AQP2 expression to a similar extent in renal cortex (low osmolality) and medulla (high osmolality) favored the assumption that solely cAMP but not tissue osmolality and ionic strength regulate AQP2 expression (Terris et al. 1996). The current study was conducted to analyze the influence of extracellular osmolality and solute composition on the AQP2 expression of primary cultured IMCD cells. By use of this cell model (Maric et al., 1998, Maric et al., 2001), potential effects of extracellular osmolality and solute composition on AQP2 expression could be studied isolated from other factors possibly induced by manipulation of medullary osmolality and osmolyte composition in the intact animal. Moreover, the use of primary cultured cells, in which expression of the AQP2 gene is governed by the endogenous promoter circumvented alterations in cell function attendant upon immortalization as dedifferentiation and increased proliferation.

The results of the study presented here underline that stimulation of the classical, AVP-triggered pathway (see introduction) is not sufficient for a high-level AQP2 expression in IMCD cells. In the presence of DBcAMP, elevated extracellular tonicity exerted by relatively membrane impermeable solutes (as sodium or sorbitol), but not simple elevation of osmolality (by urea), was found to be required for sustained AQP2 expression (Fig. 12). Extracellular tonicity and solute composition similarly affected AQP2 mRNA and protein levels (Fig. 2 and 3). No effect of extracellular tonicity and solute composition on AQP2 protein levels of WT-10 cells, an MDCK cell line transfected with the human AQP2 coding region under the control of a viral promoter, was evident (Fig. 4). It is therefore well conceivable that the effects of extracellular tonicity and solute composition on IMCD cell AQP2 mRNA levels were brought about by altered AQP2 transcription, rather than by altered AQP2 mRNA stability *per se*. Osmolality and solute composition did not affect CREB phosphorylation (see Fig. 11), indicating that the effect of media osmolality and solute composition on AQP2 expression is not mediated by cAMP, but is exerted *via* an alternative pathway. The finding that DBcAMP concentrations above 500 μM reduced AQP2 protein levels but increased CREB phosphorylation suggests that higher DBcAMP levels lead to the activation of factors repressing AQP2 transcription, translation or

protein stability (see Figs. 9 and 10). The non-linear relationship between CREB phosphorylation and AQP2 expression underlines that Ser-133 phosphorylation of CREB is not the only determinant of AQP2 expression.

In this respect it is notable, that within each, rat, murine, and human AQP2 proximal promoter region, the consensus sequence for the tonicity (osmolality)-responsive-element (TonE/ORE; Ferraris et al. 1996, Moriyama et al. 1989, Miyakawa et al. 1998) was located at approximately 600 bp downstream of the transcription initiation site (Fig. 17A). Moreover, a second TonE/ORE was found approximately 1.3 kb downstream of the human AQP2 gene (Fig. 17B). In the following, the term TonE will be used, because elevation of effective osmolalic activity (tonicity) exerted by relatively membrane impermeant solutes, but not mere hyperosmolality was found to stimulate TonE-dependent transcription (Miyakawa et al. 1999, [2]Handler and Kown 2001). The TonE element found in the proximity of the human AQP6 gene is most likely not involved in the gene´s transcriptional regulation. Promeneur and co-workers reported that AQP6, which is present in intracellular vesicles within renal collecting duct intercalated cells type A, but not in AQP2 expressing principal cells, is up-regulated in water loaded rats (Promeneur et al., 2000). The conservation of the TonE in the regulatory region of the AQP2 gene amongst the three species examined, as well as the conservation of its position, support a potential relevance of the TonE in AQP2 gene regulation in response to altered tonicity.

Organic osmolyte transporters are regulated by tonicity: TonE and TonEBP. A number of genes involved in the intracellular accumulation of osmoprotective solutes in response to extracellular hypertonicity such as aldose reductase (AR), sodium-myo-inositol cotransporter (SMIT), and sodium/chloride/betaine cotransporter (BGT-1) were shown to contain TonE motifs in their regulatory regions (Ko et al., 1997; Rim et al., 1998; Miyakawa et al., 1998). It has been reported that multiple TonE elements act in synergy to promote transcription of the AR and BGT-1 gene in response to hypertonicity (Ko et al., 1997; Miyakawa et al., 1998). These elements may be spread over 50 kb downstream of the transcription initiation site, as was reported for the sodium/myo-inositol cotransporter gene (SMIT; Rim et al., 1998). The putative TonE consensus sequence (TGGAAANN(C/T)N(C/T); Rim et al., 1998, Miyakawa et al., 1998) is recognized by the recently cloned transcription factor TonE-binding protein/nuclear factor of activated T-cells (TonEBP; Miyakawa et al., 1999; NFAT5; López-Rodríguez et al., 1999). TonEBP is a member of the Rel family of transcriptional activators, but is clearly distinct from other members as nuclear factor κB (NF-κB) or NFAT1-4 in the amino acid sequence of its DNA binding domain (López-Rodríguez et al., 1999; Stroud et al., 2002).

AQPs and tonicity. An influence of tonicity has been implicated in expressional regulation of some members of the aquaporin family. Nonetheless, none has been confirmed to be regulated by the

TonE/TonEBP pathway. Hypertonicity was shown to induce AQP1 expression, presumably via a novel hypertonicity-responsive element, involving the mitogen-activated-kinase (MAPK) cascade (Umenishi and Schrier, 2002; 2003). In addition, Leitch and co-workers revealed that AQP1 protein degradation is attenuated by hypertonicity (Leitch et al., 2001). A transient rise in AQP3 mRNA and protein levels was detected in MDCK cells in response to hypertonicity, but the mechanism involved is yet unclear (Matsuzaki et al., 2001). A similar observation was made in human keratinocytes (Sugiyama et al., 2001). AQP5, an aquaporin expressed in lung, salivary and lacrimal glands, was reported to be induced by hypertonicity through an ERK-dependent pathway (Hoffert et al., 2000). Osmolality and solute composition are strong regulators of AQP2 expression in IMCD cells (Storm et al., 2003), and the study in hand substantiates the evidence that AQP2 is a target gene of the TonE/TonEBP pathway.

About the activation of TonEBP. TonEBP is ubiquitously abundant, as it was found strongly expressed in diverse tissues including brain, heart, kidney, and in T-cells (Miyakawa et al., 1999; Trama et al., 2000). The stimulus for increased TonEBP action was shown to be hypertonicity, but not urea-derived hyperosmolality (Miyakawa et al., 1999; [1]Ferraris et al., 2002; Woo et al., 2002). Upon increases in tonicity, TonEBP abundance is up-regulated and TonEBP translocates rapidly to the nucleus where it activates tonicity-responsive genes. Hypotonic challenge decreases the nuclear localization of TonEBP and the protein´s overall abundance (Miyakawa et al., 1999; [1]Woo et al., 2000). Under isotonic conditions TonEBP is partially active and distributed in the cytoplasm and the nucleus ([1]Ferraris et al., 2002; [1]Woo et al., 2000). It was proposed that intracellular ionic strength (essentially determined by the sum of Na^+, K^+, and Cl^-) is a determinant of TonEBP action ([1]Woo et al., 2000). A recent work of Neuhofer and co-workers corroborated this hypothesis. They revealed that TonEBP-dependent transcription correlated with the intracellular ionic strength regardless of extracellular tonicity. Inhibition of Na^+/K^+-ATPase as well as high extracellular K^+ concentrations decreased TonEBP action despite elevated intracellular ionic strength. Moreover, dilution of the cytosol due to an increase in cellular water content suppressed TonEBP action despite high intracellular ionic strength (Neuhofer et al., 2002). Nevertheless, the molecular mechanisms underlying the activation of TonEBP in response to hypertonicity are yet poorly understood. It is known that TonEBP is a constitutive homodimer that forms a circle around the DNA (Stroud et al., 2002). TonEBP contains a transactivation domain (TAD), whose activity increases dose-dependently with extracellular sodium concentrations ([1]Ferraris et al., 2002). Activity of the TAD was induced by hypertonically-activated catalytic PKA-subunits (cPKA), but not by elevation of intracellular cAMP. Hypertonic TAD activation was inhibited by the PKA inhibitor H89. Reciprocal immunoprecipitation revealed that cPKA associated with TonEBP ([2]Ferraris et al., 2002).

Elevated tonicity: a prerequisite for robust AQP2 expression in IMCD cells. The present study shows that robust AQP2 mRNA and protein expression relied on elevated tonicity in IMCD cells, in addition to stimulation by the membrane-permeating cAMP analogon DbcAMP. In cells exposed for 6 days to medium with 300 mosmol/l (300N), only a very faint AQP2 expression was detectable despite the continuous presence of 500 μM DBcAMP. This is in agreement with the observations of Woo and co-workers, who revealed that TonEBP exhibited a basal activity under isotonic conditions in MDCK cells. Hypertonic induction of TonEBP expression was saturated at a tonicity of approximately 450 mmol/l ([1]Woo et al., 2000), and a similar saturation point appears to be in effect in the renal medulla (Cha et al., 2001). In the IMCD cells studied here, a clear and significant increase in AQP2 protein expression was induced when 300N medium (which itself contains approximately 110 mmol/l NaCl) was elevated to 400 mosmol/l by addition of 50 mM NaCl. AQP2 expression increased dose-dependently with sodium concentrations up to a medium tonicity of 600 mosmol/l (Fig. 13). Nonetheless, AQP2 expression elicited by mere sodium-derived hypertonicity fell slightly short of the expression level exhibited by control cells kept in medium elevated with 100 mM NaCl and 100 mM urea to 600 mosmol/l (600N, control). Consequently, the effects of extracellular urea, NaCl, and sorbitol concentrations on AQP2 expression were tested. Elevation of simple osmolality by the membrane-permeating compound urea was clearly not sufficient to increase AQP2 expression (Fig. 12), which is in correspondence with the failure of urea-derived hyperosmolality to increase the expression of TonEBP target genes (Woo et al., 2002). However, urea addition appeared to enhance the promoting effect of tonicity on AQP2 expression (Fig. 12, 13 and 14). Compared to controls, cells cultured in medium elevated to 400 mosmol/l by the addition of 50 mmol/l NaCl exhibited 50 % AQP2 expression, whereas AQP2 expression was up to 75 % when the medium additionally contained 200 mmol urea (Figs 12 and 13). AQP2 expression declined gradually when medium tonicity was raised above 600 mosmol/l by NaCl (Fig. 13), an effect clearly attenuated by the addition of urea (Fig. 12; 900N, 900 250/100). There is yet no direct evidence for a stimulating effect of a combination of hypertonicity and urea on TonEBP action. A promoting effect of urea supplementation in hypertonicity elicited AQP2 expression in IMCD cells, was also evident in cells adapted to sorbitol-derived hypertonicity. Elevation of medium tonicity to 600 mosmol/l by addition of sorbitol (600S) clearly increased AQP2 expression, compared to cells grown at 300 mosmol/l. Nevertheless, AQP2 expression in cells cultured in 600S reached only ~50 % of controls (600N), which was increased to ~75 % in cells kept in 600S/U (containing 200 mmol sorbitol and 100 mmol urea; Fig. 14) and to a similar extent in 600Na/S (containing 200 mmol sorbitol and 50 mmol NaCl; Fig. 12). Sorbitol failed to increase AQP2 expression at all when used in a concentration of 600 mmol/l (as in 900S, Figs. 2,3 and

7). This suggests that sorbitol exerted a specific inhibitory effect on AQP2 expression in a concentration-dependent manner.

In the aggregate the data show that in IMCD cells, extracellular hypertonicity was substantial for a robust AQP2 expression, with hypertonic NaCl being more potent than sorbitol. Urea-derived hyperosmolality itself was not sufficient to stimulate AQP2 expression, but increased the promoting effect of hypertonicity on AQP2 expression.

Na^+/K^+-ATPase: effect on intracellular ionic strength and TonEBP activity. In hypertonically challenged MDCK cells, inhibition of Na^+/K^+-ATPase by ouabain or high extracellular K^+ concentrations strongly attenuated the induction of mRNAs of the TonE/TonEBP target genes AR, BGT-1, and SMIT. When challenged with 75 mM NaCl, intracellular ionic strength rose in those cells from ~190 mmol/kg wet wt to ~245 mmol/kg wet wt within 4 h and was as high as ~230 mmol/kg wet wt after 8 h. The intracellular ratio of K^+ to Na^+ concentrations (~9 :1) appeared unaffected. Ouabain-treatment lead to a clear increase in intracellular ionic strength and the ratio of K^+ to Na^+ concentrations was reversed (~1 : 7) (Neuhofer et al., 2002). Within a typical mammalian cell, Na^+, K^+, and Cl^- are the main constituents of intracellular ionic strength, with concentrations of ~5-15 mM Na^+, 140 mM K^+, and 5-15 mM Cl^-, whereas extracellular concentrations are in the range of ~145 mM Na^+, 5 mM K^+, and 110 mM Cl^-. The transmembrane sodium gradient constitutes the driving force for diverse secondary sodium-coupled transport processes and is established by Na^+/K^+-ATPase activity ([1]Alberts, 2002). In the kidney, the extracellular sodium concentration is far higher, which requires enhanced extrusion of Na^+ entering the cell down its concentration gradient. In collecting duct cells, the expression of the α, and β subunits of Na^+/K^+-ATPase increased with sodium- or mannitol derived tonicity (Ohtaka, 1996). This is in agreement with data presented by Capasso and co-workers, who reported that Na^+/K^+-ATPase α, and β subunit expression increased strongly in IMCD cells adapted to hypertonicity, as did *myo*-inositol uptake and HSP-70 expression. Na^+/K^+-ATPase α subunit expression in water-loaded or dehydrated mice (for 36 h) was unchanged in the renal cortex compared to euhydrated controls, but was significantly decreased upon water-loading in the papilla ([1]Capasso et al., 2001). Moreover, Na^+/K^+-ATPase γ-subunit was not expressed in isotonically cultured mIMCD3 cells. When these cells were acutely challenged with sodium-derived hypertonicity a strong increase in Na^+/K^+-ATPase γ-subunit expression was elicited. Highest Na^+/K^+-ATPase γ-subunit expression was observed in mIMCD3 cells adapted to hypertonicity. In contrast, mannitol-derived hypertonicity barely induced the Na^+/K^+-ATPase γ-subunit. In the inner medulla of water-loaded mice, Na^+/K^+-ATPase γ-subunit expression was found significantly decreased ([2]Capasso et al., 2001). It could be speculated whether the relatively weak AQP2 expression elicited by sorbitol in IMCD cells

might have its cause in inadequate intracellular ion composition or reduced intracellular ionic strength due to faint Na^+/K^+-ATPase γ-subunit expression. Taken together, these findings strongly suggest that adequate expression and activity of Na^+/K^+-ATPase, which is required to keep up the physiological intracellular ratio of K^+ to Na^+ concentration, is also essential for the hypertonic induction of TonEBP regulated genes.

Correlation between TonEBP activation and AQP2 expression in IMCD cells. In IMCD cells, a close relationship between TonEBP localization and AQP2 expression was observed. TonEBP was highly abundant in the nuclei of IMCD cells cultured in 600N medium (control). Under these conditions AQP2 expression was high. With the onset of hypotonicity, TonEBP abundance weakened in the nuclei and appeared in the cytoplasm (Fig. 19). Quantification of TonEBP signal intensities in nuclei and cytoplasm of IMCD cells revealed that hypotonicity significantly reduced nuclear TonEBP within 16 h and increased the portion of the (inactive) transcriptional enhancer in the cytoplasm (Fig. 20). In response to hypotonicity, AQP2 expression dropped to ~50 % of controls within 16 h. In the same time frame, DBcAMP withdrawal failed to decrease AQP2 expression significantly (Fig. 16). This again underlines that the reduction in AQP2 expression elicited by either hypotonicity or DBcAMP-depletion are mediated by different pathways.

When IMCD cells adapted to 300N medium were challenged with hyperosmolar urea, no increase in nuclear abundance of TonEBP was observed (Fig. 21). Hyperosmolar urea also failed to induce an increase in AQP2 expression (Fig. 12). In contrast, elevation of extracellular tonicity and urea concentrations for 19 h lead to a clear shift in TonEBP abundance to the nuclear compartment (Fig. 21). Correspondingly, AQP2 protein expression started to increase drastically with a lag-time of ~15 h after the onset of hypertonicity (Fig. 15). This is in agreemetn with data presented by Miyakawa and co-workers who reported that full activation of TonEBP in response to hypertonicity required more than 10 h (Miyakawa et al., 1999). The close temporal relationship between nuclear TonEBP localization and the levels of AQP2 protein detected, strongly support an involvement of TonEBP action in the regulation of AQP2 expression by tonicity.

In addition, it is illustrated that TonEBP protein expression was regulated by osmolality and solute composition in IMCD cells (Fig. 18). A clear reduction in TonEBP expression was evident when IMCD cells were cultured in 300N medium compared to controls (cultured in 600N medium). TonEBP expression increased comparing cells cultured at 300 mosmol/l to cells cultured at 350 mosmol/l. No further increase in TonEBP expression was elicited when sodium was added up to a final tonicity of 450 mosmol/l. Unexpectedly, the expression of TonEBP in IMCD cells was also promoted by a mere increase in osmolality by urea (Fig. 18).

This result stands somewhat in contrast to the finding that urea-pretreatment inhibited TonEBP mRNA expression and TonEBP action in mIMCD3 cells (Tian and Cohen, 2001). Aware of the fact that inhibition of TonEBP action by urea would be an obviously maladaptive event, and given that organic osmolyte uptake relies on TonEBP action in the urea-rich renal medulla, the authors suspected additional, urea-responsive events to obviate the need for maximally up-regulated TonEBP-dependent signalling *in vivo*.

Senescent rats: linking up AQP2 expression and medullary osmolality/tonicity. Evidence for an osmolality/tonicity-dependent regulation of AQP2 expression *in vivo* is provided by studies of Preisser et al. and Combet et al. on senescent rats. Senescent rats exhibited a strongly reduced urine concentrating ability and a reduced medullary osmolality. AQP2 expression was reduced by 80 % compared to adult animals, but papillary cAMP levels remained unchanged (Preisser et al., 2000). The impaired urine concentrating ability of senescent rats was restored by administration of dDAVP for 6 days. This treatment strongly increased papillary osmolality (736+/-41 mosmol/l [untreated] vs. 1,208 +/-42 mosmol/l [dDAVP-treated]) and AQP2 expression of senescent rats. dDAVP-treatment increased the abundance of UT-A1 urea transporter in the initial inner medullary collecting duct and the overall abundance of UT-A2, and increased the urea fraction of total papillary osmolytes. In adult rats, dDAVP-treatment did not further increase AQP2 expression and elicited only a modest increase in papillary osmolality (1,038+/-41 mosmol/l vs. 1,339 +/-76 mosmol/l). The authors proposed that restoration of papillary osmolality, not a specific action of dDAVP via V_2-receptors, was responsible for the recovery of AQP2 expression in dDAVP-treated, senescent rats. Measurements of papillary osmolality and urea concentration revealed that the non-urea fraction responsible for increased osmolality nearly doubled in dDAVP treated, senescent rats (Combet et al., 2003). With urea and sodium being the main interstitial osmolytes in the renal medulla, medullary tonicity was also clearly increased. This might have been brought about by increased apical sodium entry caused by enhanced epithelial sodium transporter (ENaC) abundance, which was shown to be elicited by dDAVP (Ecelbarger et al., 2000). Increased intracellular Na^+ concentrations in turn should increase expression and activity of Na^+/K^+-ATPase, and increase the PKA mediated increase of transactivation activity of TonEBP.

TonEBP and urea. Interestingly, the TonE/TonEBP pathway was shown to participate in medullary urea accumulation and in the protection of renal cells from the detrimental effects of high urea concentrations. Of the four isoforms encoded by the UT-A gene, UT-A1, UT-A3 and UT-A4 share the same promoter shown responsive to regulation by TonEBP (Nakayama et al., 2000), indicating that urea accumulation increases with TonEBP activity. During dehydration, the urea concentration is increased even more than the sodium concentration (Valtin et al., 1966;

Garzia-Perez and Burg, 1991), i.e. the balance of the primary interstitial medullary osmolytes is shifted in favor of urea, which is also due to the action of AVP on UT-A activity (Bagnasco et al., 2000; Combet et al., 2003). TonEBP protects from the denaturing effect of urea by promoting the accumulation of counteracting organic osmolytes (Yancey et al., 1982), and enhancing expression of the heat-shock protein HSP-70 (Woo et al., 2001). HSP-70 was found more abundant in the inner medulla compared to the cortex (Müller et al., 1996; Müller et al., 1998). Over-expression and down-regulation of HSP-70 protected medullary cells from cell death caused by high urea concentrations (Neuhofer et al., 1999; Neuhofer et al., 2001). So far, no evidence supporting a promoting effect of urea concentrations on TonEBP-dependent transcription is available but, considering that urea concentrations swing far more with the hydration status than tonicity, the question was raised whether urea-signaling might participate in determination of the subcellular localization of TonEBP (Woo et al., 2002). However, the importance of urea in the urine concentrating mechanism, was first reported decades ago. Protein deprivation was shown to decrease urine concentrating ability in animals and humans, which was rapidly restored by urea infusion, a procedure leading to an increase in the medullary urea concentration (Crawford et al., 1959; Levinsky et al., 1959; Penell et al., 1975). Schmidt-Nielsen and co-workers have shown that a low-protein diet (8% protein in chow instead of 24%) abolished the increase in urea concentration from medullary base (~700 mM urea) to papillary tip (~1000 mM urea) in the rat inner medulla, without affecting the sodium gradient. The decrease in medullary osmolality, caused by a low-protein diet, was therefore solely due to the reduction in medullary urea content (Schmidt-Nielsen et. al., 1985). More recently, the protein deprivation-induced decrease in urine concentrating ability has been linked to a decrease in AQP2 protein expression in the tip region of the inner medulla (Sands et al. 1996). In conjunction with the promoting effect of urea on the tonicity-elicited AQP2 expression (this study), these associations point to a crucial role of tonicity and urea in the regulation of AQP2 expression *in vivo*.

Does furosemide treatment abrogate TonEBP action? The washout of the medullary osmolyte gradient by the loop-diureticum furosemide favored the notion that neither tonicity nor osmolality participate in the regulation of AQP2 expression, because inner medullary AQP2 expression was unaffected (Terris et al., 1996; Marples et al., 1996, Marples et al., 1998). In these studies, only AQP2 protein levels were assessed. Therefore it was not ruled out whether furosemide might have decreased inner medullary AQP2 mRNA levels, but increased AQP2 protein stability or translation, thereby masking a putative negative effect on AQP2 transcription. Urine osmolality of furosemide treated rats fell from ~1200 mosmol/l to ~410 mosmol/l (Marples et al., 1996), indicating that medullary osmolality was still above 400 mosmol/l. Interestingly, SMIT signals in

the inner medullary collecting duct have been shown to be hardly affected by furosemide treatment (Yamauchi et al., 1995). The expression of BGT-1 mRNA was shown to decrease with furosemide treatment in the thick ascending limbs of henle, whereas no dramatic decrease was evident in the inner medullary collecting ducts (Miyai et al., 1996). In rats made chronically diuretic by a 6-day furosemide treatment, DdAVP administration lead to normalization of urine concentrating ability within 3 days. Medullary tonicity in form of Na^+, Cl^-, and K^+ concentrations was increased in response to DdAVP administration but intracellular tonicity (Na^+, Cl^-, and K^+ concentrations) was similar in chronic diuresis or after 3 days of recovery (Sone et al., 1995), suggesting that intracellular ionic strength was upheld during furosemide treatment. The peritubular hypotonicity elicited by furosemide treatment may activate transport processes, which keep up or even elevate intracellular ionic strength in the tubular epithelium itself. In thick ascending limb epithelium an exemplary mechanism is activated by hypotonicity. Upon peritubular hypotonicity, NHE3 activity is strongly up-regulated leading to luminal extrusion of H^+ concomitant with Na^+ uptake. The hypotonicity-elicited increase in NHE3 activity was not attenuated by furosemide (Watts et al., 1999). Moreover, basolateral sodium-urea countertransport in initial IMCD was shown to increase five-fold in rats made diuretic with furosemide (Kato and Sands, 1998). This should also contribute to preservation of intracellular ionic strength despite peritubular hypotonicity. In addition, furosemide triggers an increase in urea uptake via UT-A1 along the middle to terminal portion of the IMCD (Sands, 2000). As mentioned above, TonEBP participates in UT-A1 expression (Nakayama et al., 2000). If furosemide treatment would severely impair TonEBP action in the IMCD, UT-A1 expression and therefore its action would decrease rather than increase. It is proposed that, though furosemide diuresis abrogated the cortico-medullary osmotic gradient and greatly reduced medullary tonicity, the maneuver may have failed to lower ionic strength in IMCD cells and therefore did not abrogate the presumed action of TonEBP on AQP2 transcription. The furosemide experiment was originally performed to exclude the possibility that the detrimental effects of hypokalemia on AQP2 expression were due to reduced medullary osmolality caused by inhibition of NaCl transport in the thick ascending loop of henle (Gutsche et al., 1984). The molecular mechanisms involved in hypokalemia-induced AQP2 down-regulations remain to be resolved (see below).

TonEBP and hypokalemia. The time course of the onset of NDI and its relationship to AQP2 expression in potassium deprived rats was further investigated in a study by Amlal and co-workers. They revealed that after 12 h of potassium deprivation cortical-, but not medullary AQP2 expression was markedly reduced. AQP2 expression decreased prior to the onset of hypokalemia, i.e. a decrease in serum potassium concentrations, and thus elicited an early urine

concentrating defect prior to polydipsia. Because no decrease in serum potassium concentrations was detected within this time frame, it was suggested, that the diminished expression of cortical AQP2 mRNA and protein levels was due to a transcellular shift in potassium concentrations leading to intracellular potassium deprivation (Amlal et al., 2000). Dietary K^+ depletion was shown to decrease Na^+/K^+-ATPase activity in rat cortical collecting ducts (reviewed in Muto, 2001). In antidiuretic (water deprived) rats fed a K^+-depleted diet, intracellular ionic strength in papillary collecting duct cells fell to 138 +/-9 mmol/kg wet wt. Papillary collecting duct cells in euhydrated and antidiuretic rats on a normal K^+ diet exhibited an intracellular ionic strength of 171 +/-5 and 179 +/-11 mmol/kg wet wt, respectively. Total organic osmolyte content was also reduced in the medullae of K^+-depleted antidiuretic rats (356 +/- 14 mmol/kg wet wt) compared to antidiuretic control animals (465 +/-11 mmol/kg wet wt) (Beck et al., 2000). As mentioned beforehand, lowered intracellular strength as well as the reversal of the intracellular ratio of K^+ and Na^+ concentrations decreased the expression of TonEBP target genes, despite elevated extracellular tonicity (Neuhofer et al., 2002). In consequence of the several lines of evidence described above, it appears possible, that the NDI brought about by a potassium deprivation elicited reduction in AQP2 expression, might have its cause in a decreased TonE/TonEBP interaction within the AQP2 promoter (Fig. 17). The down-regulation of AQP2 expression in response to potassium deprivation might therefore be an example, for the clinical relevance of the TonE/TonEBP pathway in renal water handling.

AVP-independent expression of AQP2 in the male reproductive tract. Apart from its renal expression site, AQP2 was detected in the vas deferens of the male reproductive tract, but the principal cells there appear to interpret the gene´s regulatory region differently (Stevens et al., 2000). Both, the vas deferens as well as the kidney collecting duct are derived from the Wolffian duct during development. An AQP2 Cre recombinase transgene containing 14 kb of the human AQP2 upstream region was expressed in kidney and male reproductive tract in transgenic mice (Nelson et al., 1998). In contrast to the kidney collecting duct, the expression of AQP2 is not regulated by AVP in the vas deferens, where the water channel is constitutively present in the apical membrane. Transepithelial fluid absorption in the vas deferens is achieved together with AQP1 which is constitutively present in the basolateral membrane. AQP2 protein expression is absent in the proximal portion of the vas deferens, and increases towards the distal part (ampulla). Water reabsorption from the luminal fluid is thought to create the proper environment for maturation of sperm, and also increases sperm concentration (Stevens et al., 2000). Along the entire male genital tract, seminal fluid osmolality is higher than that of serum (Rossato et al., 2002). Interestingly, AQP2 mRNA in the vas deferens strongly increased with time of dehydration, whereas protein levels remained unchanged (Stevens et al., 2000). Therefore, it is

intriguing to speculate whether TonEBP action might participate in the transcriptional control of AQP2 in the vas deferens. Hypothetically, this might be a reminiscence to the common embryonic origin of collecting duct and vas deferens. It is yet unclear, how the dehydration-elicited increase in AQP2 mRNA is blunted on the protein level, a secondary mechanism which probably evolved to maintain fertility during dehydration, because marked hyperosmolality decreases sperm motility (Rossato et al., 2002).

The effects of agents impairing TonEBP action on AQP2 expression. A number of agents reported to interfere with TonE dependent transcription were tested for their influence on AQP2 expression.

Proteasome inhibition. Woo et. al. reported, that proteasome inhibition interfered with redistribution of TonEBP to the nuclei in MDCK cells challenged with hypertonicity, which led to a decrease in SMIT and BGT-1 mRNA levels compared to controls (Woo et.al., 2000). The present study shows that proteasome inhibition reduced the expression of AQP2 in IMCD cells kept in 600N medium to 50 % of controls and completely abolished the hypertonicity-induced recovery of AQP2 protein levels of cells expressing only faint levels of AQP2 due to prolonged cultivation in 300N medium (see Fig. 22). Proteasome inhibition decreased AQP2 protein degradation in a mouse cortical collecting duct cell line ($mpkCCD_{c14}$), but also abolished the AVP-induced expression of AQP2 mRNA (Hasler et al., 2002). This corroborates the assumption that inhibition of proteasomal function affects AQP2 expression on the transcriptional level. Using the same cell line, proteasome inhibition was shown to prevent the cAMP-independent activation of PKA by increased intracellular Na^+ concentrations, which impaired Na^+/K^+-ATPase targeting to the basolateral membrane (Vinciguerra et al., 2003). TAD activity of TonEBP increased with extracellular Na^+ concentrations which relied on hypertonically, but not cAMP-activated PKA ([1,2]Ferraris et al., 2002). These findings suggest that proteasome inhibition impairs TonEBP activity by a dual action. The impairment of nuclear localization observed in lactacystin or MG-132-treated and hypertonically challenged MDCK cells ([2]Woo et al., 2000) suggests that TonEBP is retained in the nucleus by an inhibitory protein which is marked for proteasomal targeting upon stimulation. The molecular mechanisms involved may be similar to that regulating the nuclear import of nuclear factor κB (NFκB). In the inactive state, NFκB is bound to IκB. Binding of tumor necrosis factor to its receptor activates IκB-kinase, which phosphorylates IκB and thereby marks the protein for ubiquitination and subsequent proteasomal degradation. This unmasks the NFκB nuclear localization signal and permits transcription of NFκB target genes ([2]Alberts, 2002). In addition, activation of PKA by hypertonicity is prevented by proteasome inhibition (Vinciguerra et al., 2003), leading to the failure of hypertonicity to activate the TAD of TonEBP. In the IMCD cells used here,

immunofluorescence studies revealed that proteasome inhibition decreased TonEBP-derived nuclear signal intensity compared to controls. This was observed when cells were continuously kept in 600N medium or challenged with 600N medium after prolonged cultivation in 300N medium (see Fig. 29 and 30). The decrease in nuclear TonEBP was not as marked as observed for cells challenged with hypotonicity, suggesting that reduced TAD activation, rather than impaired nuclear localization of TonEBP was responsible for the effect of proteasome inhibition on AQP2 expression.

Rottlerin. Rottlerin, a PKC δ inhibitor was shown to attenuate TonEBP-dependent transcription, presumably in a PKC δ – independent manner, because the negative effect on TonEBP action was not brought about by general PKC inhibitors (Zhao et al., 2002). The influence of rottlerin treatment on hypertonicity-induced AQP2 expression of IMCD cells was therefore tested. No effect of a 72 h rottlerin treatment (10 μM) on AQP2 expression was observed when IMCD cells were continuously kept in 600N medium (see Fig. 24). In contrast, the up-regulation of AQP2 expression in response to hypertonicity was completely abolished by a 48 h treatment with rottlerin (see Fig. 23). Data by Chou and co-workers indicated that PKC δ was highly abundant in isolated rat inner medullary collecting ducts (Chou et al., 1998). Hypertonicity was shown to induce membrane targeting of PKC δ to the plasma membrane of NIH/3T3 fibroblasts, which was necessary for ERK activation (Zhuang et al., 2000). Nevertheless, nuclear targeting of TonEBP appeared unaffected by rottlerin treatment of hypertonically challenged IMCD cells (see Fig. 30). The strong reduction in actin stress fiber abundance in IMCD cells challenged with hypertonicity in the presence of rottlerin is suggestive for a role of the actin cytoskeleton in the hypertonicity-elicited up-regulation of AQP2. Rottlerin was shown to potently inhibit PRAK (p38-regulated/activated kinase) and MAPKAP-K2 (MAPK-activated protein kinase 2) in in vitro assays (Davies et al., 2000). This opens up a number of pathways by which rottlerin could possibly interfere with TonEBP-dependent transcription.

MAPK-inhibition. Members of the mitogen-activated protein kinase (MAPK) family have been implicated to participate in the adaptation of cells challenged with hyper- or hypotonicity (Wojtaszek et al., 1998). Sheikh-Hammad et al. reported that p38 function is crucial for the expression of TonEBP-dependent genes in MDCK cells (Sheikh-Hammad, 1998). This finding stands in contrast to the observation of others. Kwon et al., reported that extracellular-signal-regulated kinase (ERK) activation was not necessary for the osmotic induction of genes encoding osmolyte transporters in MDCK cells (Kwon et al., 1995). In mIMCD3 cells, transcription driven by TonEBP was not affected by p38 inhibition or by expression of dominant-negative isoforms of MKK3 and MKK4, both activators of p38 (Kultz et al., 1997). Hoffert and co-workers reported that AQP5, the water channel expressed in lung, salivary, and

lacrimal gland epithelia, is induced by hypertonicity in MLE-15 cells (mouse lung epithelial cells) through an ERK-dependent pathway, inasmuch as AQP5 induction was prevented by PD 098059 or U0126 treatment (both inhibitors of ERK activation). Hypertonicity failed to induce activity of a 1.5 kb proximal promoter of AQP5, which did not bear a TonE. The authors suggest that, in a fashion similar to the SMIT-gene, TonE-elements are to be found outside this region (Hoffert et al., 2000). Recently, AQP1 expression was reported to be induced by hypertonicity involving a HRE (hypertonicity-responsive-element) in the promoter region, which is distinct from TonE. If the critical sequence (-54 to -46 of the human AQP1 gene) was changed from GCTCCCCCC to GCTTTCCCC, promoter activity was attenuated (Umenishi and Schrier, 2002). A further analysis revealed that p38 inhibition attenuated AQP1 promoter activity in response to hypertonicity (Umenishi and Schrier, 2003). Within the rat AQP2 promoter, an element closely matching the proposed HRE (GCTCCCCaC) was located at -248 to -239 (data not shown). Nevertheless, neither inhibition of p38 (by SB28059) nor inhibition of ERK activation (by PD 098059) interfered with the increase in AQP2 expression elicited by hypertonic challenge in the IMCD cells studied here. This indicates that the putative HRE is not involved in the regulation of AQP2 expression by tonicity.

On the contrary, both inhibitors alone -or in combination promoted the hypertonicity-elicited up-regulation of AQP2 protein levels in IMCD cells adapted to 300 mosmol/l (see Fig. 26). In IMCD cells continuously kept in 600N medium, no effect of the p38 inhibitor SB28059 was evident, but the MEK1 inhibitor PD098059 slightly increased AQP2 protein levels. Moreover, the magnitude of AQP2 down-regulation in response to hypotonicity was clearly reduced in the presence of the p38 inhibitor SB28059, or the MEK1 inhibitor PD098059, and to a lesser extent by the solvent DMSO. It was reported that glycerol and DMSO were effective in rescuing the transport of NDI-causing, misfolded AQP2 mutants to the plasma membrane. Without chemical chaperones, the misfolded AQP2 mutants were retained in the endoplasmatic reticulum and exhibited a reduced half-time due to degradation (Tamarappoo and Verkman, 1998). The attenuation of AQP2 down-regulation in response to hypotonicity in the presence of DMSO could therefore be due to stabilization of the native conformation of AQP2 leading to a reduction in AQP2 protein degradation. The stabilizing effect of DMSO, SB28059, or PD098059 on AQP2 protein levels was detectable after 24 h of hypertonic challenge (Fig. 27). Nevertheless, AQP2 expression continued to decrease when IMCD cells were challenged up to 120 h with hypotonicity in the presence of DMSO or SB28059 (Fig. 28), although baseline AQP2 levels were not reached (Fig. 15C). In the presence of PD098059 AQP2 down-regulation appeared to come to a halt after ~30 h (yielding ~55 % AQP2 protein of controls), and was increased to ~70 % of controls after 120 h (see Figs. 27 and 28).

Immunocytochemistry data show that long-term exposure of IMCD cells to hypo- or hypertonicity together with a combination of PD098059 and SB203580, lead to an increase in nuclei-bound TonEBP signal intensity compared to controls (see Fig. 30 and 31). This suggests that the p38 and/or the ERK pathway may be involved in the nuclear export of TonEBP. The actin appearance of the cytosceleton was not affected by treatment with PD098059 and SB203580.

Inhibition of the hypertonically activated kinases p38 or Fyn was reported to impair TonEBP transactivation activity (Ko et al., 2002). In contrast, Lee and co-workers reported recently that neither p38 nor Fyn inhibition consistently interfered with TonEBP transactivation activity. Transactivation activity was found to be mediated by five discrete domains of TonEBP that cooperate in activation of transcription. Two of the five domains mediated transactivation activity in response to hypertonicity. Transactivation activity appeared independent of TonEBP phosphorylation by protein kinases (Lee et al., 2003). These contradictory findings may have their cause in the different cell types used in these studies. Capasso and co-workers found no difference in JNK1, JNK2, ERK, and p38 expression comparing mIMCD3 cells grown at 300 mosmol/l or adapted to 600 mosmol/l. Comparable increases in tonicity yielded only a blunted increase in Jun-kinase and p38 kinase activity in cells adapted to 600 mosmol/l. The ability of adapted cells to activate these kinases was not impaired as indicated by a full response in Jun-kinase and p38 kinase activity upon hypotonic challenge. It was suggested that these MAP kinases, known to be involved in the initial response to hyper- and hypotonicty as regulatory volume decrease and increase, do not play a major role in the chronic adaptive response ([1]Capasso et al., 2001). Thus, it appears questionable whether TonEBP activity in medullary cells, which are constantly exposed to chronic but fluctuating hypertonicity, should be dependent upon p38 or ERK action.

In the IMCD cells investigated here, p38 and MEK1 inhibition increased the promoting effect of hypertonicity on AQP2 protein expression and attenuated the decrease in AQP2 protein expression elicited by hypotonicity. Although p38 and MEK1 inhibition appeared to increase the nuclear localization of TonEBP, it remains to be elucidated whether the effect of MAPK inhibition on the regulation of AQP2 expression in response to alterations in tonicity is exerted at the transcriptional level. Nonetheless, the presented results indicate that MAPK signalling may participate in the regulation of renal collecting duct water permeability.

Regulation of AQP2 by tonicity: a potential physiological benefit. A coupling of interstitial sodium-derived tonicity and urea concentrations along the collecting duct with the expression of AQP2 would make good sense physiologically and economically, considering that water reabsorption across the collecting duct can only occur, when the osmolality in the tissue surrounding the collecting

duct is high enough to provide a driving force for the reabsorption of water from the pre-urine. To allow for concentration of pre-urine along the collecting duct, medullary osmolality increases from the outer medulla towards the papilla. Moreover, the steepness of the osmolality gradient is increased during dehydration to allow further concentration of the urine. As mentioned above, the nuclear localization of TonEBP in the terminal part of the inner medulla is not altered by water loading or dehydration in rats, whereas the hydration status clearly affected the nuclear localization and activity of TonEBP within the outer medulla and the initial part of the inner medulla. The expression SMIT mRNA increased in this region with dehydration (Cha et al., 2001). During the swings between water diuresis and antidiuresis urea concentrations in the renal medulla change far more than do sodium concentrations (Valtin et al., 1966). Woo and co-workers therefore suggested that urea may participate in the signalling which determines the activity of TonEBP in the renal medulla (Woo et al., 2002). The finding that under hypertonic conditions, elevated urea had a stimulating effect on AQP2 expression in IMCD cells also points in that direction, but surely needs further proof.

AVP and TonEBP - major determinants in renal osmolyte accumulation, salt handling, and water conservation.

It appears that the intertwined actions of AVP and TonEBP are major determinants in renal osmolyte accumulation, salt handling, and water conservation. Final adjustments in renal Na^+ reabsorption take place in the renal collecting duct. In principal cells apically localized epithelial sodium channels (ENaCs) allow the entry of luminal Na^+, which is extruded via basolaterally localized Na^+/K^+-ATPase. AVP was shown to increase the expression of renal epithelial sodium channels (Ecelbarger et al., 2000) and activity of UT-A urea transporters (Combet et al., 2003), emphasizing the hormone´s importance in renal sodium and urea accumulation. It was shown that a rapid recruitment of Na^+/K^+-ATPase to the basolateral membrane compartment is stimulated in cortical collecting duct cells by cAMP (Gonin et al., 2001). This implicates that AVP action participates in modulating intracellular ionic strength and ion composition in renal cells, which in turn affects the activity of the TonE/TonEBP pathway. TonEBP mediates the intracellular accumulation of organic osmolytes required to maintain intracellular ionic strength, despite fluctuations in tonicity due to the renal concentrating mechanism. The expression of UT-A transporters was shown to be regulated by TonEBP, which thereby participates in the accumulation of high medullary urea concentrations required for water reabsorption. TonEBP also protects medullary cells from the denaturing effects of urea by increasing the expression of heat shock proteins.

The study presented here strongly suggests that TonEBP participates in renal water conservation *via* regulation of AQP2 expression. The finding that cPKA increased the transactivation activity of TonEBP upon hypertonic stimulation, a process not elicited by

elevated cAMP concentrations, but dose-dependently affected by extracellular sodium concentrations (Ferraris et al., 2000; Ferraris et al., 2000a), raises the question whether the TonE/TonEBP pathway might merge with the classical AVP-triggered pathway at the level of PKA activation *in vivo*. Though hypertonicity appeared unable to further activate DBcAMP-stimulated PKA, as suggested by unaltered CREB phosphorylation in IMCD cells grown at different tonicities (Fig. 11), hypertonicity might participate in the stimulation of PKA and thus activation of the CRE/CREB pathway when intracellular cAMP levels are low. This hypothetical setting might manifest itself in the dehydration-induced increase in AQP2 expression observed in rats treated with V_2R antagonists, provided that Ser-133 phosphorylation of CREB might partially be accomplished by hypertonically activated PKA. In addition, a diminished action of TonEBP at its binding site in the 5´regulatory region of the AQP2 gene might be the cause for the blunted AQP2 expression caused by potassium deprivation, which leads to a reduction in intracellular ionic strength and presumably to disturbances in intracellular ion composition. The data presented strongly suggest that elevated tonicity and an upheld intracellular ionic strength constitute a prerequisite for high-level AQP2 expression. Moreover, the TonE-element(s) present in the AQP2 promoter might participate in limiting the gene´s expression to tissues with intracellular tonicities above plasma levels, as is the case in the epithelium of the vas deferens and the kidney collecting duct.

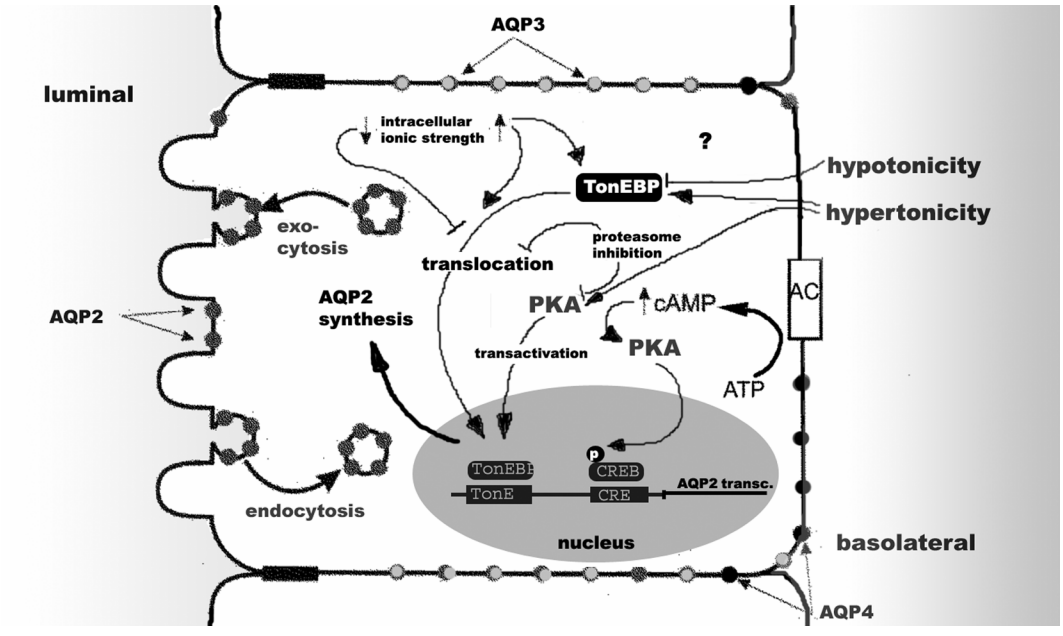

Fig. C. Extended model of AQP2 regulation in a renal principal cell including the TonE/TonEBP pathway. Arrows symbolize a positive and blunt ends indicate an inhibitory effect. Hypertonicity and elevated intracellular ionic strength increase the abundance of TonEBP and leads to its translocation to the nucleus. Hypotonicity and decreased intracellular ionic strength on the other hand

decrease TonEBP synthesis and the protein´s nuclear abundance. The sensor responding to extracellular hypertonicity or elevated intracellular ionic strength and triggering TonEBP action is yet unknown. PKA activated by hypertonicity (or increased intracellular ionic strength), increases the transactivation activity of TonEBP. TonEBP and pCREB appear to act in concert in setting off AQP2 transcription.

Based on the evidence accumulated in this study, I would like to propose a model for the long-term regulation of AQP2 including the TonE/TonEBP pathway (see Fig. C). Considering that in the inner medulla of rats TonEBP nuclear abundance was shown to be unaffected by water loading or water deprivation, it is most likely that hypertonic stimulation of AQP2 expression in this collecting duct segment is saturated, i.e. the classical AVP-triggered pathway determines AQP2 expression. This is different in the cortical and outer medullary segments of the collecting duct, where sodium-derived tonicity swings with the hydration status in a range affecting the nuclear abundance of TonEBP (Cha et al., 2001). Within these collecting duct segments, TonE/TonEBP interaction may set the margins for the effect of the AVP-triggered signalling cascade on the CRE/CREB interaction within the AQP2 gene´s regulatory region.

A TonEBP knockout mouse model, engineered after the work in hand was finished, corroborates the thesis that TonEBP participates in the expressional regulation of AQP2 (Storm et al., 2003). In the TonEBP deficient mutant mice, AQP2 expression in inner and outer medullary collecting ducts was markedly inhibited compared to control animals (López-Rodríguez et al., 2004).

In the aggregate, these findings broaden the knowledge of the long-term regulation of AQP2 and may prove useful in the treatment of diuretic states.