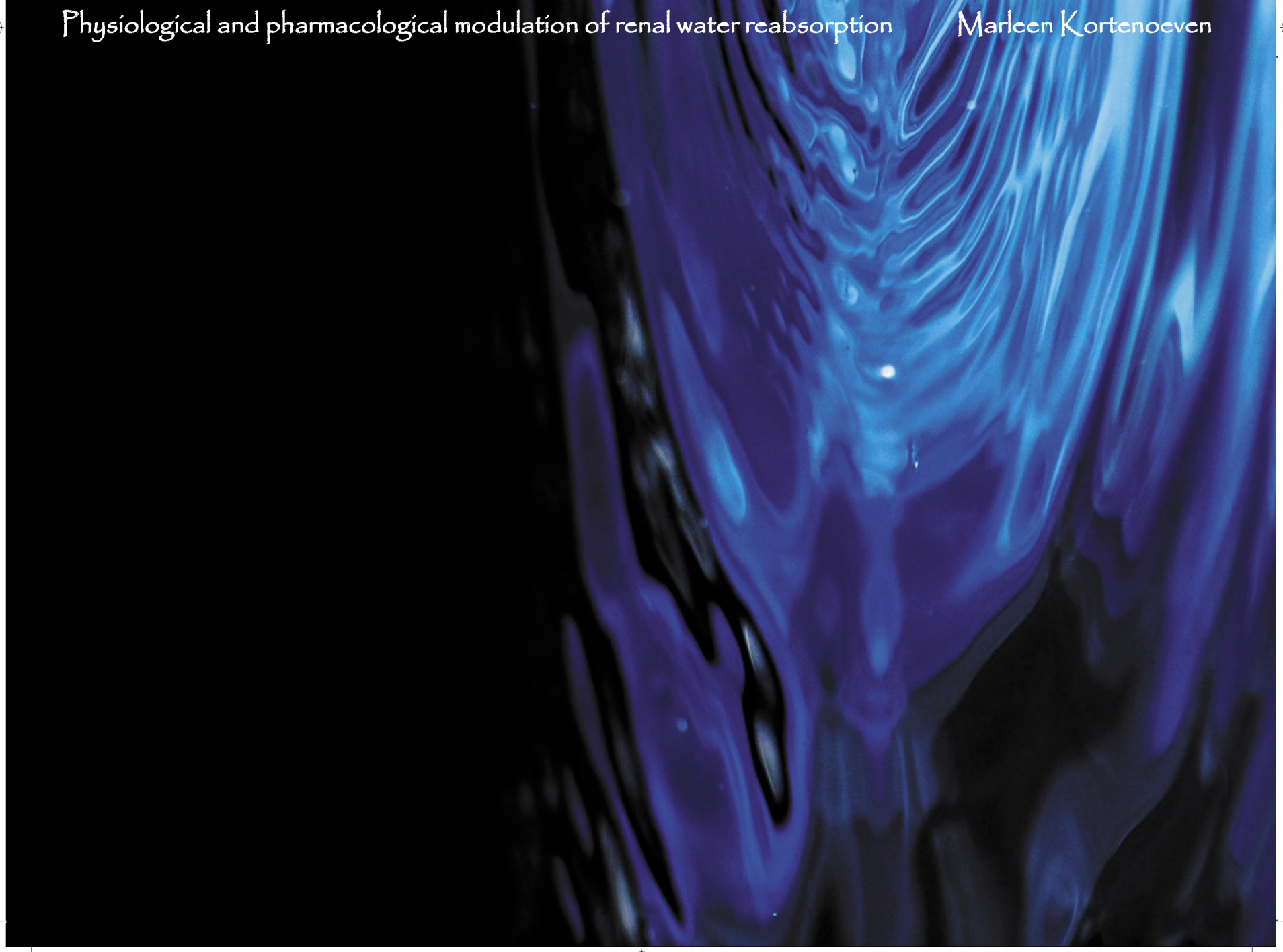
Physiological and pharmacological modulation of renal water reabsorption

Marleen Kortenoeven



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Physiological and pharmacological modulation of renal water reabsorption

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Medical Sciences

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CHAPTER 1

General introduction

General introduction

Water homeostasis

Maintaining water homeostasis by controlling both the blood osmolality and blood volume is essential for most physiological processes in the human body. Every day, water is lost through breathing, sweating, and in the urine and feces. Water is obtained through metabolism and food and water intake (1). While several of these processes occur autonomic, body water homeostasis is tightly controlled by regulating both water intake and urinary water excretion.

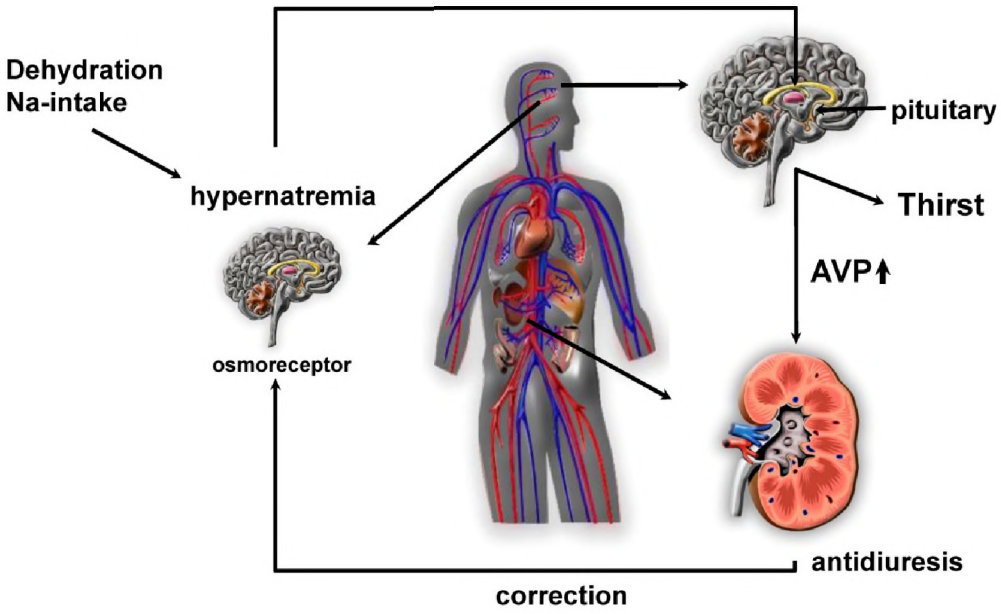

Figure 1. Body Osmoregulation. Osmoreceptors in the brain trigger physiological responses such as thirst, thus regulating water intake, and release of AVP from the pituitary, thereby reducing water excretion in the kidney.

A 1-2% rise in plasma osmolality or extracellular sodium concentration is detected by osmoreceptors located in the subfornical organ (SFO) and the organum vasculosum lamina terminalis (OVLT), two circumventricular organs of the hypothalamus (2; 3). In states of hypernatremia, these receptors are activated, which results in the sensation of thirst and subsequent water intake. Furthermore, neurons from the OVLT and SFO connect to the arginine vasopressin (AVP) producing magnocellular neurons of the supraoptic nucleus of the hypothalamus. Magnocellular neurons containing AVP project axons to the posterior pituitary, where they release AVP into the bloodstream upon activation (4) (see Fig. 1).

Besides osmolality, blood volume is tightly regulated as well, mainly by the renin-angiotensin-aldosterone system. In this process, regulation of renal sodium excretion plays an important role. However, body volume is also regulated by water intake and excretion. Thirst and AVP

secretion are influenced by blood volume and blood pressure changes, sensed by high and low pressure baroreceptors present in the aorta and the left atrium of the heart, and are also regulated indirectly by volume-mediated regulation of angiotensin II (2; 3; 5) (see Fig. 2). AVP secretion is far more sensitive to small changes in plasma osmolality than to changes in blood volume, as reductions in plasma volume of 10-15% are required before AVP is released. However, volume-induced regulation of AVP secretion is exponential, and severe reductions in blood volume will result in very high AVP levels (3; 5).

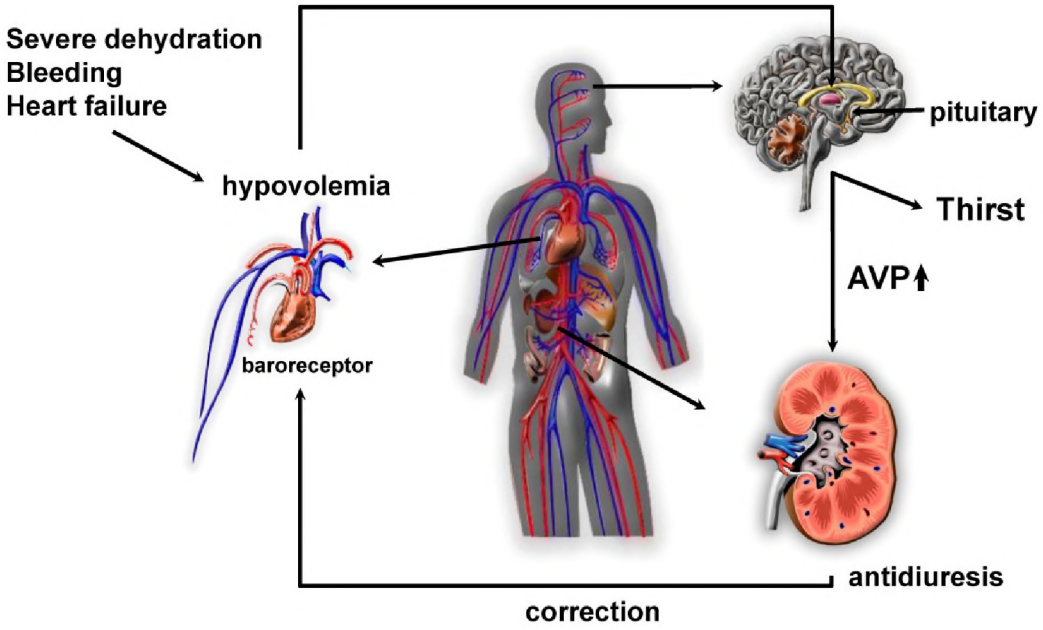

Figure 2. AVP in volume regulation. Baroreceptors in the heart and blood vessels trigger thirst, thus regulating water intake, and the release of AVP from the pituitary, thereby reducing water excretion in the kidney.

AVP raises the blood pressure by inducing moderate vasoconstriction, and it enhances blood coagulation. Besides this, AVP also increases the release of adrenocorticotropic hormone and influences learning, memory and social behavior (6; 7).

The most important role of AVP however, is to regulate the body's retention of water; it causes the kidneys to conserve water, thus concentrating the urine and reducing urine volume. AVP regulates the urinary concentrating process by increasing water permeability, urea permeability, and sodium transport (3). In the kidney, 180 liter of blood plasma is filtered by the human glomeruli each day. Less than 1% of this huge volume is excreted in the urine. Approximately 67% of the filtered water is reabsorped in the proximal tubule and 15% in the descending limb of Henle's loop, which both are constitutive processes. Depending on the body's needs, the remaining fluid can be reabsorbed in the connecting tubule and collecting duct, defining the final urine concentration. This process is tightly regulated, which allows the body to adapt to periods of water load or water restriction. The adjustment of water reabsorption mainly depends on the release of AVP (8).

Physiological regulation of AQP2

Regulation by vasopressin

AVP-regulated maintenance of the water balance in the renal nephron occurs mainly via modulating cell surface expression of the water channel aquaporin-2 (AQP2). AVP binds to the vasopressin type-2 receptor, present in the basolateral membrane of renal collecting duct principal cells and connecting tubule cells (9). This induces a signaling cascade, involving Gs protein mediated activation of adenylate cyclase, a rise in intracellular cAMP, activation of protein kinase A (PKA), and subsequent phosphorylation of AQP2 at Ser256. This results in the redistribution of AQP2 water channels from intracellular vesicles to the apical membrane, greatly increasing the osmotic water permeability, leading to a concentrated urine (10-13) (see Fig. 3). A study in oocytes showed that phosphorylation of at least three of four monomers of an AQP2 tetramer is needed to redistribute AQP2 tetramers from storage vesicles to the apical membrane (14). Driven by the transcellular osmotic gradient of sodium and urea, water will enter principal cells through AQP2 and leave the cells via AQP3 and

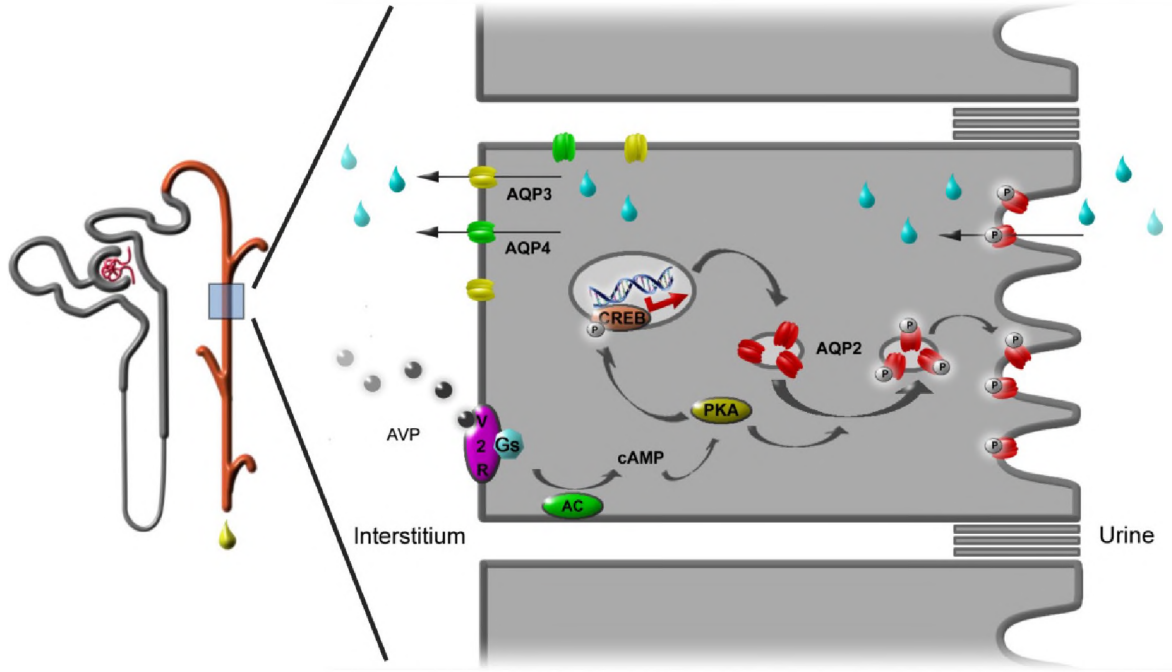

Figure 3. Regulation of AQP2-mediated water reabsorption. AVP binds to the vasopressin type-2 receptor, present on the basolateral membrane of renal collecting duct principal cells and connecting tubule cells. This induces a signaling cascade, involving Gs protein mediated activation of adenylate cyclase, a rise in intracellular cAMP, activation of protein kinase A (PKA), and subsequent phosphorylation of AQP2 at Ser256. This results in the redistribution of AQP2 from intracellular vesicles to the apical membrane. Driven by the transcellular osmotic gradient, water then will enter principal cells through AQP2 and enters the blood via AQP3 and AQP4 water channels, which are constitutively expressed in the basolateral membrane, resulting in concentrated urine. On the long term, vasopressin also increases AQP2 expression via phosphorylation of the cAMP responsive element binding protein (CREB), which stimulates transcription from the AQP2 promoter.

AQP4 water channels, which are constitutively expressed in the basolateral membrane, resulting in concentrated urine (15; 16). Once the water balance is restored, AVP levels drop and AQP2 is internalized via ubiquitination at Lys270 (see Fig. 4) (17).

In addition to its effect on AQP2 localization, cAMP also increases AQP2 expression via phosphorylation of the cAMP responsive element binding protein (CREB), which stimulates transcription from the AQP2 promoter (18-20). The increase in transcription is chronic, requiring hours to take effect (21; 22).

Recently, it has been shown by phosphoproteomic analysis of isolated rat renal inner medullary collecting duct cells, that besides the phosphorylation at Ser256, AQP2 can also be phosphorylated at Ser261, Ser264 and Ser269 (Thr269 in human AQP2, see Fig. 4) (23). While Ser264 and Ser269 phosphorylation are, like Ser256, increased upon administration of the AVP analogue dDAVP to AVP-deficient Brattleboro rats, phosphorylation of Ser261 is decreased (24-26). AQP2 phosphorylated at either site shows distinct subcellular localizations. Phosphorylated Ser261-AQP2 is mainly found in intracellular vesicles and seems to be absent from the plasma membrane (24). Ser264-AQP2 resides in intracellular vesicles and the plasma membrane, and upon treatment with dDAVP there is a change in distribution from predominantly intracellular vesicles to the plasma membranes (25). Finally, phosphorylated Ser269-AQP2 is solely found in the apical plasma membrane and not in intracellular vesicles. Madin-Darby canine kidney cells expressing an Ser269Asp "phosphomimic" AQP2 mutant showed constitutive localization at the plasma membrane suggesting that Ser269 phosphorylation enhances apical plasma membrane retention of AQP2 (26). However, the exact function of these phosphorylation sites remains to be established.

Tonicity

AQP2 expression is not only regulated by AVP but also by other factors. For example osmolality not only affects plasma AVP, but also appears to have direct effects on AQP2 expression and urine concentrating ability. In the syndrome of inappropriate antidiuretic hormone secretion (SIADH), for example, levels of AVP are inappropriately high relative to plasma osmolality, resulting in free-water retention and hypotonicity. Under these circumstances, however, the free-water excretion is considerably higher than would be expected from the AVP concentrations (27). This phenomenon, called vasopressin escape, indicates that there are mechanisms counteracting AVP action. In a rat animal model, it has been shown that the onset of this escape coincides with a decrease in the AQP2 expression in the renal collecting duct (28). Hypotonicity and/or volume expansion have been proposed to mediate this AVP-independent direct regulation of AQP2 expression (29).

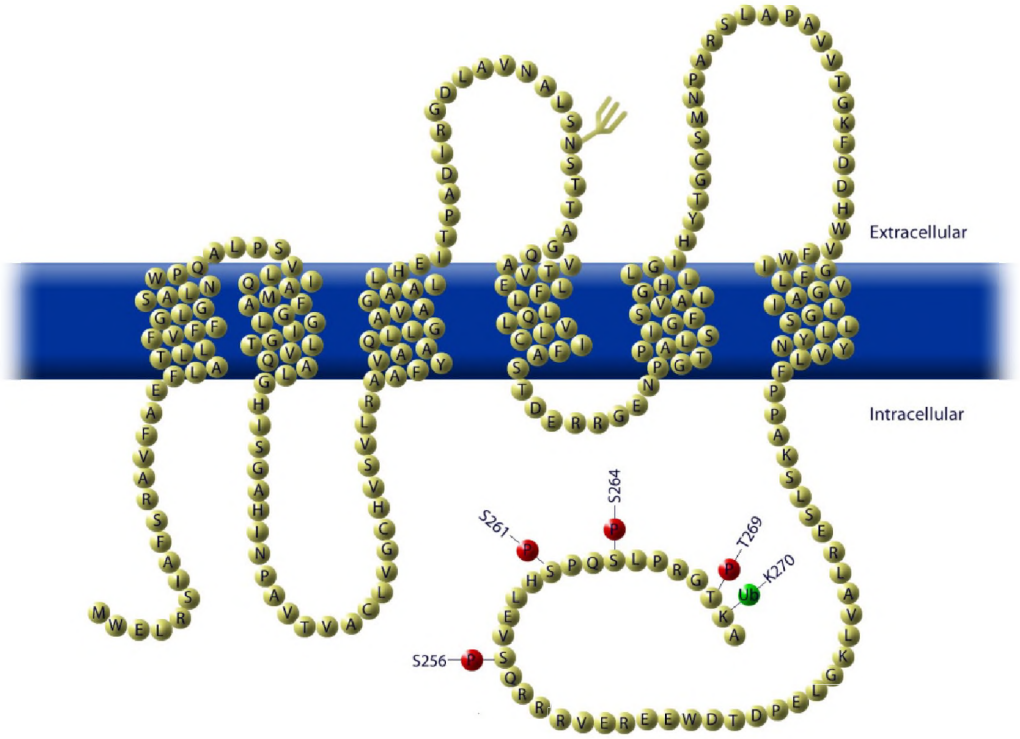

Figure 4. Schematic representation of the human AQP2 protein. AQP2 is a 271 amino acid protein that is predicted to have six transmembrane domains with its N and C-terminus intracellular. The ubiquitination site (Ub) and phosphorylation sites (P) in the C-terminus are indicated.

Alternatively, an AVP-independent increase of AQP2 expression has been suggested to occur with *hyper*tonicity. Valtin and Edwards reported that water deprivation of AVP-deficient Brattleboro rats leads to hypertonicity, increased papillary interstitial osmolality, and concentration of the urine (30). Similarly, Li et al found that hypertonicity due to hyperglycemia or increased NaCl levels increases AQP2 expression in Brattleboro rats (31).

In mouse collecting duct principal cells (mpkCCD$_{c14}$), Hasler *et al.* showed that hypertonicity decreases the abundance of AQP2 on the short term, but increases its abundance and mRNA levels on the long term (32). Here, hypertonicity did not affect the stability of AQP2 mRNA or protein, indicating that hypertonicity increases AQP2 abundance by increasing its transcription.

A protein suggested to be involved in the hypertonicity response is the tonicity-responsive enhancer binding protein (TonEBP). TonEBP is upregulated with hypertonicity in cultured cells and in the kidney medulla (33; 34). In renal epithelial cells, acute hypertonic challenge triggers a rapid increase in expression of genes involved in the cellular accumulation of osmolytes, such as sodium/myo-inosytol co-transporter and the sodium/chloride/betaine co-transporter, leading to cell survival in hypertonic conditions (35). Expression of these genes is increased by TonEBP (35).

However, TonEBP might also have effects on urinary concentration as TonEBP knockdown or inhibition, but also mutation of the tonicity responsive element (TonE) in the AQP2

promoter, reduced AQP2 expression in mpkCCD cells (36; 37). Moreover, in TonEBP$^{-/-}$ mice and mice transgenic for dominant-negative TonEBP, AQP2 expression was decreased (38; 39). However the severe atrophy of the renal medulla in these mice complicates the interpretation of the direct involvement of TonEBP in AQP2 regulation. Kasono *et al.* observed a hypertonicity-induced increase in AQP2 transcription in MDCK cells only when the AQP2 promoter was -6,1 kb or longer and therefore did not involve TonE, which localizes at 489 bases upstream of the AQP2 transcription start site (29), suggesting that TonE and TonEBP are not involved in AQP2 regulation.

In addition to TonEBP, activation of the nuclear factor of activated T cells c (NFATc) pathway has been suggested to be involved in hypertonicity. Li *et al.* reported that NFATc translocates to the nucleus upon hypertonicity and that NFATc binding sites downstream of TonE are also important for hypertonicity-induced AQP2 expression in mpkCCD cells (37).

Prostaglandins

Besides AVP, several other hormones and chemical messengers regulate the water balance by antagonizing the AVP-induced water transport, like extracellular purines and dopamine (40) (see Fig. 5). Prostaglandins are also involved in regulation renal water transport. In particular, prostaglandin E_2 (PGE_2) has been shown to decrease AVP-stimulated water reabsorption in perfused collecting ducts (41-43). Of the four E-prostanoid receptor subtypes, both EP1 receptor activity, inducing calcium mobilization, and EP3 receptor activity, inhibiting cAMP generation via Gi, can inhibit AVP-stimulated water reabsorption (42; 44-46). One mechanism of PGE_2 counteracting AVP is by retrieving AQP2 from the plasma membrane, as shown in MDCK cells and inner medulla suspensions (47; 48). Furthermore, PGE_2 modulates the cytoskeleton. It has been shown in primary rat inner medullary collecting duct cells that PGE_2 binding to its EP3 receptor activates Rho, thereby inhibiting water permeability by preventing depolymerization of the actin cytoskeleton, necessary for AQP2 translocation (49).

Paradoxically, although PGE_2 decreases water reabsorption in the presence of AVP, it increases the osmotic water permeability in collecting ducts in the absence of AVP (42; 43). The stimulatory effect of PGE_2 on basal water permeability is most likely mediated via the EP4 receptor, as this receptor couples to Gs, thereby activating adenylate cyclase and increasing cAMP levels.

Besides PGE_2, also $PGF_{2\alpha}$ affects water homeostasis, as it has been shown to inhibit AVP-stimulated water permeability in the collecting duct (50; 51). Activation of FP receptor leads to calcium mobilization in most cell types, but in the collecting duct, FP receptor activation seems to inhibit cAMP generation via Gi proteins, thereby inhibiting water reabsorption (50).

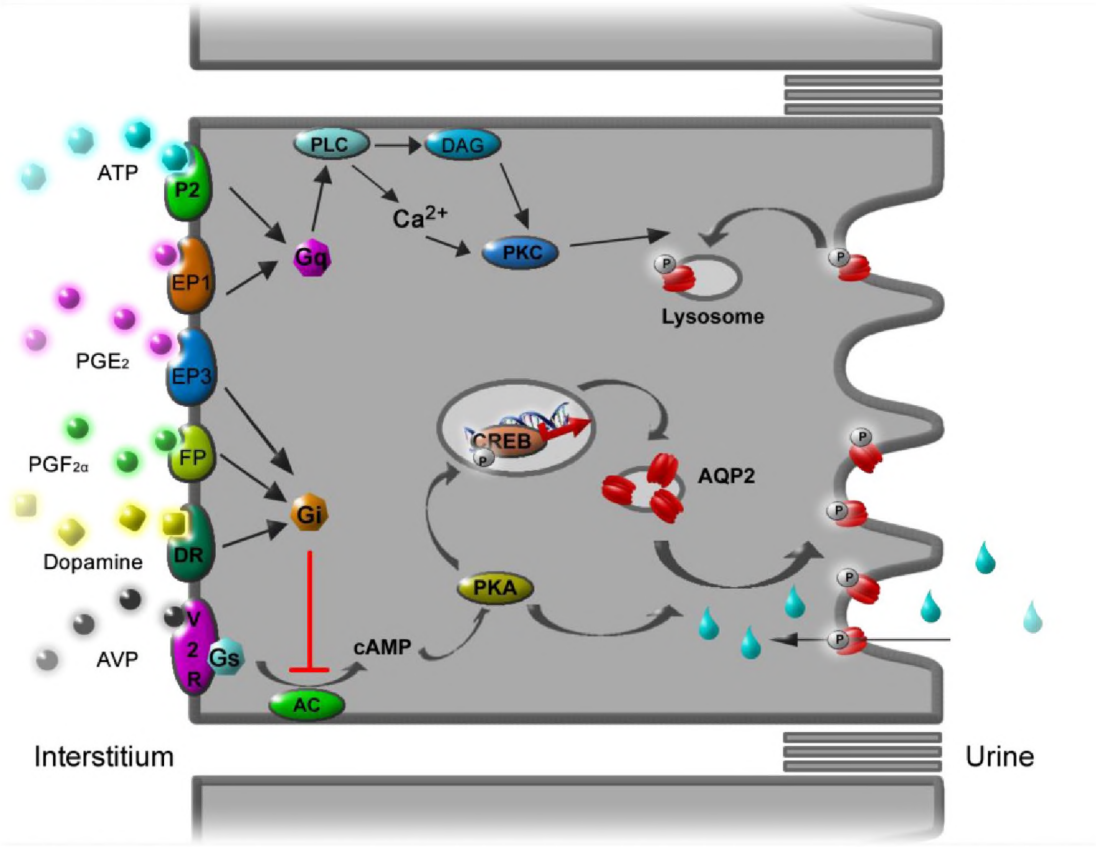

Figure 5. Hormonal inhibition of AQP2-mediated water reabsorption. Several hormones can antagonize AVP-induced water transport. Indicated are adenylate cyclases (*AC*), aquaporin-2 (*AQP2*), vasopressin *(AVP)*, cyclic adenosine monophosphate *(cAMP)*, Diacylglycerol *(DAG)*, dopamine receptor *(DR)*, prostaglandin E receptor 1 (*EP1*), prostaglandin E receptor 3 (*EP3*), prostaglandin F receptor (*FP*), *P2* purinergic receptor (*P2*), prostaglandin E_2 *(PGE_2),* prostaglandin $F_{2\alpha}$ *($PGF_{2\alpha}$)*, phospholipase C *(PLC)* , vasopressin V2 receptor *(V2R)*.

Dopamine

Dopamine has been shown to decrease AVP-stimulated water permeability and cAMP production in isolated cortical and inner medulla collecting ducts (52-54). Dopamine is synthesized in renal proximal tubular cells and possibly in the inner medulla collecting duct. Also renal dopaminergic nerves may contribute to the amount of renal dopamine (55). In the cortical collecting duct, the diuretic effect of dopamine is mediated by activation of D4 receptors, coupling to Gi (53; 56). In the inner medullary collecting duct, the inhibitory effect of dopamine is suggested to be mediated through α_2-adrenergic receptors, which can also be activated by high concentrations of dopamine (52). Like PGE_2, dopamine also causes AQP2 internalization from the plasma membrane into intracellular storage vesicles in MDCK cells (48).

Extracellular purines

Extracellular purines, ATP and UTP, have been shown to decrease AVP-induced water

permeability in isolated inner medulla collecting tubules (57; 58). Extracellular purines appear to attenuate the AVP-triggered increase of intracellular cAMP, which is thought to be conferred by increasing intracellular Ca^{2+} levels and the activation of a counteracting protein kinase C (58). There are also indications that ATP may affect AQP2-mediated water permeability indirectly by stimulating the release of PGE_2 (59).

Extracellular purines can mediate their action via the ionotropic P2X receptors as well as the metabotropic, G-protein-coupled P2Y receptors. Various P2X and P2Y receptors have been identified in the collecting duct (60; 61).

The involvement of the P2Y2 receptor in water reabsorption is suggested by a recent study on P2Y2 receptor knockout mice showing that, although net urinary reabsorption was not different compared to wild-type mice, these knockout mice showed increased renal medullary expression of AQP2, as well as elevated urinary cAMP excretion (62). A recent study in oocytes showed that, besides P2Y2, activation of P2X2 and P2Y4 receptors by ATP decreases AQP2 plasma membrane expression as well (63). Whether other P2 receptors contribute to the purinergic effect on AVP-mediated water reabsorption is unclear.

Water homeostasis-associated pathologies

Increased AQP2

Disturbances of water balance occur frequently, and are characterized by hyponatremia, hypernatremia or polyuria, and can be mediated by too high or too low levels of AQP2. Hyponatremia with high AQP2 abundance can be caused by osmoregulation disorders, but is most frequently caused by diseases with low effective circulating volume. Since in these conditions, maintaining volume balance overrules osmoregulation, development of hyponatremia is the mere consequence of the body's goal to maintain circulating volume. Congestive heart failure (CHF) is characterized by high levels of AVP which contribute to hyponatremia and increased extracellular volume (64). In CHF rat models, increased AQP2 levels and a marked redistribution of AQP2 to the apical plasma membrane were found (65; 66). Both AQP2 protein and AQP2 mRNA levels were increased (66).

Hepatic cirrhosis is another chronic condition associated with water retention, hyponatremia and increased AVP levels (64). However, unlike CHF, the changes in expression of AQP2 protein levels vary considerably between different experimental models of hepatic cirrhosis. In several rat models of liver cirrhosis, increased AQP2 protein and mRNA levels were found (67; 68). In another study, however, total AQP2 levels were not changed, but an increase in plasma membrane expression of AQP2 was observed (69). Other rat models of liver cirrhosis

displayed decreased AQP2 levels and impaired water reabsorption (70; 71). An explanation for the differences found between these rat models is at present lacking.

There are several disorders of disturbed osmoregulation. In the syndrome of inappropriate secretion of antidiuretic hormone (SIADH), AVP levels are abnormally increased, leading to excessive renal water reabsorption, which might result in life-threatening hyponatremia (72). It was shown in a rat model of SIADH that AQP2 levels are markedly increased and that AQP2 plays an important role in water retention and development of hyponatremia in SIADH (68). SIADH patients are normovolemic or slightly hypervolemic. The most common causes of SIADH are neoplasia, neurological diseases, lung diseases and a wide variety of drugs (73). Most commonly, SIADH patients show excessive release of AVP which is totally unrelated to plasma osmolality, caused by for example ectopic AVP production by neoplasms, or patients continue to regulate water excretion but do so around a lower plasma osmolality setpoint compared to normal. The underlying mechanism of this is unclear (73).

Another cause of normovolemic hyponatremia is the Nephrogenic Syndrome of Inappropriate Antidiuresis (NSIAD). The clinical presentation of NSIAD resembles those of SIADH and consist of hyponatraemia and the lack of urinary dilution. However, in contrast to SIADH, the AVP levels in plasma are undetectable or very low (74). NSIAD is a rare disorder in water balance caused by an activating mutation in the AVP receptor type 2 (V2R), causing the constitutive activation of the receptor. The basal ability of the mutant V2R to induce cAMP, as measured by cAMP-inducible luciferase reporter in COS-7 cells, was shown to be increased compared to the wild-type V2R (75). This gain of function due to constitutive activation of V2R in NSIAD patients explains the inappropriate antidiuresis.

Decreased AQP2

In contrast to the conditions mentioned above, Diabetes Insipidus (DI) is characterized by an impaired renal water reabsorption, leading to polyuria and, consequently, polydipsia. DI can present with hypernatremia, but patients most often have a normal plasma osmolality, provided the thirst mechanism is normal and there is adequate access to fluid. In central DI, normal AVP production is impaired. Central DI can be caused by mutations in the AVP gene, but is usually occurring as a consequence of head trauma or diseases in the hypothalamus or pituitary gland (76). In a model of central DI, the AVP-deficient Brattleboro rat, it was shown that these rats have decreased expression levels of AVP-regulated AQP2, and the AQP2 deficit was reversed by chronic AVP infusion, suggesting that patients lacking AVP are likely to have decreased AQP2 expression (77). Administration of the synthetic AVP homolog dDAVP is usually able to drastically decrease urine output in central DI patients.

A second form of DI can occur during pregnancy, due to an abnormal increase in vasopressinase, an AVP-degrading enzyme produced by the placenta. Because dDAVP is resistant to degradation by vasopressinase, it is an effective treatment for this form of DI (78).

The last form of DI is called nephrogenic DI (NDI), and is caused by the inability of the kidney to respond to AVP stimulation. NDI can be congenital or acquired. Congenital NDI can be divided into X-linked, autosomal recessive and autosomal dominant NDI.
The X-linked form of congenital NDI is caused by loss-of-function mutations in the *AVPR2* gene, encoding the V2R (79). More than 90% of all congenital NDI patients suffer from X-linked NDI. Mutations in the AVPR2 gene interfere with receptor signaling, thus making the principal cells of the collecting duct insensitive to AVP. This results in a severe urine concentration defect. The molecular mechanism underlying this insensitivity differs among mutants. The most common mechanism is misfolding of the protein and retention in the endoplasmic reticulum. Other mechanisms include defective processing or unstable mRNA, diminished binding of the Gs protein, reduced affinity for AVP, and misrouting of the V2R to different organelles in the cell (80).
Approximately 10% of the patients diagnosed with NDI have mutations in the *AQP2* gene. In more than 90% of patients there is evidence of autosomal recessive inheritance. Nearly all mutations cause misfolding of the protein, which is consequently trapped in the ER, followed by rapid proteasomal degradation (80). The healthy parents of patients with recessive NDI express both mutant and wild-type AQP2 proteins. However, since these AQP2 mutants are not able to form heterotetramers with wild-type AQP2, this leaves only the formation of the functional wild-type AQP2 homotetramers, likely explaining the healthy phenotype of the parents (81).
The least occurring form of hereditary NDI, autosomal dominant NDI, is caused by mutations in the C-terminal tail of AQP2. These AQP2 mutants form heterotetramers with wild-type AQP2 and are, due to the mutation, missorted to other cellular organelles (80). Since wild-type AQP2 is retained in mixed tetramers and also missorted, water reabsorption is severely affected, explaining the dominant mode of inheritance of NDI in these patients.

In contrast to the rare inherited forms of NDI, acquired forms of NDI are much more common. It has been demonstrated that hypokalemia and hypercalcemia, two common electrolyte disorders, cause NDI.
In rat models of hypokalemia it was shown that this condition is associated with a decreased AQP2 expression (82). Besides AQP2, hypokalemia also decreases the expression of renal

urea and sodium transporters, which may contribute to the urinary concentrating defect (83; 84).
Hypercalcemia does decrease AQP2 abundance in rats as well, but does not affect AQP2 mRNA levels. This suggests that there is no effect on AQP2 transcription, but that hypercalcemia increases AQP2 degradation or decreases AQP2 translation. Hypercalcemia also decreased the fraction of AQP2 at the apical membrane, suggesting an effect on AQP2 targeting (85). Hypercalcemia also reduces the expression of the water channels AQP1 and AQP3, and decreases the expression of renal sodium transporters, likely contributing to the decreased urine concentrating ability (86; 87).

A relatively common condition associated with long-term impairment of urinary concentrating ability is obstruction of the urinary tract. Urinary tract obstruction is a serious clinical condition, which in children is usually due to congenital abnormalities, whereas in adults it is mostly caused by stones, enlargement of the prostate, and urinary tract neoplasms. Experimental bilateral obstruction of the ureters was found to be associated with markedly reduced expression of AQP1, AQP2 and AQP3 (88; 89) In addition, bilateral ureteral obstruction is associated with marked down-regulation of sodium transporters and urea transporters (90; 91). Following release of the obstruction, there is a marked polyuria during which period AQP2 and AQP3 levels remain down-regulated, but are restored to normal in time, providing an explanation at the molecular level for the observed postobstructive polyuria (89).
Recently, bilateral obstruction has been demonstrated to be associated with increased cyclooxygenase-2 (COX-2) expression and prostaglandin release from interstitial cells. Treatment of rats with COX-2 inhibitors prevented down-regulation of AQP2 (92; 93).

Lithium-treatment
The most common form of NDI is lithium-induced NDI. Lithium is regularly used to treat psychiatric diseases, such as bipolar disorders, schizoaffective disorders and depression. Lithium is a frequently prescribed drug and used by 1 in 1000 of the population (94; 95). Unfortunately, approximately 20% of patients develop NDI (96-98). Lithium-NDI patients are at risk for dehydration-induced lithium toxicity, and prolonged lithium treatment might lead to end stage renal disease (94). However, since the symptoms of the underlying psychiatric disorder have a high impact on the quality of life, cessation of lithium therapy is not an option for many patients.
Studies in rats showed that lithium-NDI develops in two stages. At the short term (10 days), lithium-NDI coincides with AQP2 and AQP3 down-regulation and natriuresis, without gross changes in renal morphology (99-101). The lithium-induced natriuresis is suggested to be

due to the reduced expression, in some renal segments, of the salt-transporting proteins NCC, the β- and γ-subunits of ENaC, and the Na-K-ATPase (99; 102; 103).
Chronic lithium treatment (4 weeks) also leads to a severe decrease in the fraction of principal cells. This is 'compensated' by an increase in the fraction of intercalated cells, which are involved in acid/base balance regulation (104; 105).
At present, it is unclear how lithium causes NDI. Some data, however, suggest that lithium may exert its effects by entering principal cells through the epithelial sodium channel, ENaC. ENaC has a higher permeability for lithium than for sodium (106), the ENaC-blocker triamterene increases lithium excretion (107) and it has been shown in a limited number of lithium-NDI patients that blocking ENaC with amiloride significantly reduces urine volume and increases urine osmolality (108-110).
Recently it was shown in mpkCCD cells, which endogenously express AQP2, that lithium does not affect AVP-induced cAMP generation, PKA-dependent phosphorylation of AQP2, or the phosphorylation of the AQP2 transcription factor CREB. Also *in vivo*, in AVP-deficient Brattleboro rats with clamped blood dDAVP levels, there was no difference in dDAVP-generated cAMP generation after lithium treatment (111).
Lithium has been shown to decrease renal medullary glycogen synthase kinase (Gsk)-3β activity, and this was temporally related to increased COX-2 expression in the kidney, consistent with a tonic *in vivo* suppression of COX-2 expression by Gsk-3 activity (112; 113). Consistent with the role of COX-2 in prostaglandin production, lithium increased excretion of PGE_2 in the urine of rats and mice (112; 114). As PGE_2 reduces AVP-stimulated water reabsorption in the collecting duct (41; 42), this could suggest an important role for PGE_2 in the development of lithium-induced NDI. This is also suggested by studies showing that blocking prostaglandin production by indomethacin reduces the urine volume of lithium-treated rats (115) as well as of patients with lithium-induced NDI (116; 117).

Pharmacological regulation of AQP2-related pathologies

Demeclocycline

Demeclocycline is a bacteriostatic antibiotic of the tetracycline group, which has been shown to cause water diuresis and NDI (118; 119). Because of the effect on water diuresis, demeclocycline is currently used to treat sustained hyponatremia in patients with SIADH (120). Demeclocycline has been shown to restore the sodium plasma concentration in SIADH patients to normal levels, permitting unrestricted water intake in these patients (121).
Wilson *et al.* showed that the aquaretic effect of demeclocycline is exerted by selective inhibition of the water reabsorption in the distal part of the nephron (122). Others have shown

that demeclocycine inhibits the AVP-induced osmotic water flow in the toad urinary bladder (118; 123; 124), a model system of the mammalian collecting duct.

V2R antagonists

Recently, several V2-receptor antagonists have been developed. In rat models of SIADH and liver cirrhosis, administration of the V2R antagonist OPC-31260 increased the urinary flow rate, lowered the urinary osmolality and attenuated the hyponatremia (125; 126). Besides that, OPC-31260 has been shown to produce water diuresis and improve hyponatremia in SIADH patients as well (127). Three other V2R antagonists, Tolvaptan, Lixivaptan and Conivaptan, were tested in patients with hyponatremia due to a variety of causes including CHF, liver cirrhosis and SIADH and were found to increase diuresis and correct hyponatremia (128-130). The V2R antagonist SR-121463B increased the urinary volume, decreased urinary osmolality and corrected the hyponatremia in patients with SIADH, suggesting that also SR-121463B is a successful treatment of hyponatremia (131). Altogether, this suggests that the V2R antagonists are promising drugs for the treatment of hyponatremia.

Diuretics

At the moment, thiazide diuretics are used to reduce excessive urine output in NDI patients. In 1960, the first study was published that showed that hydrochlorothiazide could decrease urine volume and increase urine osmolality in patients with congenital NDI (132). Since then, thiazides have become an important component in the treatment of patients with congenital diabetes insipidus. Also in lithium-induced nephrogenic diabetes insipidus, thiazide diuretics have been used successfully (119).

The precise mechanisms by which thiazide diuretics elicit their paradoxical anti-diuretic effect are largely elusive. The distal convoluted tubule, where the thiazide-sensitive NaCl-cotransporter is located, is water impermeable; therefore, the water-preserving effect of thiazides is unlikely related to a direct effect on this tubule. Thiazides antidiuretic action might by secondary to an increased renal sodium excretion, causing a decreased extracellular volume leading to an activation of the renin-angiotensin-aldosterone system, a decrease in glomerular filtration rate and an increased proximal sodium and water reabsorption. As a result, less water is delivered to the distal tubules, and less is excreted in the urine. Recently it has also been shown that thiazide directly enhances water permeability of isolated collecting ducts (133), and that thiazide treatment increases the expression of AQP2 in lithium-induced NDI (134), suggesting a direct effect of thiazide on the collecting duct.

The combined administration of hydrochlorothiazide with a prostaglandin synthesis inhibitor such as indomethacin was shown to be more effective in reducing urine volume than the

thiazide-diuretic alone (135-137). Similarly, combining administration of hydrochlorothiazide with the ENaC-blocker amiloride was shown to be more effective than the thiazide-diuretic alone as well (138-140). Besides this, the addition of amiloride also prevents hypokalemia and metabolic alkalosis, common side-effects of thiazide therapy (138; 139).

Aim of the thesis

Regulation of AQP2 is critical to osmoregulation and the maintenance of body water homeostasis. However, the exact molecular mechanisms which regulate AQP2 in different conditions are still unknown. This thesis aims to improve the understanding of the physiological and pharmacological modulation of renal water reabsorption and AQP2 expression.

In the first chapters, the physiological regulation of AQP2 was investigated. In **chapter 2**, the pathways involved in the inhibition of AQP2 mediated water transport by ATP and dopamine were studied in a cell model as well as in kidney.

Prostaglandins counter-act AVP-induced water reabsorption, but can also stimulate water reabsorption in the absence of AVP. To further investigate this, the effects of prostaglandins on AQP2 expression, as well as the effect of AVP on prostaglandin production and receptor expression were investigated, as described in **chapter 3**.

Osmolality not only affects plasma AVP, but also has a direct effect on AQP2 expression and urine concentrating ability. These effects were further investigated in **chapter 4,** using a collecting duct cell model.

In **chapter 5**, the long term AQP2 regulation after sustained AVP stimulation, in contrast to the effects after immediate stimulation, was investigated.

AQP2 expression can also be modulated by several medications. One of these, demeclocycline, is currently used to treat sustained hyponatremia in patients with SIADH. The mechanism by which demeclocycline decreases renal water reabsorption is unknown, and is investigated in **chapter 6**.

Lithium-induced NDI, the most common form of NDI, is associated with a decreased AQP2 expression. The exact mechanism by which lithium causes this AQP2 downregulation is investigated in **chapter 7**. At present it is unclear whether lithium-induced NDI can be attenuated. In **chapter 8** the effect of the ENaC blocker amiloride on lithium-induced NDI is described, using a cell model as well as lithium-NDI rats.

Finally, a general discussion is presented in **chapter 9**.

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CHAPTER 2

Counteracting vasopressin-mediated water reabsorption by ATP, dopamine and phorbol esters: mechanisms of action

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Abstract

Water homeostasis is regulated by a wide variety of hormones. When in need for water conservation, vasopressin, released from the brain, binds renal principal cells and initiates a signalling cascade resulting in the insertion of AQP2 water channels in the apical membrane and water reabsorption. Conversely, hormones, including extracellular purines and dopamine antagonize AVP-induced water permeability, but their action mechanism is largely unknown, which was investigated here.

Addition of these hormones to mpkCCD cells decreased total and plasma membrane abundance of AVP-induced AQP2, partly by increasing its internalization to vesicles and lysosomal degradation. This internalization was ubiquitin-dependent, because the hormones increased AQP2 ubiquitination, and the plasma membrane localization of AQP2-K270R, which cannot be mono-ubiquitinated, was unaffected by these hormones. Both hormones also increased AQP2 phosphorylation at S261, which followed ubiquitination, but was not essential for hormone-induced AQP2 degradation. A similar process occurs *in vivo*, as incubation of dDAVP-treated kidney slices with both hormones also resulted in the internalization and S261 phosphorylation of AQP2. Both hormones also reduced cAMP and AQP2 mRNA levels, suggesting an additional effect on AQP2 gene transcription. Interestingly, phorbol esters only reduced AQP2 through the first pathway.

Together, our results indicate that ATP and dopamine counteract AVP-induced water permeability by increasing AQP2 degradation in lysosomes, preceded by ubiquitin-dependent internalization, and by decreasing AQP2 gene transcription by reducing the AVP-induced cAMP levels.

Introduction

Vasopressin (AVP)-regulated maintenance of the water balance in the renal collecting duct occurs via alternating cell surface expression of the water channel aquaporin-2 (AQP2), and depends on the body's need for water conservation. In states of hypernatremia and hypovolemia, AVP is released into the bloodstream, interacts with its renal type 2 receptors and initiates a signal transduction cascade (1). This includes activation of adenylate cyclase, a rise in intracellular cAMP and calcium levels, activation of protein kinase A (PKA) and its recruitment to AQP2 containing vesicles, and subsequent phosphorylation of AQP2 at serine 256 (S256) (2-4). This phosphorylation is essential for the re-distribution of tetrameric AQP2 from intracellular storage vesicles to the apical plasma membrane (5). In addition, cAMP also increases AQP2 expression via phosphorylation of the cAMP responsive element binding

protein (CREB), which activates the AQP2 promoter (6; 7). Due to the increased plasma membrane expression of AQP2, water is able to pass the apical membrane passively along an osmotic gradient and enters the blood via AQP3 and AQP4 water channels, which are constitutively expressed in the basolateral membrane. Once the water balance is restored, AVP levels drop and AQP2 is internalized via ubiquitination at Lys270 (8).

Besides AVP, several other hormones regulate the water balance by antagonizing the AVP-induced water transport (9), but the underlying mechanism is poorly understood. Extracellular purines, ATP and UTP, decrease AVP-induced water permeability, which is at least partially mediated via the P2Y2 receptor, located in the basolateral membrane of principal cells (10-13), but may also involve P2X2 and P2Y4 receptors (14). Moreover, dopamine, carbachol, an acetylcholine analogue, and endothelin-1 inhibit the AVP-induced water permeability (15-20).

Intracellularly, the action of these hormones shows overlap, but also differs. ATP/UTP and dopamine appear to attenuate the AVP-triggered increase of intracellular cAMP, which is thought to be conferred by activation of a counteracting protein kinase C (PKC, ATP/UTP; (11)), or coupling of the hormone receptor to the inhibitory G (Gi) protein (dopamine; (15). In contrast, carbachol reduced the AVP-induced water transport via a PKC-dependent pathway, but did not impair the AVP-induced cAMP production (20; 21).

At present, however, it is unknown whether the reduced AVP-induced water permeability induced by these hormones involves AQP2 degradation and, if so, whether this occurs via AQP2 ubiquitination, internalization and degradation, as we recently reported to occur with forskolin removal or addition of phorbol ester 12-tetradecanoylphorbol-13-acetate (TPA), a drug that can activate PKC (8; 22). Moreover, it is unknown whether any of these hormones employs phosphorylation of AQP2 at any other site than S256, as it has recently been shown that AQP2 can also be phosphorylated at S261, S264 and S269, and that pS261-AQP2 is mainly found in intracellular vesicles (23; 24).

Therefore, to obtain more insight into the mechanism by which hormones counteract the action of AVP on AQP2, we here analyzed in detail the effect and underlying mechanism of ATP and dopamine on the AVP-induced AQP2 abundance.

Materials and Methods

Chemicals and reagents

[deamino-Cys^1, D-arg^8]-vasopressin (dDAVP), ATP, UTP, dopamine, forskolin, cycloheximide and chloroquine were purchased from Sigma (St. Louis, MO, USA). dDAVP was

administered to the basolateral side only. All other compounds were administered to both the apical and basolateral side.

Ex vivo immunofluorescence

Female Sprague Dawley rats were anesthetized with ether and sacrificed by decapitation. Kidneys were quickly removed and sections of approximately 0.5 mm were made and divided in four groups. The sections were equilibrated for 10 min in a buffer containing 118 mM NaCl, 16 mM HEPES, 17 mM Na-HEPES, 14 mM glucose, 3.2 mM KCl, 2.5 mM $CaCl_2$, 1.8 mM $MgSO_4$, and 1.8 mM KH_2PO_4 (pH 7.4). AQP2 trafficking was stimulated in the same buffer and at 37°C with 1 nM dDAVP for 15 minutes followed by a 30 minutes incubation with dDAVP alone or in combination with either 0.1 mM dopamine or ATP, or 100 nM TPA. Next, the kidney sections were overnight fixed in 4% paraformaldehyde at 4°C, infiltrated with 30% sucrose in PBS for 24 hours, embedded in Cryomatrix (DDK Srl Milano, Italy) at dry ice and cut with a cryostat to obtain 5μm sections.

To stain for AQP2, the kidney sections were washed three times with PBS, blocked with 1% PBS-BSA for 1 hour, incubated with our affinity-purified rabbit 1:1000-diluted antibodies raised against the 20 amino acids N-terminal of the poly-phosphorylated region of human AQP2 (CLKGLEPDTDWEEREVRRRQ; pre-C tail) for 2 hours, and washed three times with PBS. After washing, the sections were incubated with 1:1000 diluted goat anti-rabbit antibodies coupled to Alexa-488 (Invitrogen, Milano, Italy), rinsed three times with PBS and mounted in mounting medium containing 50% glycerol in 0.2 M Tris-HCl, pH 8.0 in the presence of 2.5% n-propyl gallate. Images were obtained with a Leica TCS SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Cell culture

MpkCCD cells (clone 14)(25) were grown in a modified defined medium (DMEM:Ham's F12 1:1 vol/vol; 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% foetal calf serum, and 20 mM HEPES (pH 7.4)).

Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell, 0.4 μm pore size, Corning Costar, Cambridge, MA, USA). 1.13 cm^2 filters were used for immunocytochemistry or immunoblotting, and 4.7 cm^2 filters for biotinylation experiments. The cells remained in culture for 8 days before being analyzed. Cells were incubated with 1 nM dDAVP for the last 4 days, to maximally induce AQP2 expression (26). Biotinylation was performed as described (27). The concentrations of the AVP-counteracting hormones ATP and dopamine were 100 μM and the concentration of TPA used was 100 nM. Each experiment was done in triplicate and was repeated at least 3 times.

For the generation of cell lines stably expressing exogenous wtAQP2 of AQP2-K270R, the expression constructs pcB6-dBamHI-AQP2 and pcB6-dBamHI-AQP2-K270R (8), which encode these respective channels, were transfected into mpkCCD cells using the calcium phosphate method as previously described for MDCK cells (27). MDCK-wtAQP2 have been described (27).

Immunocytochemistry

Immunocytochemistry and confocal laser scanning microscopy (CLSM) of cells grown on semi-permeable filters were performed as described (27). For AQP2 detection, the filters were incubated with affinity-purified rabbit anti-AQP2 antibodies (1:100; (28)) and goat anti rabbit antibodies coupled to Alexa 488 (Molecular Probes, Eugene, OR, USA; 1:100).

Immunoblotting

Immunoblotting was performed as described (27). As antibodies, affinity-purified rabbit 7 anti-AQP2 antibodies (1:3000; (28)), guinea pig anti-AQP2 (1:4000) antibodies, rabbit anti-AQP2-pS261 antibodies (1:2000; kindly provided by Dr. M. A. Knepper, Bethesda, USA), mouse anti-ubiquitin (P4D1; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000) or mouse anti-β-actin (Sigma, St. Louis, MO, USA; 1:25000) were used. As secondary antibodies, goat anti-rabbit, goat anti-guinea pig, or sheep anti-mouse antibodies coupled to horseradish peroxidase (HRP; Sigma, St. Louis, MO, USA; 1:10000) were employed.

For immunoblotting of kidney sections, this tissue was lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1mM sodium orthovanadate, 25 mM Hepes (pH 7.4) and subjected to immunoblotting studies. Total AQP2 was detected with 1:1000 dilution of our affinity-purified preCtail antibodies. AQP2-pS261 antibodies (1:1000) were purchased from DBA (Segrate, Milano, Italy). As secondary antibodies, goat anti-rabbit HRP-coupled secondary antibodies were used.

Quantitative-RT-PCR

MpkCCD cells were grown as described (26) and total RNA was isolated using TriZol extraction reagent (Gibco BRL, Life Technologies, Rockville, MD), according to the manufacturer's instructions. To remove potentially contaminating DNA, total RNA was treated with DNase (Promega, Madison, WI) in DNase buffer, incubated for 1 hr at 37 °C, extracted with phenol/chloroform and precipitated using sodium acetate (3M, pH 5.2) and 100% ethanol. 1.5 µg RNA was reverse-transcribed into cDNA using MMLV Reverse Transcriptase with random primers (Promega, Madison, WI). SYBR Green Real-time quantitative PCR was performed on an iQ5 Real-Time PCR Detection System from Bio-Rad by utilizing the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), in

combination with mouse AQP2 primers (5'-CTCCACAACAATGCAACAGC-3' and 5'-GAGCAGCCGGTGAAATAGAT-3'). As an internal standard, RNA of ribosomal 18S was amplified in parallel using the primers 5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGTAGCG-3'.

cAMP assay

MpkCCD cells were grown on 0.33 cm^2 filters for 8 days, with dDAVP for the last 4 days. At the last day, cells grown with dDAVP were incubated with culture medium supplemented with 1 nM dDAVP and 250 µM 3-Isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO, USA) for 10 min to prevent cAMP degradation by phosphodiesterases, followed by 15 min incubation with the AVP-counteracting hormones mentioned above in the presence of dDAVP and IBMX. The filters were rapidly excised from their plastic support, lysed in 150 µl of 0.1 M HCl, and incubated for 15 min at room temperature to complete lysis. To remove debris, the scraped samples were then centrifuged at 600 g for 5 min at room temperature. Subsequently, 100 µl supernatant was used to determine the cAMP levels using a direct cAMP enzyme immunoassay kit (Sigma, St. Louis, MO, USA).

Immunoprecipitation

20 µl protein A agarose beads (Kem-En-Tec A/S, Copenhagen, Denmark) per sample were washed three times in lysis buffer (1% Triton X-100, 150mM NaCl, 25mM Hepes (pH 7.4)). Per sample, 1 µl of rabbit anti-AQP2 or guinea pig anti-AQP2 antibodies was added to 1 ml lysis buffer with protease inhibitors (1 mM PMSF, 5 µg/ml pepstatin, 5 µg/ml leupeptin, and 5 µg/ml a-protinin) and rotated for 4h at room temperature. Before use, the antibody-coupled beads were washed three times with lysis buffer. Cells were treated as described and lysed in 1 ml lysis buffer containing protease inhibitors and 20 mM *N*-ethylmaleimide (NEM). The samples were centrifuged at 12,000X G for 10 minutes and the supernatant was incubated for 16 hours with the antibody-coupled beads at 4°C. The beads were washed four times with lysisbuffer, carefully dried and resuspended in 30 µl of 1x Laemmli buffer with 0.1M DTT. Subsequently, the samples were analyzed by immunoblotting for ubiquitin, AQP2-phospho-S261, or total AQP2 as indicated.

Statistical analyses

Films were scanned using a GS-690 Imaging Densitometer (Bio-Rad, CA, USA) and analyzed using Bio-Rad software. Statistical comparisons were made using one-way analysis of variance (ANOVA) and Bonferroni's post hoc correction. $P < 0.05$ was considered significant.

Results

Effect of ATP and dopamine on AQP2 abundance

To study the role of hormones counteracting AVP *in vitro*, we used the mouse cortical collecting duct (mpkCCD) cells as a model, because these cells show an AVP-induced expression of endogenous AQP2 (25). To determine whether AVP-counteracting hormones counteract AVP, mpkCCD cells were grown to confluence for 8 days, incubated for the last 4 days with dDAVP to generate a steady-state endogenous AQP2 abundance (26), and then incubated for the last 8 hours with 100 µM ATP or dopamine in the continued presence of dDAVP. TPA, which is known to decrease AQP2 abundance in mpkCCD cells (8), was taken along as a control. Subsequent immunoblotting showed that both hormones and TPA decreased AQP2 abundance (Fig. 1). These data indicated that mpkCCD cells can be used as a model to study the mechanism by which ATP and dopamine counteract AVP. Although lower concentrations of ATP and dopamine also affected AQP2 abundance, the above-mentioned concentrations of AVP-counteracting hormones gave more consistent results and were therefore selected for the experiments below.

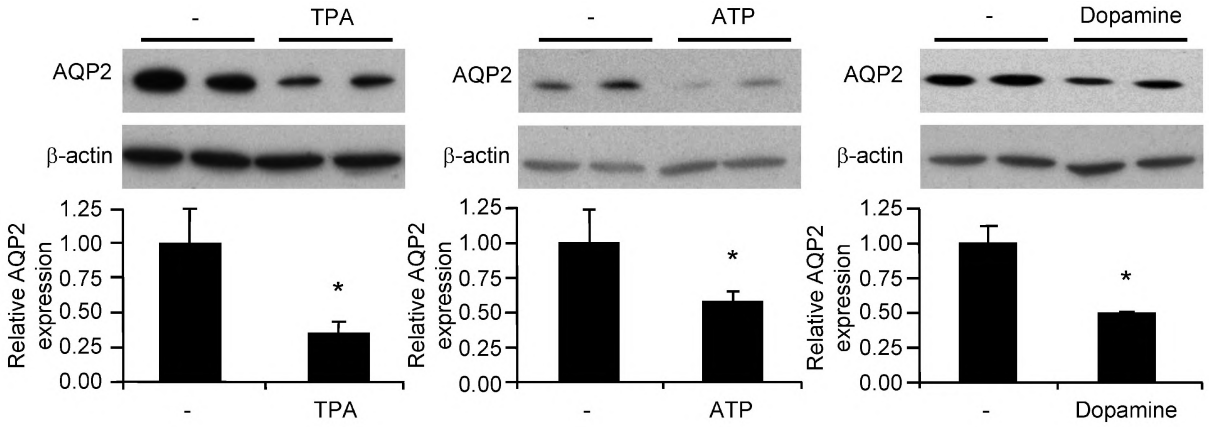


Figure 1. Effect of TPA, ATP and dopamine on dDAVP-induced AQP2 abundance. MpkCCD cells were grown for 4 days, incubated with 1 nM dDAVP for an additional 4 days and treated with 100 nM TPA, 100 µM ATP, or 100 µM dopamine in the presence of dDAVP for the last 8 hours. Subsequently, the cells were lysed and immunoblotted for AQP2. The signals were semi-quantified using densitometry. Samples significantly ($P<0.05$) different from controls are indicated by asterisk. One out of three independent experiments is shown. The quantification shown here is pooled data from all three experiments.

Effect of ATP and dopamine on AQP2 internalization

The observed reduction in AVP-induced AQP2 abundance in the 8 hours time frame can be due to increased AQP2 internalization and degradation, which is a relative fast process, and/or reduced AQP2 transcription, which usually is a slow process. To determine whether these hormones employ AQP2 internalization, dDAVP-induced mpkCCD cells were left untreated or treated with TPA, ATP or dopamine for 2h, and subjected to apical cell surface

biotinylation assays. Immunoblotting of the biotinylated proteins and normalization for the respective total amounts of AQP2 revealed a significantly-reduced AQP2 abundance in the apical membrane with TPA, ATP and dopamine (Fig. 2). As anticipated for the short incubation time, none of the treatments significantly affected the total amount of AQP2.

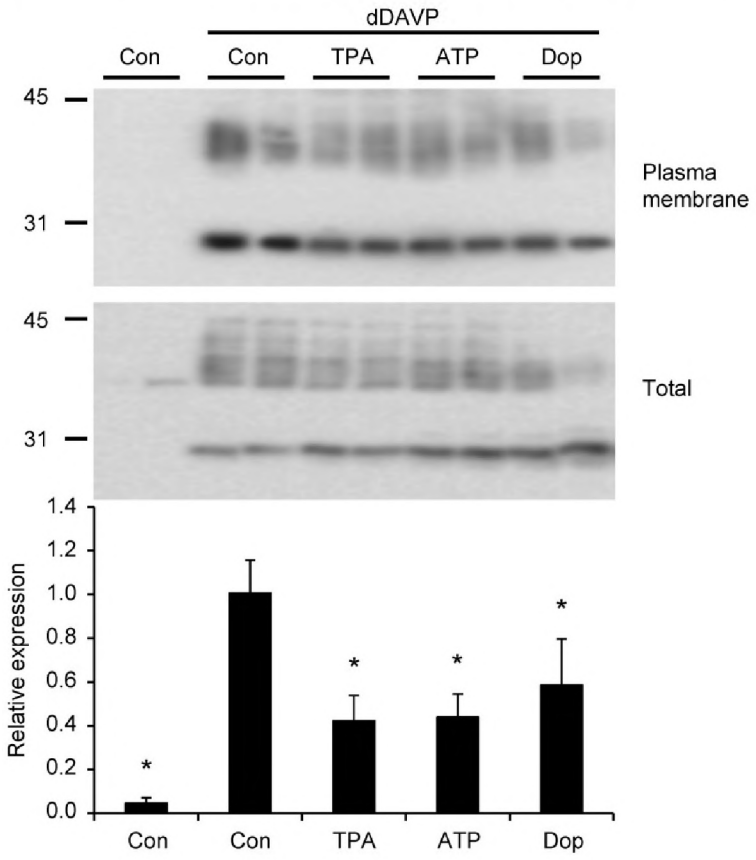


Figure 2. Effect of TPA, ATP and dopamine on AQP2 internalization
MpkCCD cells were grown and treated as described in the legend of Fig. 1, except that the treatment with TPA, ATP and dopamine was for 2 hours. Subsequently, cells were subjected to cell surface biotinylation and lysed. Biotinylated proteins were pulled-down from the remaining solution and immunoblotted for AQP2 (plasma membrane). In addition, total lysates were immunoblotted for AQP2 (Total). The signals were semi-quantified using densitometry. Samples significantly ($P<0.05$) different from controls are indicated by asterisk. Triplicate samples were analyzed and independent experiments were performed in threefold, of which a representative experiment is shown. Graphs show pooled data from three experiments.

Effect of ATP and dopamine on AQP2 ubiquitination

Recently, we showed that TPA induces short chain ubiquitination of AQP2 *in vitro* and *in vivo* and that this ubiquitination precedes, and is essential for, AQP2 internalization and degradation upon TPA treatment in MDCK-AQP2 cells (8). As ubiquitination is more readily detectable in MDCK then in mpkCCD cells, we initially reverted to MDCK-AQP2 (29) cells for this purpose. Following forskolin incubation for 45 minutes, these MDCK-AQP2 cells were left untreated (control) or incubated with TPA, ATP or dopamine for 15 minutes (optimal period for detecting ubiquitination in these cells), lysed and subjected to AQP2 immunoprecipitation. Subsequent immunoblotting for ubiquitin revealed the typical strong 43 and 50 kDa and the weaker 58 kDa ubiquitinated AQP2 bands in all lanes, which were clearly increased for cells co-incubated with ATP and dopamine and, to a higher extent, with TPA, as compared to control cells (Fig. 3). Detection of immunoprecipitated equivalents with AQP2 antibodies (Fig. 3; lower panel) revealed that similar amounts of AQP2 were loaded.

To assess the involvement of ubiquitination in the ATP and dopamine-induced internalization of AQP2 from the plasma membrane in mpkCCD cells, these cells were stably-transfected with wtAQP2 or the mutant AQP2-K270R, which cannot be short-chain ubiquitinated (8). As shown in figure 4, incubation with forskolin alone results in apical plasma membrane localization of wtAQP2 and AQP2-K270R. In line with the biotinylation data above, co-incubation with TPA, ATP or dopamine induced internalization of wtAQP2 into vesicles. In contrast, the apical localization of AQP2-K270R remained unaffected by any of these compounds, demonstrating that ubiquitination of K270 is essential for hormone-induced internalization of AQP2 in mpkCCD cells.

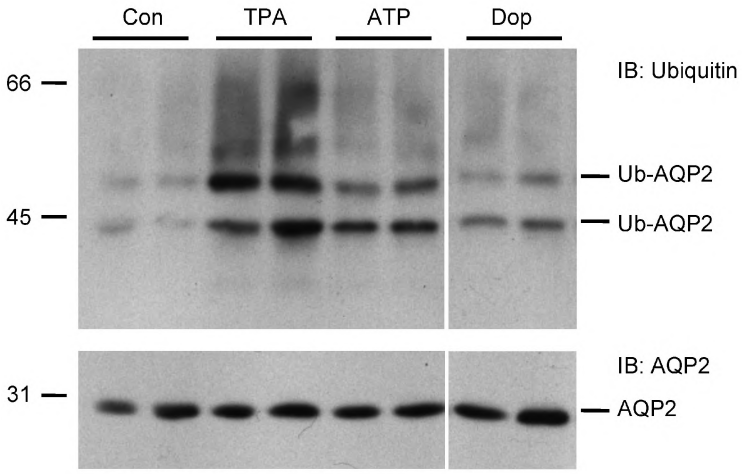


Figure 3. Effect of TPA, ATP and dopamine on AQP2 ubiquitination. Confluent monolayers of MDCK-AQP2 cells were incubated with $5x10^{-5}$ M forskolin followed by co-incubation with 100 nM TPA, 100 µM ATP, or 100 µM dopamine for the last 15 minutes. Cells were lysed and subjected to immunoprecipitation using rabbit anti-AQP2 antibodies. Samples were immunoblotted and blots were incubated with mouse anti-ubiquitin (upper panel) or guinea pig anti-AQP2 (lower panel) antibodies. Protein masses (in kDa) are indicated on the left.

Effect of ATP and dopamine on S261 phosphorylation

Besides phosphorylation of AQP2 at S256 by PKA, three additional phosphorylation sites were identified in the C-terminal tail of AQP2, S261, S264, and S269 (23; 24). Interestingly, whereas AVP application increased phosphorylation at S256, AQP2-pS261 mainly localized in vesicles and AQP2 phosphorylation at S261 was decreased upon AVP incubation (24). To investigate whether the AVP-counteracting hormones affect the phosphorylation state of AQP2 at S261 and how this relates to ubiquitination of AQP2, mpkCCD-AQP2 and AQP2-K270R cells were left untreated or pre-incubated with 100 nM dDAVP, followed by co-incubation with TPA, ATP, or dopamine for 15 minutes. Consistent with *in vivo* data, dDAVP reduced the pS261 29 kDa signals for wtAQP2 (Figure 5A, top panel, left lanes). Upon co-incubation of mpkCCD-AQP2 cells with TPA, ATP or dopamine two forms of data were obtained. Often, TPA, ATP and dopamine did not change the signal for AQP2-pS261 at 29 kDa, but induced the appearance of a signal at 43 kDa (Fig. 5A, upper left panel), a band that also appeared in the blot representing total AQP2 (Figure 5A, bottom left panel). In other experiments, AQP2-pS261 of 29 kDa was also observed (not shown). For AQP2-K270R, the basal level of AQP2-pS261 also decreased upon incubation with dDAVP (Fig. 5A, upper right

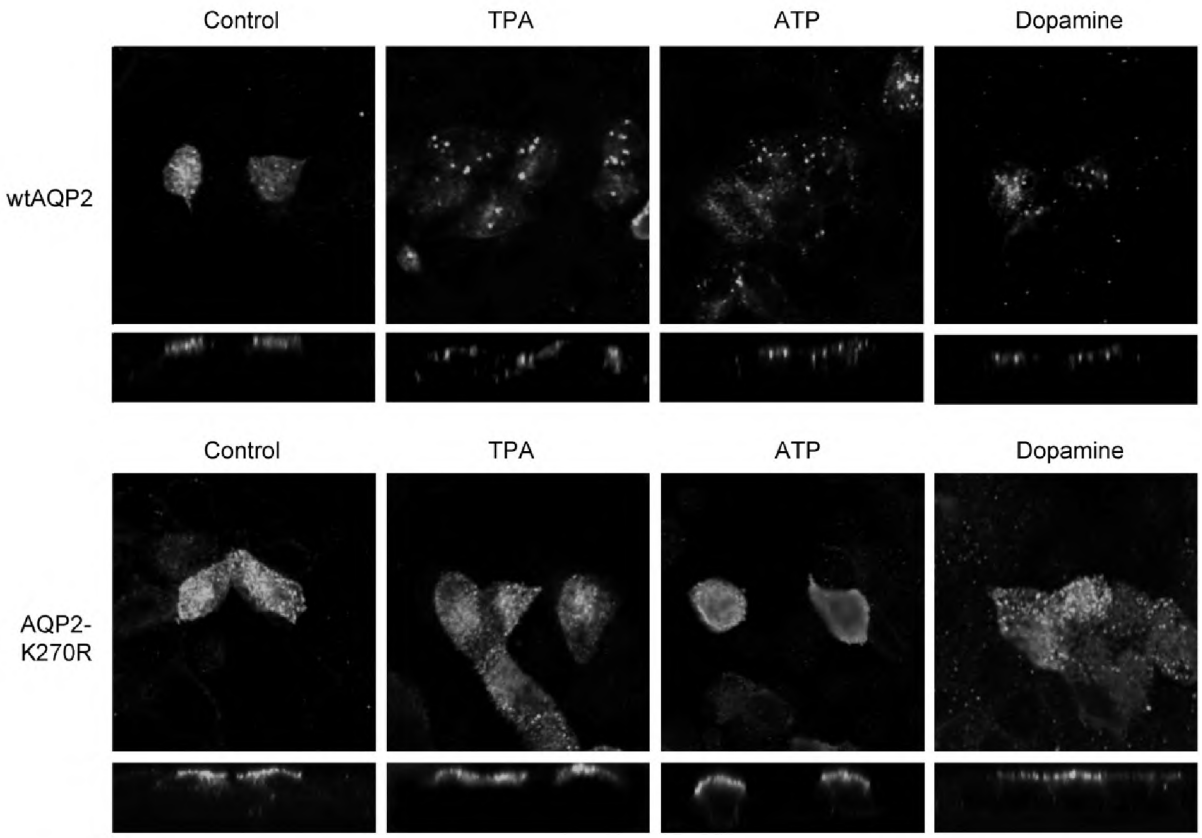


Figure 4. Effect of TPA, ATP and dopamine on the subcellular localization of AQP2 and its ubiquitination-mutant AQP2-K270R. MpkCCD-wtAQP2 and AQP2-K270R cells were grown for 8 days, incubated with $5x10^{-5}$ M forskolin for 45 minutes to induce apical localization of AQP2, and were then left untreated (control), or treated with dDAVP in the presence of 100 nM TPA, 100 µM ATP, or 100 µM dopamine for 30 minutes. Subsequently, cells were fixed and subjected to immunocytochemistry for AQP2. Top view (XY) confocal images and their corresponding cross sections (XZ) are shown.

panel). Co-incubation with TPA, ATP and dopamine neither led to an increase of the 29 kDa signal, but also did not result in the appearance of the 43 kDa signal as observed for wtAQP2. Total levels of AQP2 protein remained unaltered during the experiment (Figure 5A, bottom panels). These data suggest that ubiquitination precedes pS261 in AQP2.

To examine this in more detail, we analyzed phosphorylation of S261 in wild-type (wt) AQP2 and AQP2-K270R expressing mpkCCD cells time-dependent, after incubation with ATP and dopamine. As shown for ATP (Fig. 5B), wt-AQP2 starts to get phosphorylated at S261 around 15 minutes after starting ATP treatment. At this time point, however, it is predominantly the ubiquitinated form of AQP2 that is phosphorylated. At 30 minutes after ATP stimulation, when the extent of AQP2 ubiquitination has dropped (8), the signal for ubiquitinated AQP2-pS261 decreases, while that of 29 kDa AQP2-pS261 increased to a level that is sustained at later time points. In contrast, AQP2-K270R does not get phosphorylated at pS261 at any timepoint measured (Fig. 5B). Similar data were obtained for dopamine (not

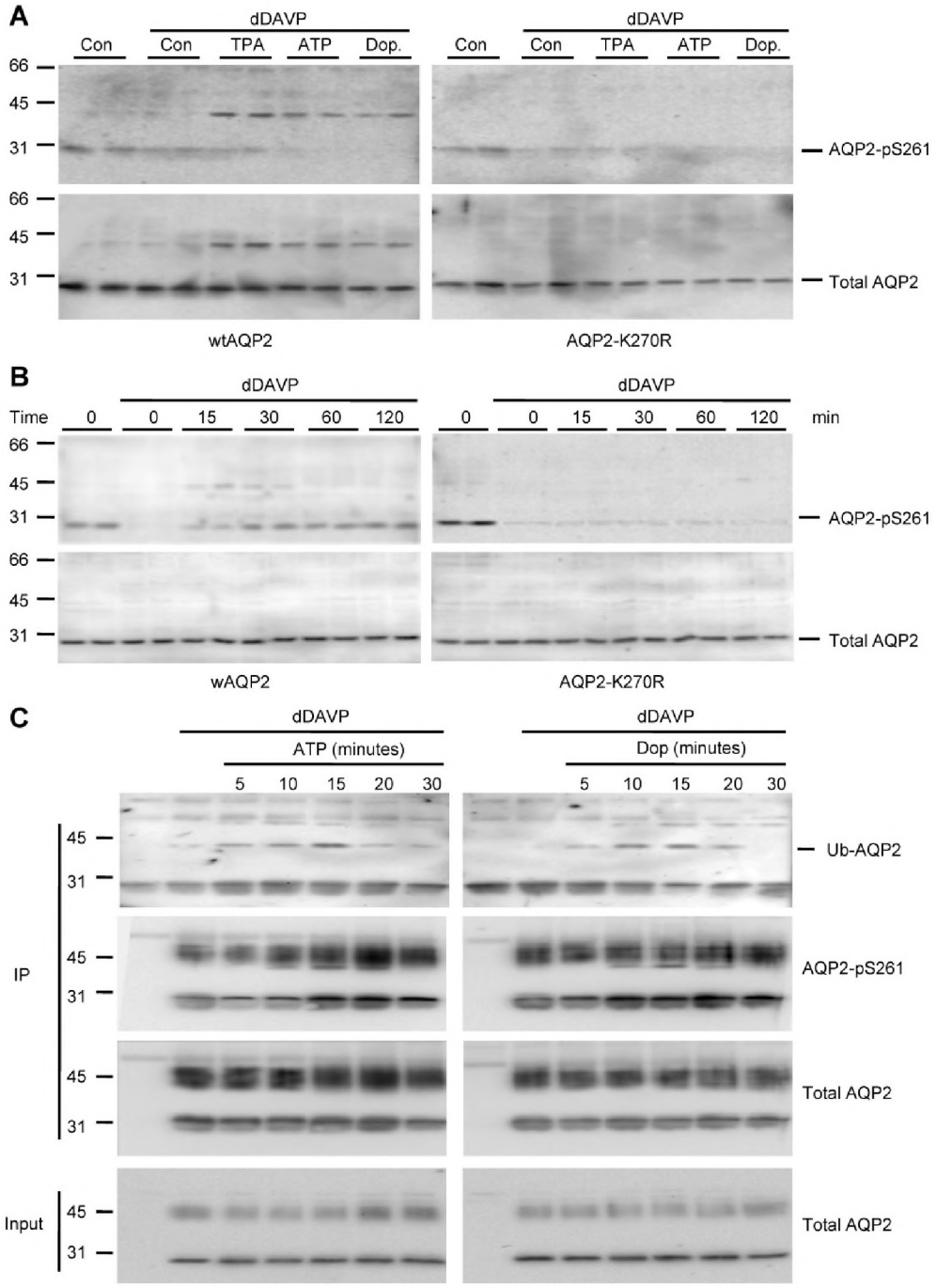
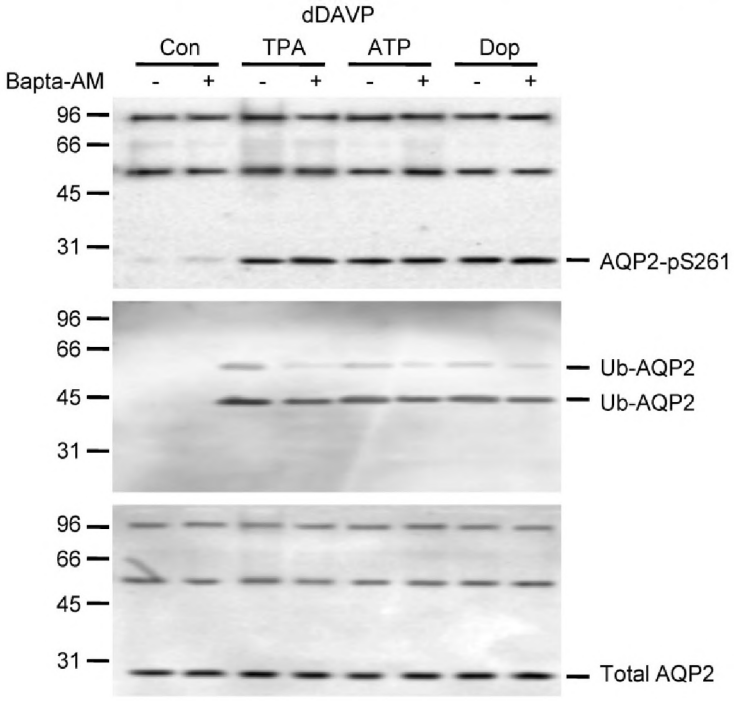


Figure 5. Effect of TPA, ATP and dopamine on S261 phosphorylation and ubiquitination of AQP2. A) MpkCCD wtAQP2 or AQP2-K270R cells were incubated with 100 nM dDAVP for 1 h 45 min, followed by co-incubation with 100 nM TPA, 100 µM ATP, or 100 µM dopamine for an additional 15 min. Cells were lysed in Laemmli buffer supplemented with DTT and de-phosphorylation and de-ubiquitination inhibitors, and analyzed on a 12% PAAG followed by immunoblotting for pS261-AQP2 (upper panel) and AQP2 (lower panel). **B)** Confluent mpkCCD cells stably expressing wtAQP2 or AQP2-K270R were incubated with dDAVP for 2 h to induce translocation of AQP2 to the plasma membrane (0, dDAVP) followed by incubation for different time periods (in minutes) with dDAVP and ATP. Cells were lysed and analyzed as described in *A*.**C)** MpkCCD cells were incubated for 4 days in the presence of dDAVP and, subsequently, treated with culture medium supplemented with 1 nM dDAVP and 100 µM ATP or 100 µM dopamine for the indicated time points (in minutes). Cells were lysed in the presence of de-phosphorylation- and de-ubiquitination blockers and AQP2 proteins were extracted by immunoprecipitation using guinea pig anti-AQP2 antibodies. Samples were analyzed by immunoblotting for ubiquitin, AQP2-pS261, or total AQP2 as indicated. Total lysates were blotted for total AQP2 to demonstrate equal AQP2 input levels. Protein mass (in kDa) is indicated on the left.

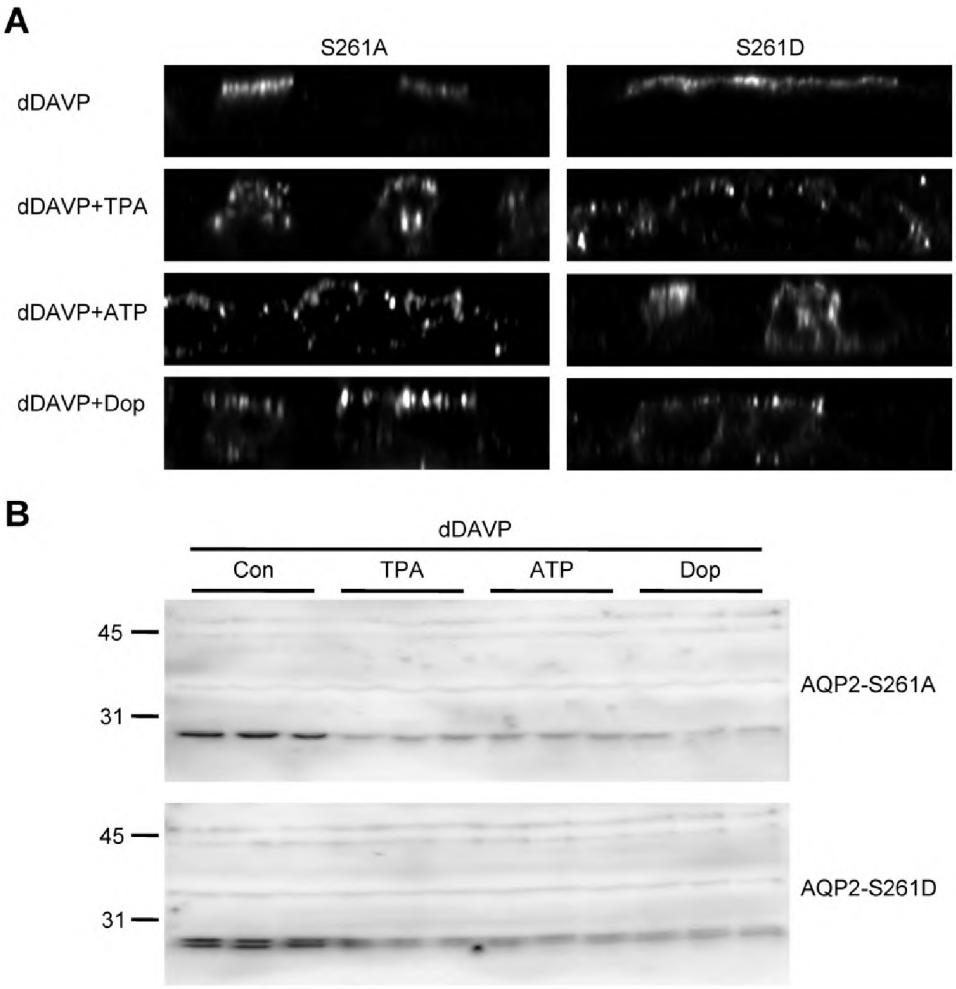
shown). Also, following incubation with ATP or dopamine at a more detailed early time frame and immunoblotting for ubiquitin revealed an increase in ubiquitinated AQP2 after 5 minutes, with a peak after 10 to 15 minutes (Fig. 5C, top panels), while pS261 signals of 29 and 43 kDa AQP2 mainly increased between 10 and 20 after hormone addition (Figure 5C, upper middle panel). [note that the 25 kDa band in the blot for ubiquitination is a background band, because it is present in mpkCCD cells not pre-treated with dDAVP (see also input, lower panel)]. The total levels of immunoprecipitated and total AQP2 remained essentially unaltered (Figure 5C, bottom two panels). Together, these data indicated that hormone-induced phosphorylation of S261 indeed follows ubiquitination of AQP2.



Supplementary Figure S1: Effect of calcium on S261-phosphorylation and degradation of AQP2. Confluent mpkCCD cells stably expressing wild-type AQP2 were incubated with 10 nM dDAVP for 2 hours to induce translocation of AQP2 to the plasma membrane, followed by 15 minutes co-treatment with TPA, ATP or dopamine. Subsequently, cells were lysed in IPP100 in the presence of de-phosphorylation and de-ubiquitination inhibitors and subjected to immunoprecipitation using guinea pig anti-AQP2 antibodies. Subsequently, samples analyzed on a 12% PAAG followed by immunoblotting using AQP2-phospho-S261, total AQP2, or ubiquitin antibodies.

In addition, we investigated the effects of intracellular calcium levels on ubiquitination and subsequent S261 phosphorylation of AQP2. However, as shown in Supplementary Fig. S1, exclusion of calcium by co-incubation with Bapta-AM did not affect ATP, dopamine, or TPA-induced ubiquitination or pS261 of AQP2. These data indicated that these processes are calcium-independent.

To test whether S261 phosphorylation is important in the hormone-induced degradation, mpkCCD cells stably-expressing AQP2-S261A, which cannot be phosphorylated, or AQP2-S261D, which mimics its constitutively-phosphorylated form, were treated with the hormones as above. Immunocytochemistry and immunoblotting (Supplementary Fig. S2), however, revealed that ATP, dopamine and TPA induced internalization and degradation of AQP2-S261A and AQP2-S261D, similar as observed for wtAQP2. These data reveal that ubiquitination, but not the phosphorylation status of S261, is critical in the process of AQP2 internalization and its subsequent degradation.



Supplementary Figure S2: Hormone-induced internalization and degradation of AQP2-S261A and AQP2-S261D. A) Confluent mpkCCD cells stably expressing AQP2-S261A or AQP2-S261D were treated with 10 nM dDAVP for 2 hours to induce translocation of AQP2 to the plasma membrane, followed by co-incubation with TPA, ATP or dopamine for 2 hours, after which cells were fixed and immunocytochemically stained using anti-AQP2 antibodies. Filters were analyzed using CLSM, of which representative cross-sections are shown. **B)** In parallel, cells were treated for 8 hours in the presence of cycloheximide and dDAVP alone, or supplemented with TPA, ATP, or dopamine. Subsequently, cells were lysed in Laemmli buffer supplemented with DTT and de-phosphorylation and de-ubiquitination inhibitors, followed by analysis on a 12% PAAG followed by immunoblotting using total AQP2 antibodies.

Effect of ATP and dopamine on AQP2 degradation

In mpkCCD cells, TPA-induced internalization of AQP2 leads to its degradation via the lysosomal pathway, which can be inhibited by chloroquine (8; 30). To test whether the hormones also affect AQP2 degradation, dDAVP pre-treated cells were co-incubated for 8 hours with ATP, dopamine or TPA in the presence or absence of cycloheximide to block protein synthesis, and in the presence or absence of chloroquine. Subsequent immunoblotting showed a significant reduction in AQP2 abundance when cells were incubated with these compounds (Fig. 6). This reduction was significantly prevented by blocking lysosomal protein degradation with chloroquine. Together, the data above reveal that ATP, dopamine (and TPA) counteract AVP-induced water permeability by increasing the

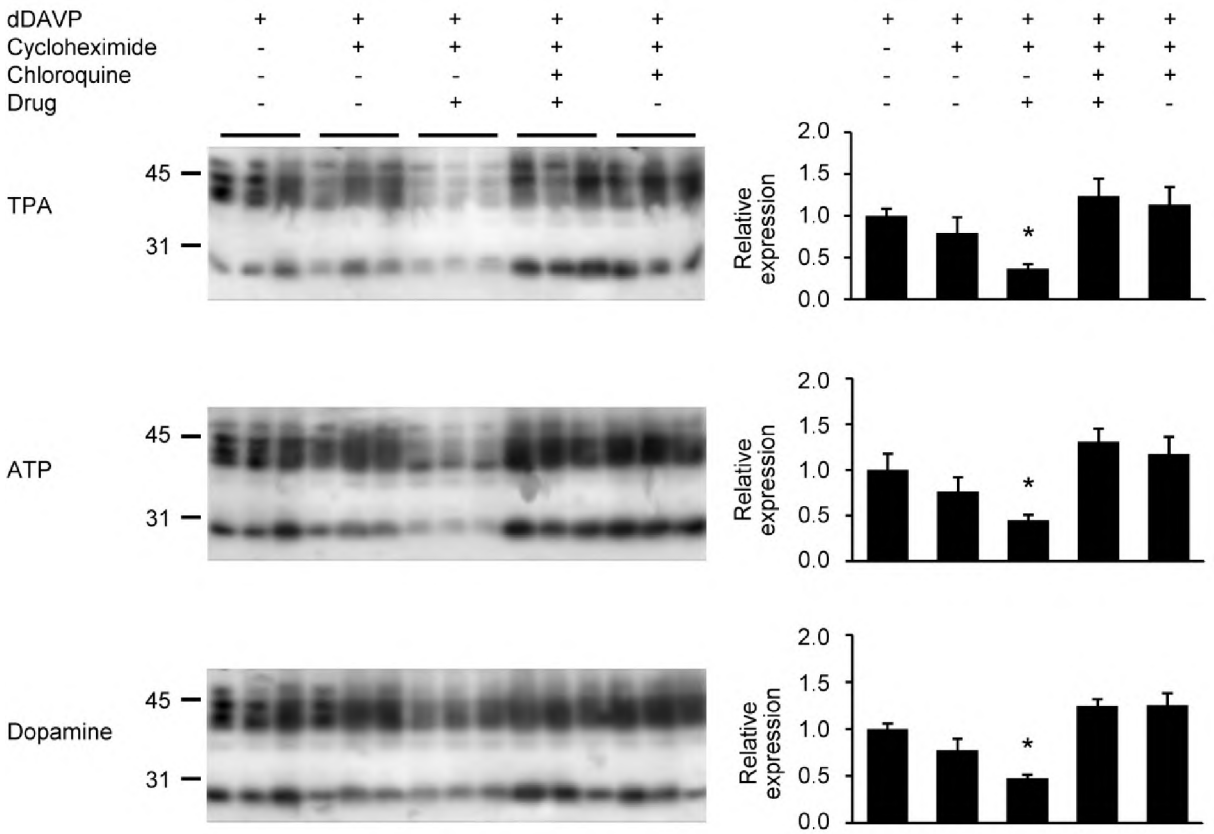


Figure 6. Effect of TPA, ATP and dopamine on lysosomal degradation of AQP2. MpkCCD cells were grown to confluence, treated with 1 nM dDAVP for 4 days, and then left untreated or incubated with 100 nM TPA, 100 µM ATP, or 100 µM dopamine with or without 100 µM chloroquine, dDAVP and 50 µM cycloheximide for 8 hours. Subsequently, cells were lysed and immunoblotted for AQP2 and signals quantified using densitometry. Quantified data represents pooled data from triplicate samples of three independent experiments. Samples significantly ($P<0.05$) different from the dDAVP alone control are indicated with an asterisk.

internalization of AQP2 from the plasma membrane in an ubiquitin-dependent manner to induce its lysosomal degradation.

Effect of ATP and dopamine on AQP2 mRNA abundance

Besides increased AQP2 internalization and degradation as shown above, the observed reduction in AVP-induced AQP2 abundance can partially be due to reduced AQP2 production. To determine whether the above-mentioned hormones affect AQP2 mRNA levels, cells were treated as above and subjected to quantitative RT-PCR. Using equal amount of starting cDNA and following normalization for 18S RNA, ATP gave a nearly 60% reduction in AQP2 mRNA, while dopamine showed a 30% reduction (Fig. 7). TPA did not reduce AQP2 mRNA levels.

Effect of ATP and dopamine on cAMP production

Binding of AVP to its V2R induces an increase in cAMP, which results via activation of CREB

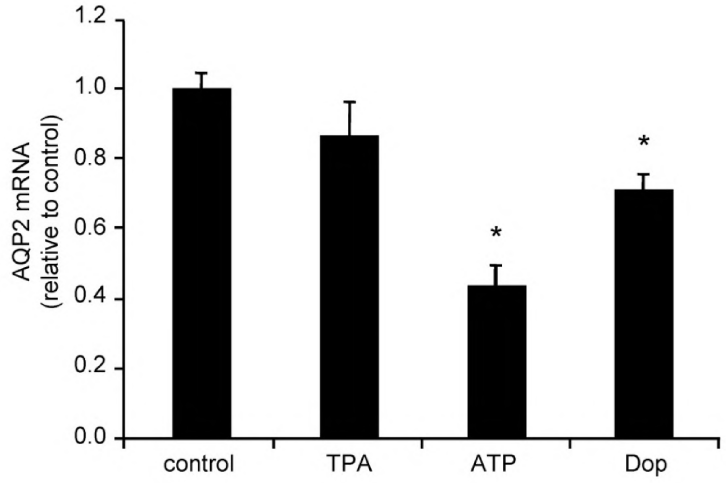


Figure 7. Effect of ATP, dopamine, and TPA on AQP2 mRNA amount. MpkCCD cells were grown and treated as described in the legend of Figure 6, except that cycloheximide was omitted. Cells were lysed, mRNA was isolated, and reverse transcribed to cDNA using random primers. Equal amounts of cDNA were subsequently subjected to Q-PCR using primers for mouse AQP2 and 18S (internal control). Following normalization against the 18S signal, the AQP2 mRNA amounts are expressed relative to control values.

in increased AQP2 gene transcription (6; 7). Considering the observed reduction in AQP2 mRNA levels with ATP and dopamine (Fig. 7), we tested whether the used AVP-counteracting hormones reduce the steady-state cAMP levels induced by dDAVP. For this, mpkCCD cells were grown and pretreated with dDAVP as above, incubated with the phosphodiesterase inhibitor IBMX for 10 minutes, followed by incubation with ATP, dopamine and TPA for 15 minutes in the continued presence of dDAVP and IBMX. Subsequent analysis of the cAMP levels revealed that ATP and dopamine significantly decreased cAMP levels, which was not observed with TPA (Fig. 8).

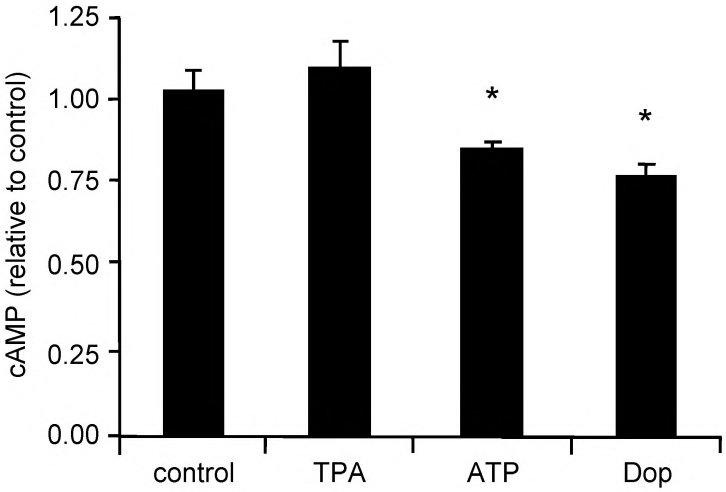


Figure 8. Effect of ATP, dopamine, and TPA on dDAVP-induced cAMP production. MpkCCD cells were grown to confluence, treated with 1 nM dDAVP for 4 days, incubated with 250 µM IBMX for 10 minutes, and then left untreated or treated with 100 nM TPA, 100 µM ATP, or 100 µM dopamine for another 15 minutes in the presence of dDAVP and IBMX. Then, cells were lysed and cAMP levels were determined with a cAMP enzyme immunoassay kit.

Effect of ATP and dopamine ex vivo

To investigate whether TPA, ATP and dopamine also induce AQP2 internalization in kidney principal cells and whether this also coincides with increased phosphorylation of AQP2 at S261, rat kidneys slices were pre-incubated with 1 nM dDAVP for 15 minutes, followed by 30 minutes with dDAVP alone or in combination dopamine, ATP, or TPA. Consistent to our mpkCCD cell line results, immunohistochemistry indeed showed that treatment with dDAVP

alone yielded a localization of AQP2 in the apical membrane, whereas co-treatment with dopamine, ATP, or TPA revealed internalized AQP2 (Fig. 9A). Moreover, immunoblotting indeed revealed that, normalized for total AQP2 levels, AQP2-pS261 levels were significantly increased upon co-incubation with dopamine, ATP, or TPA (Fig. 9B,C).

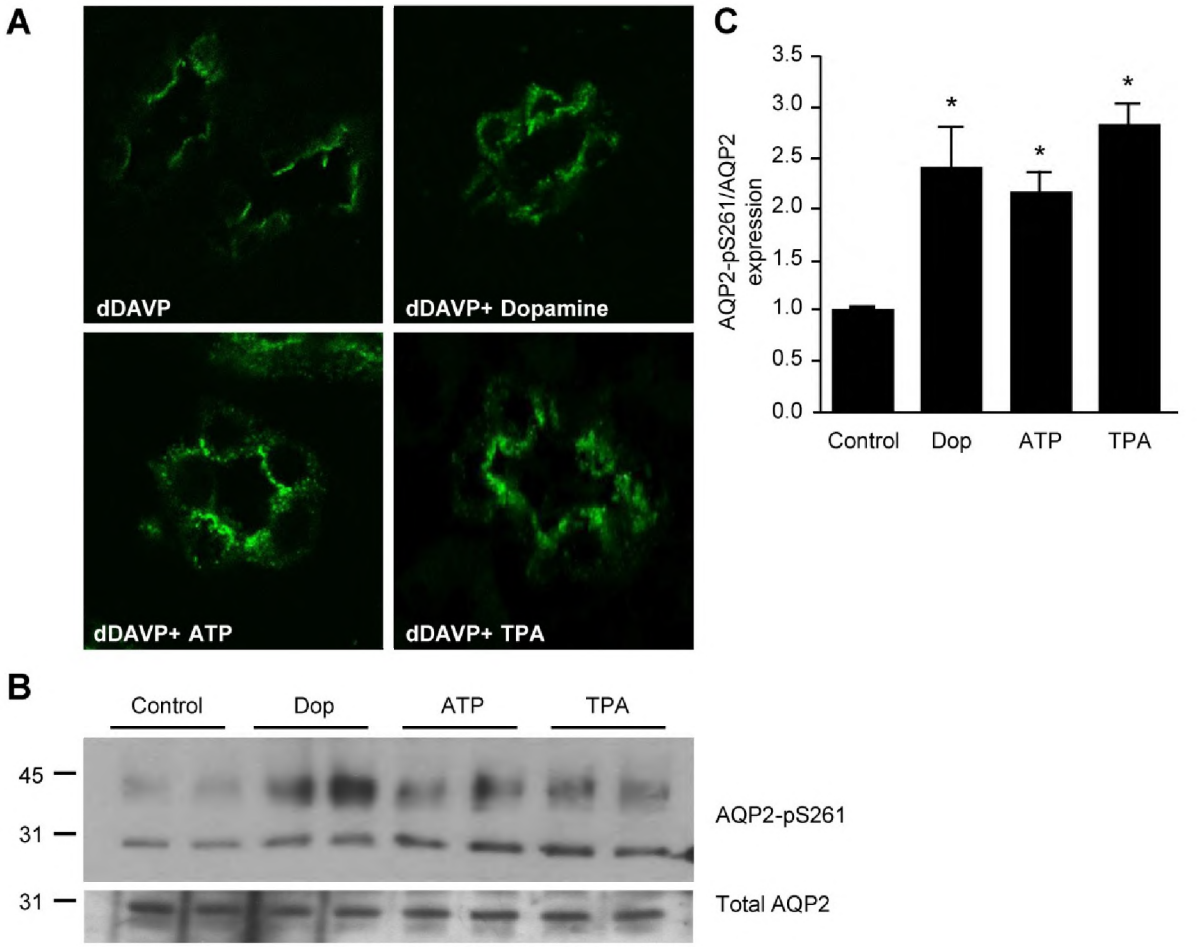


Figure 9. Effect of ATP, dopamine, and TPA on AQP2 localization and S261 phosphorylation *ex vivo*. Rat kidney slices were pre-incubated at 37°C with 1 nM dDAVP for 15 min, followed by 30 min incubation with dDAVP alone or together with 100 µM dopamine, 100 µM ATP or 100 nM TPA. Then, the kidney sections were **(A)** stained for AQP2 and subjected to confocal laser scanning microscopy, or **(B)** lysed in a 1% Triton X-100 buffer and subjected to immunoblotting for total AQP2 and AQP2-pS261. **(C)** The signals of (B) were semi-quantified using densitometry. Samples significantly ($p<0.05$) different from controls are indicated by asterisk.

Discussion

ATP and dopamine counteract AVP-action by inducing ubiquitin-dependent AQP2 internalization and lysosomal degradation.

Several studies have demonstrated a role for extracellular purines ATP and UTP, and phenetylamine dopamine in the inhibition of AVP-induced water reabsorption (10; 11; 15; 16;

31; 32). Our present study reveals that the mechanism of action by which these hormones counteract vasopressin consists of two parts:

The first part involves the short-term ubiquitin-dependent internalization and lysosomal degradation of AQP2. Using mpkCCD cells stimulated with AVP to induce total and plasma membrane expression of endogenous AQP2, ATP and dopamine induced internalization of AQP2 from the apical membrane (Fig. 2) followed by lysosomal degradation (Fig. 6). Importantly, using kidney slices we could show that these short term effects on AQP2 internalization mimicked the *in vivo* effects of ATP and dopamine on AQP2 localization (Fig. 9A). As we have shown for TPA-induced internalization of AQP2 (8), our data reveal that this internalization of AQP2 by ATP and dopamine is dependent on the ability to ubiquitinate AQP2 (Fig. 4). Using MDCK-AQP2 cells, we found that both hormones increase AQP2 ubiquitination, resulting in two main bands of 43 and 50 kDa, a weaker higher band of around 58 kDa and some smear (Fig. 3). This smear may represent glycosylated, K63-ubiquitinated AQP2 or possibly K29-poly-ubiquitinated AQP2. Poly-ubiquitination via K29 in ubiquitin is thought to be a general mechanism to degrade membrane proteins via the ER-associated degradation (ERAD) pathway. These different ubiquitination bands were not observed in precipitates of mock-transfected MDCK cells and are consistent with the addition of 2-4 ubiquitin moieties onto AQP2 (8). Possibly because of increased expression of de-ubiquitinating enzymes in dDAVP-induced mpkCCD cells, ubiquitinated AQP2 is not readily detectable in mpkCCD cells (see also below). However, our finding that wt-AQP2, but not the constitutively-de-ubiquitinated AQP2-K270R (8), is internalized in mpkCCD cells with both hormones (Fig. 4), indicates that ubiquitination is an important regulatory mechanism in mpkCCD cells as well and that ubiquitination of AQP2 is needed for both hormones to internalize AQP2.

The second part involves the long term effects on AQP2 mRNA expression. As shown in figure 8 and consistent with *in vivo* data using perfused inner medullary collecting ducts (11; 21), ATP and dopamine also significantly reduce the steady state cAMP levels. As AQP2 gene expression is increased when the PKA-phosphorylated cAMP-regulatory element binding protein (CREB) binds to the CRE present in the AQP2 promoter (6; 7), the reduced AQP2 mRNA levels observed with both hormones (Fig. 7) is likely a consequence of the reduced cAMP levels.

The reduction in cAMP with dopamine and ATP seems rather low, but it has to be taken into account that it is considered and even shown for AQP2 that intracellular cAMP levels are locally regulated, in which the kinase anchoring proteins (AKAPs) play an important role (33; 34). AKAPs bind PKA, PKA substrates, phosphatases and PDEs, and target these proteins, and thus cAMP-induced signalling cascades to various subcellular compartments to their

unique targeting domains (3; 35; 36). It will be interesting to test whether ATP and dopamine do decrease local pools of intracellular cAMP.

Short-chain ubiquitination of AQP2 precedes phosphorylation at S261

While the essentiality of S256 phosphorylation for AQP2 translocation to the apical membrane is unchallenged, several data reveal that internalization of AQP2 does not necessarily require S256 de-phosphorylation, as activation of PKC by TPA (37), but also dopamine and prostaglandin E_2 (PGE_2)-induced internalization of AQP2 to intracellular vesicles occurred without reducing S256 phosphorylation (38; 39). Recently, we have shown that TPA-induced AQP2 endocytosis involves its short-chain ubiquitination at K270 (8), but it is unclear if any of the three newly identified additional phosphorylation sites in the C-terminal tail of AQP2 may be involved, being S261, S264, and S269 (23). Interestingly, Hoffert *et al* showed that S261 phosphorylation of IMCD AQP2 was decreased upon AVP incubation (24). Here, we demonstrated that, in line with *in vivo*, dDAVP decreases phosphorylation of AQP2 at S261 (Fig. 5). More importantly, however, our data reveal that also internalization of AQP2 with ATP and dopamine coincides with an increase in S261 phosphorylation (Fig. 5B).

In mpkCCD cells stably transfected with AQP2, ATP and dopamine application initially results in an increased pS261 signal of a 43 kDa band, while the 29 kDa band was not or only weakly increased (Fig. 5A). In two different time series, it appeared that 43 kDa AQP2-pS261 preceded AQP2-pS261 of 29 kDa (Fig. 5B,C). This pS261-labelled 43 kDa band likely represents AQP2 bound by 2 ubiquitin moieties, because its mass is consistent with this (8), and the band is not observed in ATP or dopamine treated mpkCCD cells expressing AQP2-K270R (Fig. 5A, B). Although we cannot exclude the possibility that our pS261 antibodies have a higher affinity for ubiquitinated than non-ubiquitinated AQP2 and/or that ubiquitinated AQP2 is more prone to S261 phosphorylation than 29 kDa AQP2, these data indicate that AQP2 ubiquitination precedes pS261 phosphorylation as an early event in the ATP/dopamine-induced AVP-counteracting pathway of AQP2 proteins from the plasma membrane to lysosomes. This phosphorylation of S261, however, was not essential for ATP/dopamine/TPA induced internalization or degradation, because internalization and degradation of AQP2 proteins mimicking constitutively phosphorylated or de-phosphorylated S261 (Fig. S2) was similar to that of wt-AQP2. Although these data may suggest that ubiquitination is needed before pS261 can occur, it is more likely that ubiquitination is a faster process or that pS261 of AQP2 occurs at an intracellular location (i.e. after ubiquitin-mediated endocytosis), because AQP2 that cannot be ubiquitinated (AQP2-K270R) is phosphorylated at S261 in unstimulated mpkCCD cells. The role of S261 phosphorylation in AQP2 thus remains to be established. Moreover, it is noteworthy that the localization of

AQP2-S261A/D in mpkCCD cells with or without application of dDAVP is similar to that found by others in different cells types (24; 40), but is different from the localization we observed in MDCK cells (unpublished data), which is likely due to expression of a different set of proteins in our MDCK type I cell model. It will be interesting to identify the protein(s) causing this difference in handling AQP2-S261A/D.

Recently, the first steps were taken to identify the kinases involved in the phosphorylation of AQP2 at S261. Although PKA is responsible for the phosphorylation of AQP2 at S256, Hoffert *et al.* demonstrated that PKA is unable to phosphorylate S261 (41). *In vitro* experiments where synthetic COOH-terminal AQP2 peptides were incubated with various purified MAP kinases, demonstrated that JNK, p38, and CDK5/9 could potentially be involved in the phosphorylation of AQP2 at S261 (42). Although JNK, p38, and CDK5/9 are putative candidates, the identity of kinases involved in the ATP and dopamine dependent S261 phophorylation of AQP2 remains to be established.

ATP and dopamine receptors involved in AQP2 degradation

Hormones can inhibit AVP-stimulated water permeability by decreasing cAMP levels, but can also exert their effects without affecting cAMP production, depending on the receptor subtype and subsequent G protein that is activated. In case the receptor couples to Gi, cAMP production will be inhibited, whereas upon Gq coupling, phospholipase C is activated and intracellular Ca^{2+} levels will increase. In AVP-stimulated mpkCCD cells, ATP and dopamine both decreased cAMP production (Fig. 8), suggesting that ATP and dopamine activate receptors that couple to Gi.

Various purinergic receptors have been identified in the collecting duct. Our recent paper suggests the involvement of P2Y4 and P2X2 receptors, in addition to P2Y2, in AQP2-mediated water permeability in mpkCCD cells (14). Alternatively, the effect of ATP may be mediated indirectly through EP receptors. Extracellular purines have been shown to stimulate P2Y2 receptor-mediated release of arachidonic acid and PGE_2 (45), which activate the Gi coupled EP receptors EP1 and EP3.

Of the dopamine receptors, D2, D3 and D4 couple to Gi. Of these, the D4 receptors may be most likely to mediate the effect of dopamine on AQP2, since these receptors are expressed in the renal collecting duct (15; 16).

Considering the fact that TPA, which is thought to activate PKCs, does counteract vasopressin action through the short term mechanism (AQP2 internalization, ubiquitination, lysomal degradation; Fig. 2, 3, and 6)), but does, in contrast to ATP and dopamine, not affect reduce cAMP levels (Fig. 8), may indicate that ATP and dopamine indeed use a bimodal mechanism (direct PKC activation through Gq and indirect reduction of cAMP through activation of prostaglandin receptors and Gi) to counteract vasopressin action. The identity of

the purinergic and dopamine receptors involved and the precise mechanism by which activation of these receptors result in reduced cAMP and AQP2 levels remains to be established.

In summary, we demonstrated that ATP and dopamine counteract AVP-induced water permeability by two mechanism, being AQP2 internalization and lysosomal degradation in an ubiquitin-dependent and pS261-related manner, and through decreased cAMP levels.

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CHAPTER 3

A change in prostaglandin receptor subtype may explain the differential effect of prostaglandin E_2 on AQP2 expression in the absence or presence of vasopressin

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Abstract

Urine concentration involves arginine vasopressin (AVP), which induces the redistribution of aquaporin-2 (AQP2) water channels to the apical membrane and an increased transcription of AQP2 in renal principal cells. Besides AVP, prostaglandins are also involved in water balance regulation. Prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) decrease water reabsorption in the presence of AVP. However, PGE_2 increases water reabsorption in the absence of AVP.
In the present study, we utilized mouse cortical collecting duct ($mpkCCD_{cl4}$) cells as a model system to delineate how prostaglandins can exert their diverse effects on AQP2. In the presence of dDAVP, blocking cyclooxygenase activity by indomethacin for 48 hours increased AQP2 levels, while both PGE_2 and $PGF_{2\alpha}$ application reduced AQP2. In the absence of dDAVP, AQP2 was increased by PGE_2. dDAVP application significantly increased PGD_2 and PGE_2 production, while $PGF_{2\alpha}$ was decreased. The prostaglandin receptors EP1, EP4, FP and TP were found to be expressed by RT-PCR. dDAVP application increased the expression of the EP1 and FP receptor, while EP4 expression was decreased.
Altogether, our study shows that in $mpkCCD_{cl4}$ cells, both PGE_2 and $PGF_{2\alpha}$ decrease dDAVP-stimulated AQP2 abundance, while in the absence of dDAVP, PGE_2 increases AQP2 levels. This difference in PGE_2 effects might be explained by the different receptor subtype expression induced by dDAVP. Besides, dDAVP significantly increased both the production of PGD_2 and PGE_2, while levels of $PGF_{2\alpha}$ were decreased.

Introduction

To prevent dehydration, a proper regulation of the water homeostasis is essential. In this process, the kidney is the main organ involved. In response to hypernatremia or hypovolemia, arginine vasopressin (AVP) is released from the posterior pituitary gland. Subsequently, binding of AVP to the basolateral vasopressin type-2 receptor in the collecting duct principal cells in the kidney results in redistribution of aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical membrane, greatly increasing the osmotic water permeability, leading to a concentrated urine (1). In addition, AVP also increases the expression of AQP2 via phosphorylation of the cAMP responsive element binding protein (CREB), which activates the transcription from the AQP2 promoter (2-4).
Besides AVP, several other signaling molecules regulate the water balance by antagonizing AVP-induced water transport (5). One such group of molecules involved are the prostaglandins.

The arachidonic acid/prostaglandin pathway involves many different enzymes and products (Fig. 1). Arachidonic acid, which is derived from membrane phospholipids, is converted to prostaglandin H_2 (PGH_2) by the enzymes cyclooxygenase (COX) 1 and 2. PGH_2 is a substrate for many different synthases to produce PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2. Besides being produced directly from PGH_2, $PGF_{2\alpha}$ is also synthesised via PGE_2 by a reaction catalyzed by the enzyme prostaglandin E 9-ketoreductase (6)

The prostanoids are released from the cells either by diffusion or by an efflux transporter such as the multidrug resistance protein 4 (MRP4) (7). Released prostanoids can bind to their unique G-protein coupled receptors (DP, FP, IP and TP), or to one or more of four different receptors as found for PGE_2 (EP1, EP2, EP3, EP4). Some of these receptors (DP, EP2, EP4, IP) are Gs-coupled and thus increase intracellular cAMP levels when activated, whereas others are coupled to Gi (EP3, FP), reducing cAMP synthesis, and/or Gq (EP1, FP, TP), inducing calcium mobilization (6; 8; 9).

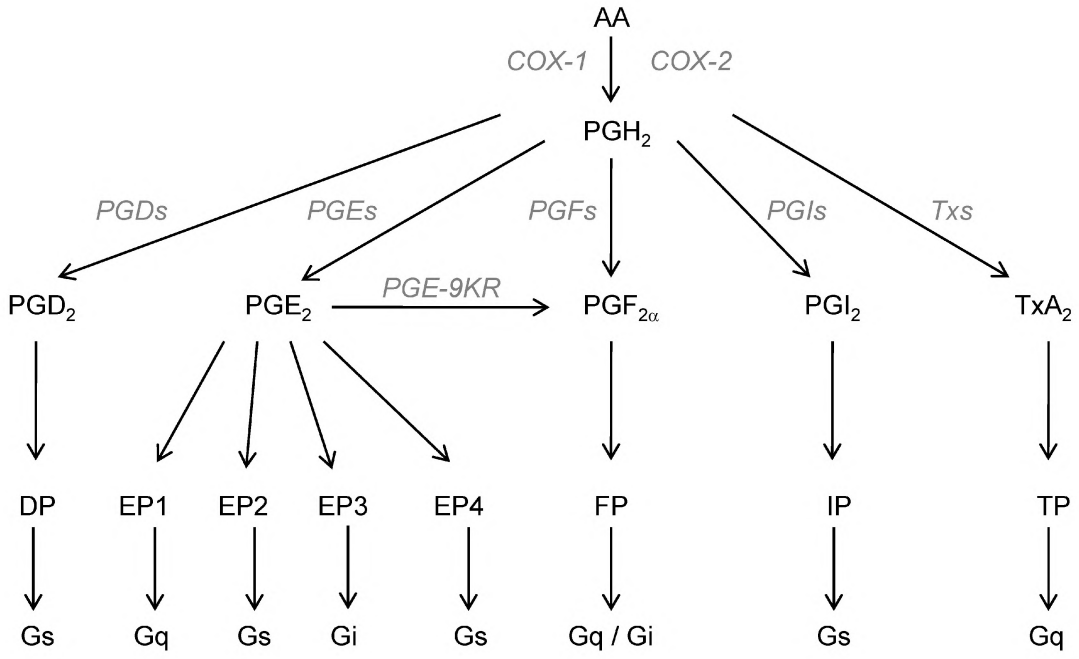

Figure 1. Synthesis of the prostaglandins. Arachidonic acid (AA) is metabolized by COX1 or COX2 to PGH_2. PGH_2 is enzymatically converted, by specific synthases (thromboxane synthase, PGE synthase, PGF synthase, PGD synthase, and PGI synthase) or prostaglandin E 9-ketoreductase (PGE-9KR), to one of five known primary prostanoids: PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$, or TxA_2. Each prostanoid interacts with distinct members of a subfamily of the G protein–coupled receptors. PGI_2 activates the IP receptor, PGD_2 activates the DP receptor, $PGF_{2\alpha}$ activates the FP receptor, and TxA_2 activates the TP receptor. PGE_2 interacts with one of four distinct EP receptors.

Of the different prostaglandins, in particular PGE_2 has been shown to decrease AVP-stimulated water reabsorption in perfused collecting ducts (10-12). In addition to its role in ambient water homeostasis, PGE_2 is also involved in pathological regulation of water reabsorption. PGE_2 has been suggested to play an important role in the development of lithium-induced nephrogenic diabetes insipidus. This is based on the observation that the

expression of COX-2 is markedly increased in the kidney of lithium treated mice, resulting in an increased urinary PGE_2 excretion (13). Similarly, in bilateral ureteral obstruction, associated with AQP2 down-regulation, COX-2 protein abundance as well as the concentration of all five primary prostanoids are increased in the kidney inner medulla (14). Administration of a COX-2 inhibitor prevents down-regulation of AQP2 in inner medullary collecting ducts and attenuates the polyuria (15). In addition, the polyuria observed in patients with antenatal Bartter's syndrome may in part be worsened by the excessive renal formation of PGE_2 (16).

Besides PGE_2, also $PGF_{2\alpha}$ affects water homeostasis, as it has been shown to inhibit vasopressin-stimulated water permeability in the collecting duct (9; 17).

Paradoxically, although PGE_2 decreases water reabsorption in the presence of AVP, it increases the osmotic water permeability in the absence of AVP (11; 12). The mechanism, however, is still unclear. In the present study, therefore, we utilized mouse cortical collecting duct ($mpkCCD_{cl4}$) cells as a model system for the renal principal cell to delineate how prostaglandins can exert their diverse effects on the expression of AQP2 in the presence or absence of AVP.

Materials and Methods

Cell Culture

Mouse $mpkCCD_{cl4}$ cells were essentially maintained as described previously (18). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were exposed to 1 nM dDAVP at the basolateral side during the last 96 hrs, to maximally induce AQP2 expression (19). Cells were incubated with 10 μM indomethacin, 1 μM PGE_2 (both Sigma, St. Louis, MO, USA) or $PGF_{2\alpha}$ (Calbiochem, San Diego, CA) during the last 48 hours.

Immunoblotting

$MpkCCD_{cl4}$ cells grown on 1.13 cm^2 filters were lysed in 200 μl Laemmli. SDS-PAGE, blotting and blocking of the PVDF membranes were done as described previously (20). Membranes were incubated for 16 hrs with 1:3000-diluted affinity-purified rabbit R7 anti-AQP2 antibodies (21) in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% w/v non-fat dried milk. Blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's (Sigma, St. Louis, MO) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

RT-PCR

MpkCCD$_{cl4}$ cells were grown on semi-permeable filters for 8 days as described above, and total RNA was isolated using TriZol extraction reagent (Gibco, Life Technologies, Rockville, MD), according to the manufacturer's instructions. To remove genomic DNA, total RNA was treated with DNase (Promega, Madison, WI) for 1 hr at 37°C, extracted with phenol/chloroform and precipitated. RNA was reverse-transcribed into cDNA using MMLV Reverse Transcriptase and random primers (Promega, Madison, WI). During cDNA production, a control reaction without the reverse transcriptase enzyme was conducted to exclude genomic DNA amplification. Primers for prostaglandin receptors were designed to bind to two different exons (see Table 1). Amplification was performed using the cDNA equivalent of 5 ng RNA for 40 cycles (95°C 45 sec, 50°C 1 min, 72°C 1.5 min). β-actin was used as a positive control for cDNA amplification. For all prostaglandin receptor primers, cDNA from tissue reported to express the particular receptor was taken along as a positive control. The proper identity of products was confirmed using restriction analysis.

SYBR Green Real-time quantitative PCR was performed on an iQ5 Real-Time PCR Detection System from Bio-Rad by utilizing the SYBR Green PCR Master Mix (Applied Biosystems Foster City, CA) and primers for the prostaglandin receptors. Signals for the ribosomal 18S (GTAACCCGTTGAACCCCATT and CCATCCAATCGGTAGTAGCG), which was amplified in parallel, were used to normalize for differences in the amount of starting cDNA.

Table 1: Overview of primer sets

Protein	Forward primer (5´-3´)	Reverse primer (5´-3´)	Product size (bp)
DP	AGGAGCTGGACCACTTTGTG	TCACAGACAGGAAACGCAAG	159
EP1	GCACGGAGCCGAGGAGC	GCAGGGGCTCATATCAGTGG	107
EP2	TCGCCATATGCTCCTTGC	TCCTCTGACACTTTCCACAAA	449
EP3	GCAGAATCACCACGGAGACG	GCGAAGCCAGGCGAACTG	190
EP4	TACGCCGCCTTCTCTTACAT	TTCACCACGTTTGGCTGATA	380
FP	CGTCACGGGAGTCACACTCT	TTCACAGGTCACTGGGGAAT	190
IP	CATGACCGTCATCATGGCCGTG	GTTGAAGGCGTTGAAGCGGAAGG	120
TP	GTGGGCATCATGGTGGTGG	CACACGCAGGTAGATGAGCAGC	168
β actin	GTATGCCTCTGGTCGTACCAC	ACGATTTCCCTCTCAGCTGTG	201

Prostanoid analysis

Samples were prepared as described (22) with minor modifications. Briefly, cell culture supernatants were spiked with ~1 ng of deuterated internal standards, and the methoximes were obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted, and the

pentafluorobenzylesters were formed. Samples were purified by thin layer chromatography, and a broad zone with R_F 0.03-0.4 was eluted. After withdrawal of the organic layer, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis on a Finnigan MAT TSQ700 GC/MS/MS (Thermo Electron Corp., Dreieich, Germany) equipped with a Varian 3400 gas chromatograph (Palo Alto, CA) and a CTC A200S autosampler (CTC Analytics, Zwingen, Switzerland).

Statistics

Students unpaired t-test was used when two groups with Gaussian distribution were compared. A *p* value of less than 0.05 is considered significant. Data are presented as mean and bars are standard error of the mean (SEM).

Results

Effect of prostaglandins on AQP2 expression

As introduced, PGE_2 has been reported to increase water reabsorption in the absence of AVP, but to decrease it in the presence of AVP (10-12). Moreover, $PGF_{2\alpha}$ has also been shown to inhibit water absorption in the collecting duct (9; 17). To analyze the effect of PGE_2 on AQP2 expression in $mpkCCD_{cl4}$ cells, cells were incubated with 1 µM PGE_2 for 48 hrs in the presence or absence of dDAVP for 4 days. Consistent with *in vivo*, PGE_2 increased the AQP2 abundance in the absence of dDAVP, but decreased it in the presence of the AVP-analogue (Fig. 2). In addition, in the presence of dDAVP, $PGF_{2\alpha}$ decreased AQP2 abundance (Fig. 2), although this effect seemed smaller than what was observed with PGE_2.

To test whether the COX inhibitor indomethacin affects dDAVP-induced AQP2 expression, cells were grown as described above, the last 4 days in the presence of dDAVP, and the last 48 hrs in the presence of 10 µM indomethacin. Subsequent immunoblotting showed increased AQP2 abundance with indomethacin (Fig. 2).

These data revealed that the effects of PGE_2 and $PGF_{2\alpha}$ on water permeability as found *in vivo* are mimicked in our $mpkCCD_{cl4}$ cells by effects on AQP2 abundance, and that dDAVP-treated $mpkCCD_{cl4}$ cells produce prostanoids which decrease AQP2 abundance.

Effect of dDAVP on prostanoid production in mpkCCD cells

To determine whether the presence of dDAVP affects the release of PGE_2 or other prostanoids in $mpkCCD_{cl4}$ cells, cells were grown as above, with or without dDAVP for the last 4 days, after which the medium was collected and analysed for the presence of prostanoids. Prostaglandin concentrations from fresh medium (before addition to cells) were

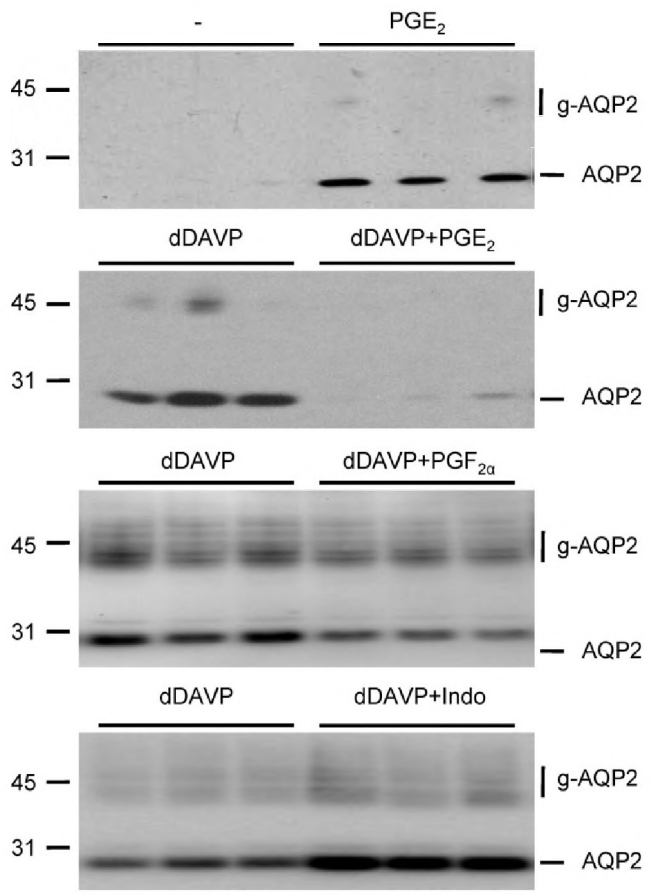

Figure 2. Effect of prostaglandins on AQP2 expression. mpkCCD$_{cl4}$ cells were grown for 8 days, either with our without 1 nM dDAVP stimulation for the last 4 days and with or without 1 µM PGE_2, 1 µM $PGF_{2\alpha}$ or 10 µM indomethacin during the last 48 hours. Cells were lysed and subjected to immunoblotting for AQP2. Molecular masses (in kDa) are indicated on the left. Non-glycosylated (AQP2) and complex-glycosylated (g-AQP2) forms of AQP2 are detected.

subtracted. The major prostanoids released from control cells were PGE_2 and $PGF_{2\alpha}$, while levels of PGD_2, 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and TxB_2 (a stable metabolite of TxA_2) were lower and close to the detection limit (Fig. 3). dDAVP incubation significantly increased the production of PGD_2 and PGE_2, while $PGF_{2\alpha}$ levels were decreased. No effect of dDAVP was observed on the release of 6-keto-$PGF_{1\alpha}$ or TxB_2.

Effect of dDAVP on prostanoid receptor expression in mpkCCD cells

The effect of prostaglandins on AQP2 expression has to be conferred by their respective G-protein coupled receptors. By RT-PCR, we determined the expression of the individual prostaglandin receptors in the mpkCCD cell system.

The lengths of the expected PCR fragments for the different receptors is given in table 1. From reverse transcribed RNA derived from dDAVP-treated cells, cDNA products of the expected size were obtained for EP1, EP4, FP and TP receptors (Fig. 4A). While EP2, EP3 or DP receptors were found to be expressed in control tissues, no expression was found in mpkCCD$_{cl4}$ cells, suggesting that these receptors are not present in this cell system. The expression of the IP receptor was inconsistent. The same receptors were found to be expressed in mpkCCD cells treated without dDAVP (not shown).

To test if the expression levels of the prostanoid receptors were influenced by dDAVP, we determined their relative expression by Q-PCR. dDAVP stimulation resulted in an increased expression of the EP1 and FP receptor subtypes, while expression of the EP4 receptor was significantly decreased. No difference was detected in the expression of the TP receptor (Fig. 4B).

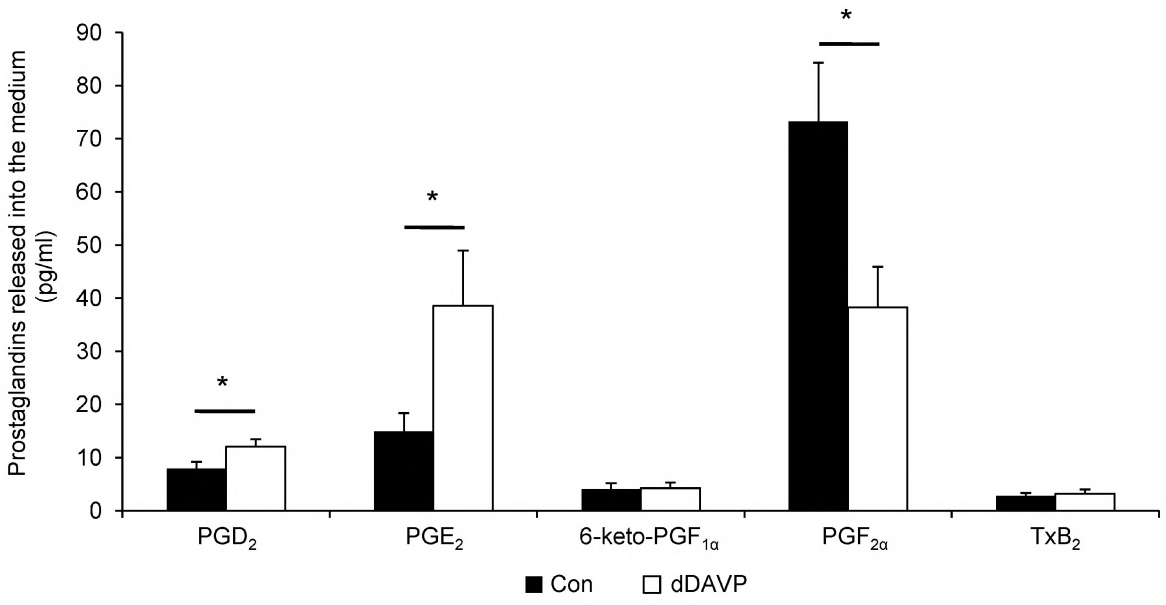

Figure 3. Effect of dDAVP on prostaglandin production in mpkCCD cells. $MpkCCD_{cl4}$ cells were grown for 8 days and incubated with or without 1 nM dDAVP for the last 4 days. Medium from both sides, incubated with the cells for 24 hrs, was collected and prostaglandin concentrations were determined. Bars are mean values of four independent filters per condition (+/- SEM). Significant differences from control ($p<0.05$) are indicated by an asterisk.

Discussion

mpkCCD cells as a model system for prostanoid regulation of AQP2 expression

In the present study, we examined the effect of prostaglandins on the expression of endogenous AQP2 in $mpkCCD_{cl4}$ cells. Prostaglandin E_2 is known to reduce AVP-stimulated water reabsorption in the collecting duct (10; 11). Similarly, in our cell system PGE_2 reduced AQP2 expression after dDAVP stimulation. In addition, PGE_2 stimulated AQP2 abundance in the $mpkCCD_{cl4}$ cells in the absence of dDAVP, which is in agreement with *in vivo* observations showing an increase in collecting duct water permeability (12).

In dDAVP-treated $mpkCCD_{cl4}$ cells, AQP2 levels were decreased after application of $PGF_{2\alpha}$, which might explain the inhibition of water reabsorption in the collecting duct observed after $PGF_{2\alpha}$ treatment (9; 17). Furthermore, blocking COX activity by indomethacin increased AQP2 abundance, showing that COX activity influences AQP2 levels and that in cells without indomethacin, dDAVP-stimulated AQP2 abundance is decreased by the action of endogenously produced prostaglandins.

The major prostaglandins produced in our cell system were PGE_2 and $PGF_{2\alpha}$, while production of PGD_2, 6-keto-$PGF_{1\alpha}$ (a metabolite of PGI_2) and TxB_2 (a metabolite of TxA_2) was low. Of the prostaglandin receptors, EP1, EP4, FP and TP receptors were found in $mpkCCD_{cl4}$ cells. No expression was found of DP, EP2 and EP3 receptors.

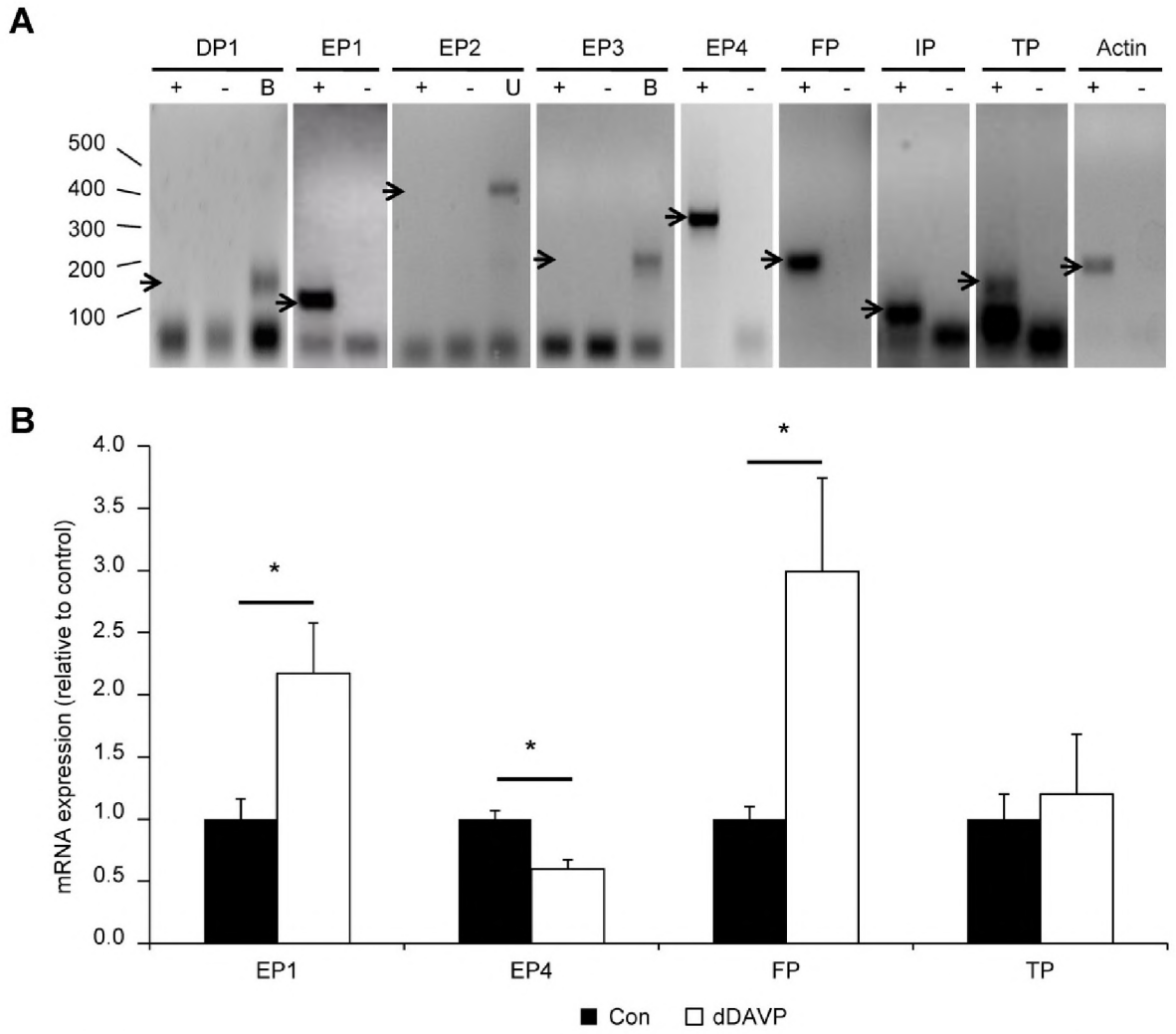

Figure 4. Prostaglandin receptor expression. A) MpkCCD$_{cl4}$ cells were grown for 8 days. Cells were lysed, total RNA was isolated and RNA was reverse-transcribed into cDNA. By RT-PCR the expression of the prostaglandin receptors was analyzed. β-actin was used as a positive control for cDNA amplification. Amplification products are visualized by agarose gel electrophoresis. +/- = with or without Reverse Transcriptase during the cDNA production. For receptors not detected in mpkCCD cells, a RT-PCR done in control tissue (B= brain, U=uterus) is shown. Arrows point at product of expected size. Sizes in bp are indicated on the left. **B)** MpkCCD$_{cl4}$ cells were grown for 8 days and treated with or without 1 nM dDAVP for the last 4 days. Cells were lysed, total RNA was isolated, RNA was reverse-transcribed into cDNA and relative expression of the prostaglandin receptors was analyzed by performing Q-PCR. Signals obtained from the house-keeping 18S were used to normalize for difference in the amount of starting cDNA. Mean values of four independent filters per condition are relative to control (+/- SEM). Significant differences ($p<0.05$) from control are indicated by an asterisk.

dDAVP-induced changes in EP1 and EP4 receptor subtype expression likely mediate the difference in AQP2 abundance in response to PGE$_2$ in mpkCCD cells

dDAVP stimulation significantly increased both the production of PGD_2 and PGE_2, while levels of $PGF_{2\alpha}$ were decreased. No effect was seen on 6-keto-$PGF_{1\alpha}$ or TxB_2. In agreement with these findings, it has been shown that vasopressin stimulates PGE_2 synthesis in isolated collecting ducts (23; 24).

Incubation of mpkCCD$_{cl4}$ cells with dDAVP resulted in an increased expression of the calcium mobilizing EP1 receptor, but decreased the expression of the EP4 receptor, coupling to Gs-

stimulated cAMP generation. As the inhibitory effects of PGE_2 on AVP-induced water reabsorption most likely occur through activation of EP1 and/or EP3 receptors (12; 25; 26), whereas the stimulatory effects of PGE_2 on basal water transport could be mediated via the EP4 receptor, this suggests that the differences in PGE_2-mediated actions on water permeability can most likely be attributed to the presence of different relative expression of the E-prostanoid receptor subtypes.

It is interesting to note that, while dDAVP increases PGE_2 production and release, the mRNA expression of EP4 receptor is reduced, whereas that of the EP1 receptor is increased. As both are bound and activated by PGE_2, these data suggest that it is not the agonist per se that determines the expression level of the receptors. Instead, our data rather indicate that the signaling cascade that is mainly activated exerts a negative feedback regulation on receptors stimulating the same pathway and a positive feedback on receptors activating an opposite pathway: dDAVP increases the cAMP-AQP2 pathway, which is also stimulated by EP4, whereas EPI, activates a pathway that leads to decreased AQP2 expression and water permeability.

Similarly to this mechanism, dDAVP increases the mRNA levels of the purinergic receptor subunit $P2Y_2$ in mpkCCD cells, and targets the subunits $P2Y_2$ and $P2X_2$ to the plasma membrane, where activation of these receptors leads to AQP2 internalisation and a decrease in water permeability (27). The same mechanism can be seen in the opposite response, where endothelin, counteracting vasopressin mediated water permeability (28) leads to an increased expression of the vasopressin V2 receptor in the inner medullary collecting duct of the rat (29).

FP receptor expression was increased by dDAVP treatment in mpkCCD cells. Activation of FP receptor leads to calcium mobilisation in most cell types, but in the collecting duct, FP receptor activation seems to inhibit cAMP generation via Gi proteins, thereby inhibiting water reabsorption (9). As FP receptor activation inhibits water reabsorption, this might be a compensatory mechanism to counteract AVP-stimulation, similar to the increase in EP1 expression, or it might be a response to the lower $PGF_{2\alpha}$ production.

As no DP receptor was detected in $mpkCCD_{cl4}$ cells the role of the dDAVP-stimulated increase in PGD_2 production after dDAVP treatment is unclear. However, PGD_2 has been shown to bind to the FP receptor with an affinity close to that for the DP receptor, indicating that PGD_2 may act on the FP receptor (30). The increase in PGD_2 might counteract the dDAVP-induced increase in AQP2 expression, although levels are low compared to the PGE_2 and $PGF_{2\alpha}$ production.

While the TP receptor is expressed in $mpkCCD_{cl4}$ cells, the expression of the IP receptor is inconclusive. Both thromboxane and PGI_2 were produced in very low amounts in $mpkCCD_{cl4}$

cells and production was not affected by dDAVP. However, if these prostanoids might have any role in water reabsorption remains unclear.

Relation of the mpkCCD cell system to the in vivo situation.

The major prostaglandins produced in our cell system were PGE_2 and $PGF_{2\alpha}$, while levels of PGD_2, PGI_2 and TxA_2 were low, which is in agreement with *in vivo* findings, showing that PGE_2 is the most abundant prostanoid in both renal cortex and medulla, followed by PGI_2 and $PGF_{2\alpha}$, with even lower amounts of thromboxane A_2 and PGD_2 (31). The synthases involved in the production of PGD_2, PGE_2 and $PGF_{2\alpha}$ are detected in the nephron (32; 33). In the nephron, production of PGE_2 and $PGF_{2\alpha}$ has been shown to occur mainly in the collecting tubule (34). As both PGI synthase and thromboxane synthase mRNA is not detected in any tubular structure, their prostanoid products seem to be mainly produced by cells outside the tubuli, like the vasculature and glomeruli, respectively (32).

The effects of prostaglandins on AQP2 expression are conferred by PG receptors. In $mpkCCD_{cl4}$ cells, EP1, EP4 and FP receptors are found, in agreement with expression in the collecting duct (8; 35). Neither DP and EP2 receptors seem to be expressed in $mpkCCD_{cl4}$ cells as well as in the kidney (8; 36). TP and IP receptors are mainly localized in the glomerulus and vasculature, respectively, but have also been located to the collecting duct (37; 38), in agreement with the expression seen in $mpkCCD_{cl4}$ cells, although results for IP were inconclusive.

Both EP1 and EP3 activity inhibits vasopressin-stimulated water reabsorption. EP1 activation, inducing calcium mobilization (8), was found in the mpkCCD cells, in agreement with *in vivo* data. However, a limitation of our cell model is the absence of the EP3 receptor, which is found *in vivo* in the collecting duct. EP3 activation inhibits cAMP generation via Gi (8), and might therefore inhibit AVP-stimulated AQP2 transcription by inhibiting the cAMP-PKA-CREB pathway. If EP3 receptor expression is also increased by dDAVP, like EP1, could not be investigated in our study. Both EP2 and EP4 receptors signal via Gs-stimulated cAMP generation. EP2 receptor mRNA is not detected in the kidney, while EP4 is expressed in the collecting duct, (8), in agreement with $mpkCCD_{cl4}$ cells expression.

In conclusion, our study shows that in $mpkCCD_{cl4}$ cells, both PGE_2 and $PGF_{2\alpha}$ decrease dDAVP-stimulated AQP2 expression, while in the absence of dDAVP, PGE_2 increases AQP2 levels. This difference in PGE_2 effects might be explained by the different receptor subtype expression induced by dDAVP treatment. In addition, dDAVP application significantly increases both the production of PGD_2 and PGE_2, while levels of $PGF_{2\alpha}$ are decreased.

Based on our data above that a negative feedback is mediated by the signaling pathways activated instead of the agonist, it is likely that *in vivo* vasopressin increases, besides AQP2,

the expression of EP1 and EP3 and decreases expression of EP4. Consequently, upon conditions with increased PGE_2 release, such as lithium-NDI or bilateral uteral obstruction, vasopressin-induced AQP2 expression would be reduced via activation of these EP1/3 receptors. This has to be established in future studies.

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CHAPTER 4

Hypotonicity-induced reduction of aquaporin-2 transcription in mpkCCD cells is independent of the tonicity responsive element, vasopressin and cAMP

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Abstract

The syndrome of inappropriate antidiuretic hormone secretion is characterized by excessive water uptake and hyponatremia. The extent of hyponatremia, however, is less than anticipated, which is ascribed to a defense mechanism, the vasopressin-escape, and is suggested to involve a tonicity-determined down-regulation of the water channel aquaporin-2 (AQP2). The underlying mechanism, however, is poorly understood. To study this, we used the mouse cortical collecting duct (mpkCCD) cell line.

MpkCCD-cells, transfected with an AQP2-promoter luciferase construct showed a reduced and increased AQP2 abundance and transcription following culture in hypotonic and hypertonic medium, respectively. This depended on tonicity rather than osmolality and occurred independent of the vasopressin analogue dDAVP, cAMP levels or protein kinase A activity. Although prostaglandins and nitric oxide reduced AQP2 abundance, inhibition of their synthesis did not influence tonicity-induced AQP2 transcription. Also, cells in which the cAMP or tonicity responsive element (CRE/TonE) in the AQP2-promoter were mutated showed a similar response to hypotonicity. Instead, the tonicity-responsive elements were pin-pointed to nucleotides -283 to -252 and -157 to -126 bp of the AQP2 promoter.

In conclusion, our data indicate that hypotonicity reduces AQP2 abundance and transcription, which occurs independent from vasopressin, cAMP, and the known TonE and CRE in the AQP2-promoter. Increased prostaglandin and nitric oxide, as found *in vivo*, may contribute to reduced AQP2 in vasopressin-escape, but do not mediate the effect of hypotonicity on AQP2 transcription. Our data suggest that two novel segments (-283 to -252 and -157 to -126 bp) in the AQP2-promoter mediate the hypotonicity-induced AQP2 down-regulation during vasopressin-escape.

Introduction

Renal water reabsorption is regulated by the hormone arginine vasopressin (AVP). AVP binding to its V2 receptor in renal principal cells induces a cAMP cascade leading to increased translocation of the water channel aquaporin-2 (AQP2) to the apical membrane, resulting in water reabsorption from the pro-urine (1). In addition to this short-term effect, cAMP increases AQP2 transcription through phosphorylation of the transcription factor CREB (cAMP Responsive Element Binding Protein), which binds to a cAMP Responsive Element (CRE) at -210 in the AQP2 promoter (2; 3). The increase in transcription is a more long-term effect, requiring hours to take effect.

AVP synthesis and release are regulated by alterations in plasma osmolality as well as non-osmotic, baroreceptor-mediated, pathways (4). Osmolality not only affects plasma AVP, but also appears to have direct effects on the urine concentrating ability and AQP2 expression. In the syndrome of inappropriate antidiuretic hormone secretion (SIADH), for example, levels of AVP are inappropriately high relative to plasma osmolality, resulting in free-water retention and hypotonicity. Under these circumstances, however, the free-water excretion is considerably higher than would be expected from the vasopressin concentrations (5). This phenomenon, called the vasopressin escape, indicates that there are mechanisms counteracting vasopressin action. In a rat animal model, it has been shown that the onset of this escape coincides with a decrease in the AQP2 expression in the renal collecting duct (6). Hypotonicity and/or volume expansion have been proposed to mediate this AVP-independent direct regulation of AQP2 expression (7).

Alternatively, an AVP-independent increase of AQP2 expression has been suggested to occur with *hyper*tonicity. Valtin and Edwards already reported that water deprivation of AVP-deficient Brattleboro rats leads to hypertonicity, increased papillary interstitial osmolality, and concentration of the urine (8). Similarly, Li *et al.* found that hypertonicity due to hyperglycemia or increased NaCl levels increases AQP2 expression in Brattleboro rats (9).

At present, it is still largely unknown how the AVP-independent expression of AQP2 is regulated at the molecular level. In mouse collecting duct principal cells (mpkCCD$_{c14}$), Hasler *et al.* showed that hypertonicity decreases the expression of AQP2 on the short term, but increases its expression on the long term (10). Here, hypertonicity did not affect the stability of AQP2 mRNA or protein, indicating that hypertonicity increases AQP2 abundance by increasing its transcription.

One of the proteins suggested to be involved in the hypertonicity response is the tonicity-responsive enhancer binding protein (TonEBP). This protein is upregulated with hypertonicity in cultured cells and in the kidney medulla (11; 12) and TonEBP knockdown or inhibition, but also mutation of its tonicity responsive element (TonE) in the AQP2 promoter, reduced AQP2 expression in mpkCCD cells (13; 14). Moreover, in TonEBP$^{-/-}$ mice and mice transgenic for dominant-negative TonEBP, AQP2 expression was decreased (15; 16). However, the role of TonEBP and its TonE element in AQP2 expression regulation is still controversial. In Madin Darby Canine Kidney (MDCK) cells, for example, Kasono *et al.* observed a hypertonicity-induced increase in AQP2 transcription only when the AQP2 promoter was -6,1 kb or longer and therefore did not involve TonE, which localizes at 489 bases upstream of the AQP2 transcription start site (7). Moreover, the severe atrophy of the renal medulla in the TonEBP$^{-/-}$ and dominant-negative TonEBP overexpressing mice complicates the interpretation of its direct involvement in AQP2 regulation.

In addition to TonE, other promoter elements have been suggested to be involved in hypertonicity-induced AQP2 expression, as Li *et al.* reported that NFATc binding sites downstream of TonE are also important for hypertonicity-induced AQP2 expression in mpkCCD cells, because following mutation of all five sites hypertonicity-induced AQP2 expression was lost to a great extent (14).

Thus far, the effects of hypotonicity on AQP2 expression and the underlying mechanisms have not been studied in great detail. Thus, it is unclear whether the regulatory changes during hypotonicity mirror the regulation under hypertonic conditions, and involves changes in TonEBP or in the classical AVP-cAMP signaling cascade elements. To decipher this, we tested and used the mpkCCD cell line, which shows dDAVP-induced expression of endogenous AQP2 (17).

Materials and Methods

Cell Culture

Mouse mpkCCD$_{cl4}$ cells (18) were grown in a modified defined medium (DMEM:Ham's F12 1:1 vol/vol; 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% foetal calf serum, and 20 mM HEPES (pH 7.4)), resulting in a medium of 355 mOsm. Cells were seeded at a density of 1.5x10^5 cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were treated for the last 96 hrs with 1 nM 1-deamino-8-D-arginine vasopressin (dDAVP) at the basolateral side, to induce AQP2 expression. When used H89 (10 μM, Calbiochem, San Diego, CA) was added to both the apical and basolateral side. Tonicity was changed by adding solute to NaCl-free defined medium (made identical to defined medium used above). Tonicity was changed at both sides of the cells. Osmolality was measured using a Knauer K-7400 semi-micro osmometer (Berlin, Germany).

Constructs

To generate an AQP2 promoter-luciferase reporter construct (pGL3-AQP2-3.0-luc), we first added the neomycine selection marker to pGL3-basic (pGL3-neo). For this, the neomycin cDNA preceded by an SV40 promoter was cut from pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA) using *Xmn*I and *Sal*I, and ligated into a blunted *BamH*I and *Sal*I site of pGL3-basic (Promega, Madison, WI, USA). Next, a 3.3 kb fragment, containing the mouse AQP2 promoter (-2970 till +60 upstream of the transcription start site) was obtained by PCR using

the primers GAAGATCTGGTCTGGGCCACGGGGGTGGCTCTTCC and GGTGCACCGAGCCTCCTCCTCAGCC on mouse genomic DNA. Following digestion with *Bgl*II, which cuts the primer (restriction site underlined) and at -2970 in the AQP2 promoter, the product was ligated into the B*gl*II site upstream of the luciferase cDNA in pGL3-neo.
For pGL3-AQP2-0.4-luc (containing -408 till +60 of the AQP2 promoter), pGL3-AQP2-3.0-luc was cut with *Sac*II and *Kpn*I, blunted and religated. Constructs lacking 31 bp segments of the -0.4 kb promoter were made by 3 points PCR using different primers (table 1) using pGL3-AQP2-0.4-luc as a template. Mutations in CRE were introduced in pGL3-AQP2-0.4-luc using site-directed mutagenesis (Stratagene, La Jolla, CA) with mutagenesis primers indicated in table 1. This mutation has been shown to inactivate the AQP2 promoter (2). Mutations in TonE, reported to reduce hypertonicity-induced AQP2 transcription (13), were introduced in pGL3-AQP2-3.0-luc using primers as shown in table 1. To generate pGL3-CRE(21)-luc, a DNA fragment containing 21 CRE-elements was cut from pCRE121-3 (19) with *Sal*I and *Xho*I and ligated into the *Xho*I site of pGL3-neo. Proper orientation and sequences were confirmed by sequence analysis.
MpkCCD cells were stably transfected using the calcium-phosphate precipitation technique as described (20). Transfected colonies were selected with G418 (0.25 mg/mL) and pooled to level out differences between individual colonies.

Luciferase Assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) following the manufacturer's instructions. Luminescence was measured for 10 seconds using an EG&G Berthold Lumat LB9507 luminometer (Bad Wildbad, Germany). To verify that equal amounts of protein per sample were used for the luciferase assay, protein concentration was determined using the BioRad (Richmond, CA) protein assay as described (21). Light absorbance at 595 nm was measured using a helios omega spectrophotometer (Thermo Scientific, Rockford, IL, US).

Immunoblotting

mpkCCD$_{cl4}$ cells from 1.13 cm^2 filter were lysed in 200 µl Laemmli/DTT buffer, sonicated and incubated for 30 min. at 37°C. PAGE, blotting and blocking of the PVDF membranes were done as described (22). Membranes were incubated overnight with 1:3000-diluted affinity-purified rabbit R7 AQP2 (23), 1:2000 rabbit CREB (Sigma, St. Louis, MO) or 1:2000 rabbit CREB-pS133 antibodies (Sigma St. Louis, MO) in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% w/v non-fat dried milk. Blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's (Sigma, St. Louis, MO) as secondary antibody coupled to

horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Densitrometric analyses were performed using Biorad quantification equipment (Bio-Rad 690c densitometer, Chemidoc XRS) and software (QuantityOne). Equal loading of the samples was confirmed by staining of the blots with coomassie blue G250 (Serva, Heidelberg, Germany).

Table 1: Overview of primer sets

Primer	forward primer (5'-3')	reverse primer (5'-3')
Del. 1 (-408 to -377 bp)	ggggtaccgtttggggtaaggcattg	cgtcttccatggtggctttacc
Del. 2 (-377 to -346 bp)	ccacatttcctcacaagccttttagtc	gactaaaaggcttgtgaggaaatgtgg
Del. 3 (-346 to -315 bp)	cttccttggccacagcctcctctgc	cagaggaggctgtggccaaggaagg
Del. 4 (-315 to -283 bp)	aggtcactggactcattgtgggggctg	agcccccacaatgagtccagtgaccttc
Del. 5 (-283 to -252 bp)	tggtgctggtgggctccatggggtaac	taccccatggagcccaccagcaccag
Del. 6 (-252 to -220 bp)	cagccctgaggcaaaacagagacgtc	gacgtctctgttttgcctcagggctgc
Del. 7 (-220 to -189 bp)	ctgaggaaaaaacgaggaattaatgaggag	ctcctcattaattcctcgtttttttcctcag
Del. 8 (-189 to -157 bp)	cttatctggagtccgctaagatggggtg	caccccatcttagcggactccagataag
Del. 9 (-157 to -126 bp)	gtcagctgtgaaacaggagcagggatg	catccctgctcctgtttcacagctgac
Del. 10 (-126 to -95 bp)	ttcgggtgggggggagcacagggttg	aaccctgtgctccccccccacccgaag
Del. 11 (-95 to -64 bp)	aagtccgccatgccaccccacgtgc	cacgtggggtggcatggcggacttg
Del. 12 (-64 to -34 bp)	caggaacgcctcctggccctataagtg	cacttatagggccaggaggcgttcctg
mutation CRE	cgaggaaaacagagtggtcaatccttatctggagtcc	ggactccagataaggattgaccactctgttttcctcg
mutation TonE	ccaagacctttgccttttaaatttgtcccaggccagccag	ctggctggcctgggacaaatttaaaaggcaaaag

cAMP assay

MpkCCD cells were seeded on filters for 8 days, either with or without 1 nM dDAVP for the last 4 days. The last 24 hrs, cells were grown in hypotonic or isotonic medium. The phosphodiesterase inhibitor 3-isobutyl 1 methylxanthine (IBMX) was added to both the apical and basolateral medium to a final concentration of 500 µM 30 min. before harvesting. In the cells grown without dDAVP, dDAVP was added to the basolateral side 15 min before harvesting. cAMP was measured using the cAMP-Glo assay (Promega, Madison, WI) according to manufacturer's instructions.

Statistical analyses

Student's T-test was applied to compare two groups with Gaussian distribution. Levene's test was used to compare variances. P-values below 0.05 were considered significant. For multiple comparisons, Bonferroni correction was applied. Data are presented as mean +/- standard error of the mean (SEM).

Results

mpkCCD-cells as a model for tonicity-induced AQP2 expression

Hypertonicity has been shown to affect AQP2 transcription (10). To develop an easy readout cellular system for AQP2 transcription and to test whether mpkCCD cells are a proper cell model for these analyses, we generated pooled colonies of mpkCCD cells stably-transfected with the pGl3-AQP2-3.0-luc construct (mpkCCD-AQP2-3.0-luc), or a construct lacking the AQP2 promoter (mpkCCD-luc). To first test whether the promoter conferred vasopressin sensitivity, the mpkCCD-AQP2-3.0-luc and mpkCCD-luc cells were grown on semi-permeable filters for 8 days of which the last 4 days with or without 1 nM dDAVP (17). Subsequent analysis revealed a 2.5-fold higher luciferase activity in mpkCCD-AQP2-3.0-luc cells with dDAVP than without dDAVP, while luciferase activity was hardly observed in mpkCCD-luc cells, and was not increased by dDAVP (Fig. 1A). As endogenous AQP2 abundance was increased in both pooled colonies treated with dDAVP (Fig. 1B), it was concluded that the dDAVP-induced luciferase activity in mpkCCD-AQP2-luc cells is due to AQP2 promoter-specific transcription. To test whether changes in osmolality affect AQP2 expression in mpkCCD-AQP2-luc cells, cells were grown for 8 days in isotonic medium (120 mM NaCl; 355 mOsm/kg H_2O). dDAVP was added during the last 4 days. In the last 24 hrs the cells were exposed to media made hypotonic or hypertonic using different concentrations of NaCl. Following lysis, immunoblotting revealed that a decreased osmolality coincided with reduced AQP2 abundance and vice versa (Fig. 1C). This was reflected in transcription from the 3-kb AQP2 promoter luciferase reporter construct, because luciferase activity increased with hyperosmolality and decreased with hypo-osmolality (Fig. 1D).

Changes in the concentration of NaCl affect both the osmolality and tonicity. To determine whether a change in osmolality or in tonicity regulates AQP2 transcription in mpkCCD cells, cells were grown and treated with dDAVP as described above. During the last 24 hrs, cells were incubated in hypotonic medium (275 mOsm) or in a medium of 355 mOsm of which 80 mOsm was made up by different solutes. Subsequent luciferase activity measurements revealed that cell impermeant solutes such as sorbitol and sucrose mimicked the effect of NaCl, whereas a cell permeant solute such as urea had no effect (Fig. 1E). Thus, these data indicate that changes in tonicity, rather than osmolality, affect AQP2 transcription in both directions and that the 3.0 kb AQP2 promoter luciferase construct is a useful read-out system. To simulate the *in vivo* situation of hyponatremia, NaCl was used to change tonicity in all further experiments.

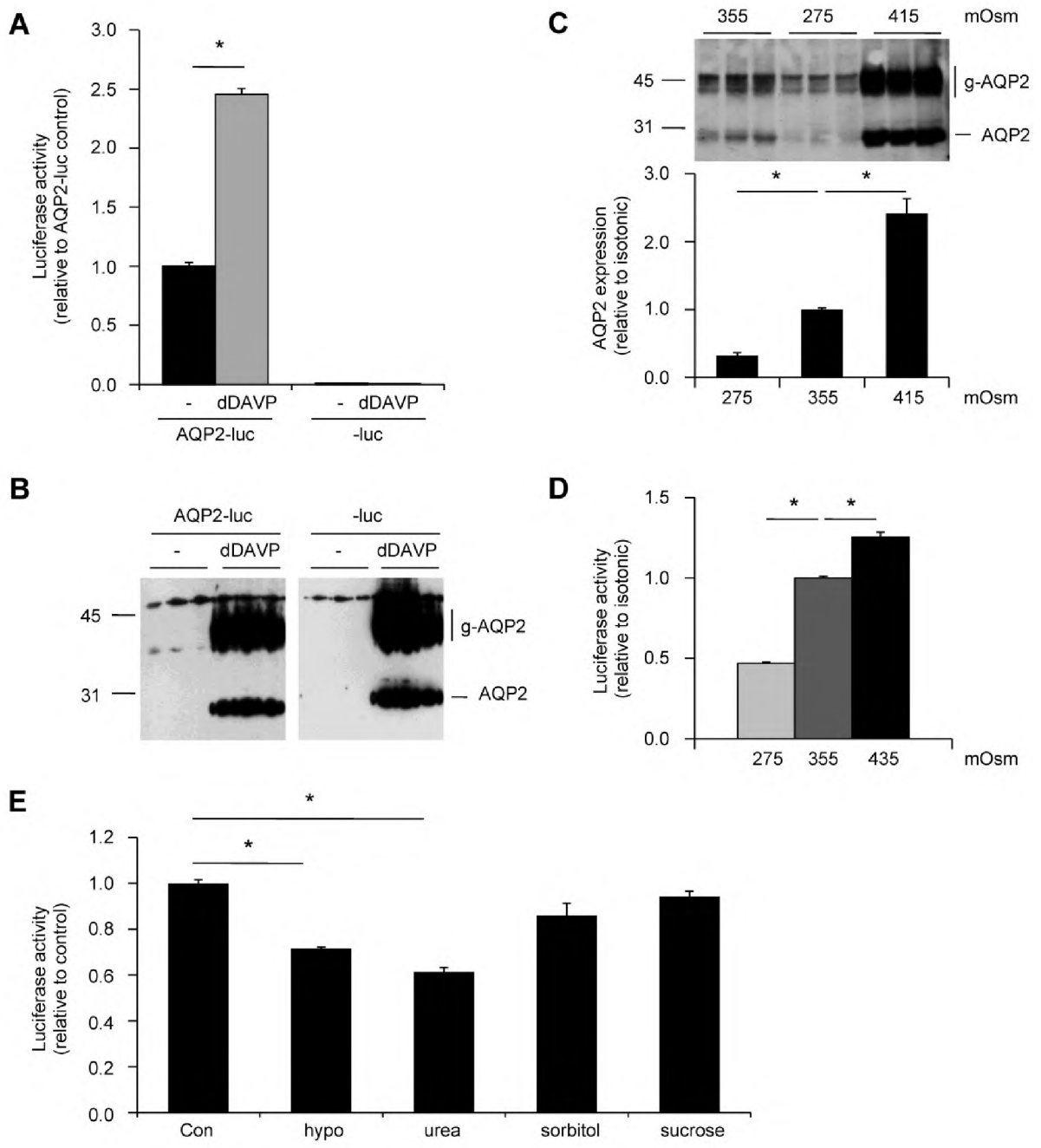

Figure 1. mpkCCD cells as a model for tonicity-induced AQP2 expression. A,B) mpkCCD-AQP2-3.0-luc and mpkCCD-luc cells were grown to confluence, the last 4 days with or without dDAVP. Cells were lysed and analyzed for luciferase activity (**A**) or for AQP2 expression (**B**). Immunoblotting the lysates for AQP2 revealed non-glycosylated (AQP2; 29 kDa) and complex-glycosylated (g-AQP2; 40-45 kDa) AQP2 in lanes of mpkCCD cells treated with dDAVP. Molecular mass (in kDa) is indicated on the left. **C,D)** MpkCCD-AQP2-3.0-luc cells were grown to confluence and treated with 1 nM dDAVP for 4 days. During the last 24 hrs cells were exposed to different osmolalities (indicated) using NaCl. Cells were lysed and analyzed for AQP2 expression (**C**) and luciferase activity (**D**). **E)** mpkCCD-AQP2-luc cells were grown as above. During the last 24 hrs, cells were grown in hypo-osmotic medium, containing 80 mM NaCl (hypo 275 mOsm), or medium with a osmolality of 355 mOsm, by adding NaCl (Con), urea, sorbitol or sucrose (indicated). Cells were lysed and luciferase activity was determined. In all experiments above, the mean values of luciferase activity per condition (+/- SEM) were determined from three independent filters per condition. Significant differences ($p<0.05$) are indicated by an asterisk.

Does the final or change in tonicity determine the AQP2 expression?

At present, it is unclear whether the change in tonicity or the tonicity itself determines AQP2 expression. To test this, dDAVP-treated mpkCCD-AQP2-3.0-luc cells were grown in medium of 355 or 275 mOsm for 8 days. In the last 24 hours medium was either unchanged or replaced by a medium of the other osmolality. Analysis of the luciferase activity revealed that under both conditions AQP2 transcription was 2-fold higher in cells grown for the last day in 355 versus 275 mOsm medium (Fig. 2). These experiments clearly indicate that the final, and not the change in, tonicity determines AQP2 expression.

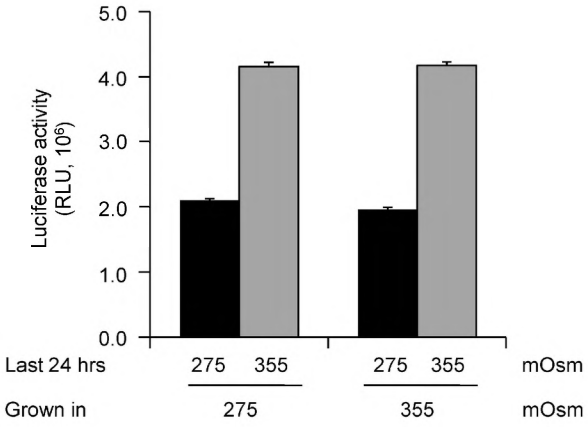

Figure 2. The final tonicity, and not the change in tonicity, determines the effect on AQP2 expression. mpkCCD-AQP2-luc cells were grown in isotonic (355 mOsm) or hypotonic (275 mOsm) medium for 8 days. In both conditions, cells were incubated in hypotonic or isotonic medium during the last 24 hours. RLU = relative light units. Mean values of luciferase activity (+/- SEM) were determined from three independent filters per condition.

Role of nitric oxide and prostaglandins

Murase *et al.* reported that endogenous nitric oxide (NO) synthesis was significantly increased in vasopressin escape rats and that inhibition of NO synthesis with L-NAME decreased urine volume and partially restored AQP2 expression (24). In addition, the excretion of prostaglandin E_2, which is known to reduce AVP-stimulated water reabsorption in the collecting duct (25; 26), is increased in vasopressin escape and prevention of this increase with the prostaglandin synthesis blocker indomethacin resulted in a delay in the onset of escape (27). To test whether inhibition of NO synthesis and prostaglandins directly affect tonicity-induced changes in AQP2 expression, mpkCCD-AQP2-3.0-luc cells were grown and treated with dDAVP as above, but for the last 24 hours changed to medium with different osmolalities in the presence or absence of 10 μM indomethacin or 1 mM L-NAME. Immunoblot analysis of AQP2 levels revealed that indomethacin and L-NAME treatment resulted in increased AQP2 protein expression levels (Fig. 3A, B). This increase with indomethacin and L-NAME was observed for every tonicity tested. However, comparison of the AQP2 expression levels revealed no differences in the tonicity response between control, indomethacin or L-NAME-treated cells. Moreover, AQP2-promoter driven transcription of luciferase was not changed with L-NAME or indomethacin at any of the tonicities tested (Fig.

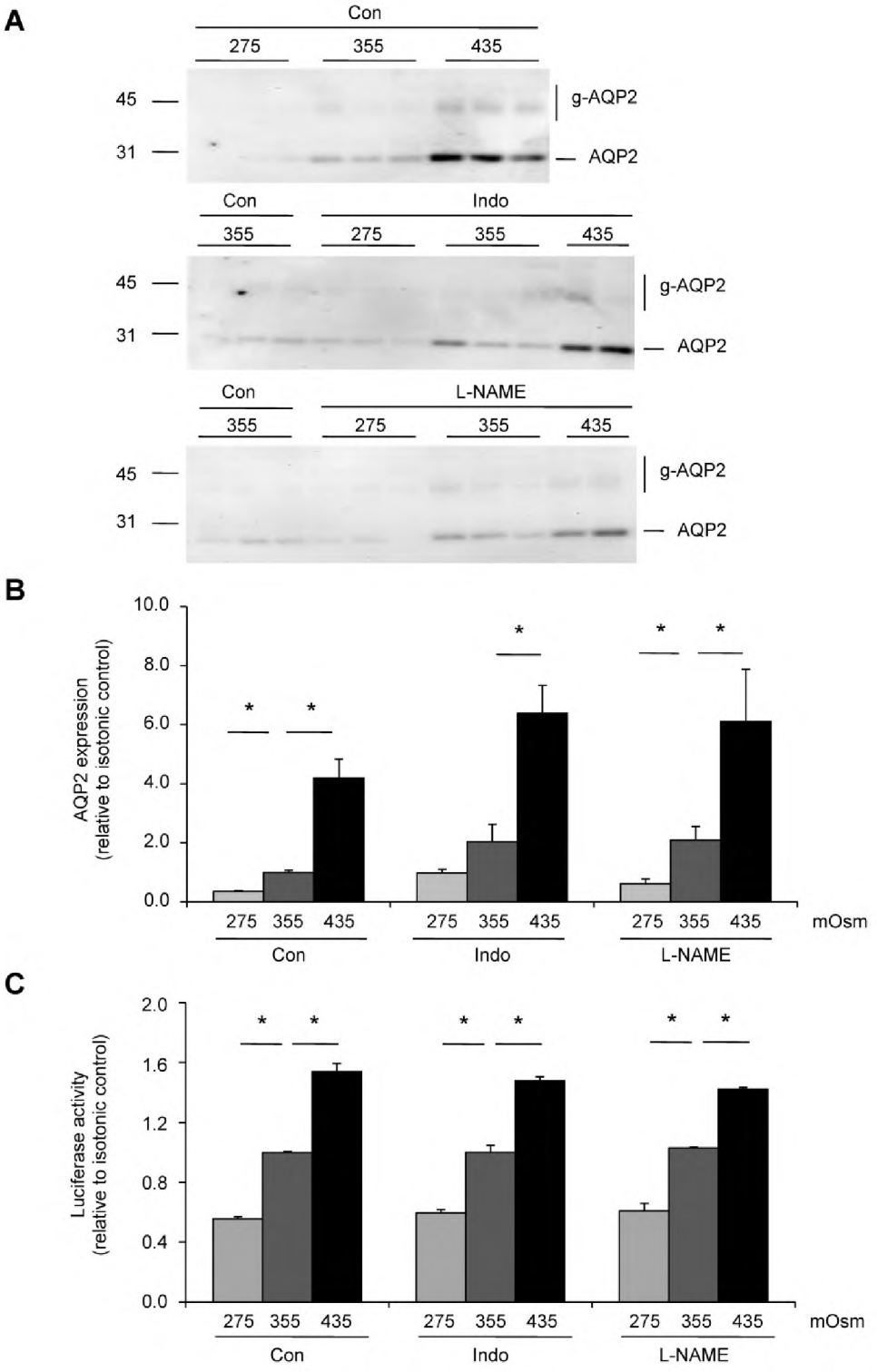

Figure 3. Role of nictric oxide and prostaglandins in tonicity-regulated AQP2 expression. A) mpkCCD-AQP2-3.0-luc cells were grown to confluence, the last 4 days with dDAVP. During the last 24 hrs, cells were exposed to different osmolalities (indicated), in the absence or presence of indomethacin or L-NAME, blockers for prostaglandin and nitric oxide synthesis, respectively. Cells were lysed and analyzed for AQP2 abundance by immunoblotting The molecular mass (in kDa) is indicated on the left. **B)** Relative abundances of AQP2 derived from figure A, normalized for AQP2 abundance of control cells grown under isotonic conditions. **C**) Of samples as described above luciferase activity was determined. Significant differences ($p<0.05$) are indicated by an asterisk.

3C). These data indicate that, although NO and prostaglandins decrease AQP2 abundance, they do not affect tonicity-induced changes in AQP2 expression.

dDAVP-dependence of tonicity-induced AQP2 transcription

Tonicity can change AQP2 abundance independent of AVP *in vivo* (7; 9). To test whether an AVP-independent effect of tonicity on AQP2 expression is also observed in our cells, mpkCCD-AQP2-3.0-luc cells were again treated with different NaCl-concentrations for the last 24 hrs, but now in the presence or absence of dDAVP for 4 days before harvesting. While luciferase levels were higher in samples treated with dDAVP, the relative changes in luciferase activity with changing tonicity in dDAVP-treated and –untreated samples were similar (Fig. 4). These data show that in mpkCCD cells, the effect of tonicity on AQP2 expression is independent of vasopressin receptor stimulation.

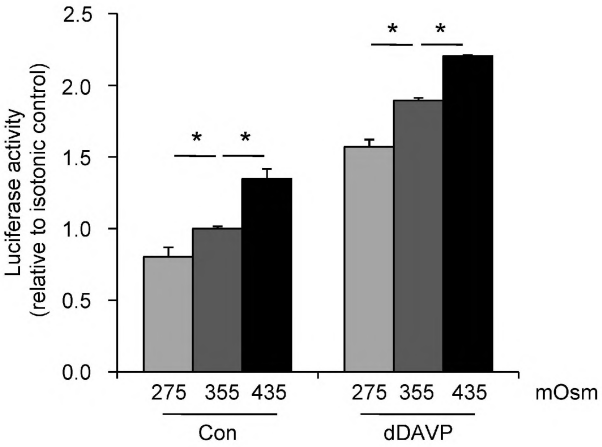

Figure 4. Role of dDAVP in tonicity induced AQP2 transcription. mpkCCD-AQP2-luc cells were grown as above, the last 4 days with or without (Con) dDAVP. During the last 24 hours, cells were grown in isotonic, hypertonic or hypotonic medium. After harvesting, luciferase activity was determined.

TonE in tonicity-induced AQP2 transcription

Regulation of AQP2 expression by tonicity through the TonE-element, located 489 bp upstream of the AQP2 transcription start site, is controversial. To test the relevance of TonE in tonicity-affected AQP2 expression under our conditions, we generated pooled colonies of mpkCCD-AQP2-3.0-luc cells in which the TonE element was inactivated as reported (13), and subjected these and the mpkCCD-AQP2-3.0-luc cells to hypo- and hypertonicity. Surprisingly, changing to hypotonic (275 mOsm) or hypertonic (435 mOsm) medium revealed a similar change in luciferase activity in the TonE mutants cells as found for mpkCCD-AQP2-3.0-luc cells (Fig. 5A).

To further investigate this, we generated mpkCCD cells stably-transfected with a construct in which luciferase expression is driven by 408 bp of the AQP2 promoter, thus lacking the TonE element (pGL3-AQP2-0.4-luc). Following subjection to hypo- and hypertonicity, qualitatively similar changes in AQP2 transcription were found for the 3.0 and 0.4 kb promoter with both changes (Fig. 5B). Combined, these data indicate that TonE is not involved in the tonicity-

induced regulation of AQP2 expression under the present conditions. In addition, our data suggest the presence of yet another tonicity-responsive element in the 408 bp segment of the AQP2 promoter.

Role of CREB in tonicity-induced AQP2 transcription

In this 408 bp segment is the CRE, located at position -210, which is involved in AVP-induced AQP2 transcription (2; 3). To test whether CRE also has a role in tonicity-induced regulation of AQP2, cells were stably-transfected with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly-placed CREs. Following growth and incubation with dDAVP as above, a reduced luciferase activity was observed with hypotonicity (Fig 6A). From this we conclude that hypotonicity does decrease CRE-regulated transcription.

As tonicity-regulated AQP2 expression appeared to be AVP-independent (Fig. 4) while CRE-mediated transcription is changed with hypotonicity (Fig. 6A), we investigated which part of the AVP-cAMP-CREB-AQP2 promoter pathway was involved.

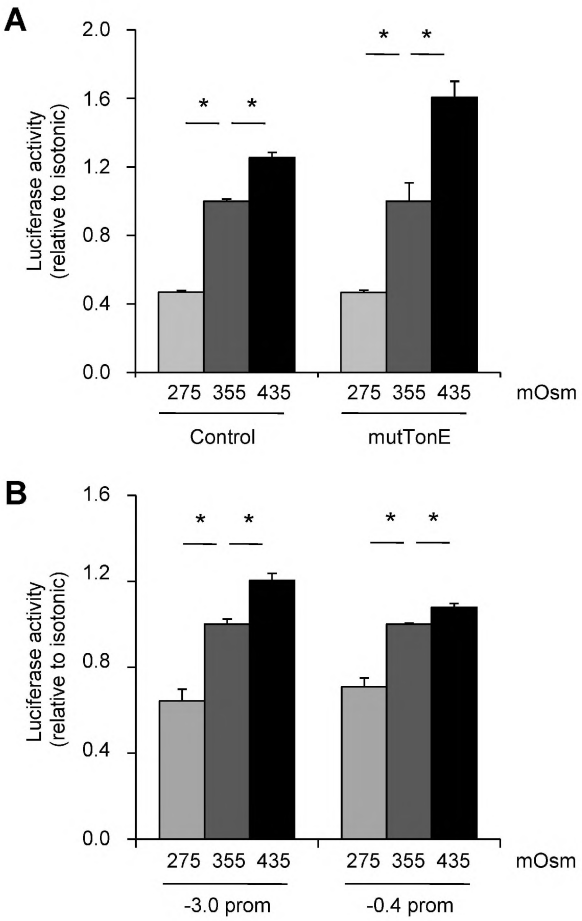

Figure 5. Role of TonE in tonicity-induced AQP2 transcription. mpkCCD cells, stably-transfected with a -3.0 kb AQP2 promoter (Control) or the same promoter, containing a mutated TonE sequence (mutTonE) (**A**), or together with mpkCCD cells transfected with a - 0.4 kb AQP2 promoter (**B**) were grown as above, of which the last 24 hours in isotonic, hypertonic or hypotonic medium. Cells were lysed and subjected to luciferase activity measurements. Mean values of luciferase activity (+/- SEM) were determined from three independent filters per condition.

As cAMP levels show a peak right after dDAVP addition, but are reduced to low levels within 120 minutes following addition (28), we tested the effect of hypotonicity on dDAVP-induced cAMP levels on the long and short term. For the long term, cells were grown as above, treated with dDAVP for 4 days, incubated in hypotonic or isotonic medium during the last 24 hours and treated with the phosphodiesterase inhibitor IBMX during the last 30 minutes. cAMP levels were not changed by hypotonicity (Fig. 6B). To investigate whether there is an effect of hypotonicity on short term cAMP generation, cells were grown without dDAVP, incubated in hypotonic or isotonic medium during the last 24 hours and subsequently treated

with IBMX for the last 30 minutes and with dDAVP for the last 15 minutes. Analysis revealed that the dDAVP-induced cAMP levels were not changed (Fig. 6B).

To test if the effect of tonicity on AQP2 transcription involves protein kinase A (PKA), we tested whether the hypotonicity-induced decrease in AQP2 transcription was still observed when we would block PKA activity with H89, a PKA-selective inhibitor. Cells were grown for 8 days and 24 hrs before harvesting, medium was replaced with iso- or hypotonic medium with dDAVP with or without 10 μM H89. H89 decreased luciferase activity in cells grown in both iso- and hypotonic medium, indicating that PKA is involved in AQP2 transcription at both tonicities. However, the H89-induced down-regulation of AQP2 transcription of about 30% was the same for both tonicities (Fig. 6C), indicating that neither PKA, nor cAMP or dDAVP are involved in the hypotonicity-induced reduction in AQP2 transcription.

A tonicity-induced decrease in transcription can be caused by a reduced CREB activity, which can be the result of a decrease in total CREB and/or CREB-phosphorylation at Ser133 (29). Indeed, immunoblotting showed a small, but significant decrease in total and CREB-pS133 with hypotonicity (Fig 6D,E). The relative changes in total and CREB-pS133 were similar, indicating that the decrease in CREB-pS133 is due to the decrease in total CREB.

To investigate the role of the CRE element in the AQP2 promoter, mpkCCD cells were stably-transfected with the pGL3-AQP2-0.4-luc construct in which the CRE-element was inactivated. Subsequently, these and mpkCCD-AQP2-0.4-luc cells were subjected to the hypotonicity assay with or without dDAVP. As anticipated, dDAVP treatment increased luciferase activity significantly in mpkCCD-AQP2-0.4-luc cells, but not in cells containing the CRE mutant (Fig. 6F). In both cell models, however, hypotonicity led to a similar decrease in luciferase activity, which indicated that the AQP2 CRE element is not essential for the response to hypotonicity.

AQP2 promoter segments involved in the decreased transcription under hypotonic conditions

Because the CRE in the AQP2 promoter was shown not to be essential for the hypotonicity-effect (Fig. 6F), we further analyzed the -0.4kb AQP2 promoter for the hypotonicity element by generating twelve AQP2 promoter deletion constructs in which in each 31 bp (three rounds of the DNA helix) was deleted (Fig. 7A), leaving the relative orientation of the transcription factor binding sites intact. Cells stably-transfected with these constructs were tested for their response to 24 hrs of hypotonicity in the presence of dDAVP. While most cells with these constructs yielded significantly-reduced luciferase activity with hypotonicity, luciferase activity was not significantly reduced in cells containing constructs lacking nucleotides -284 to -254 (segment No. 5) and -158 to -127 (No. 9) (Fig. 7B).

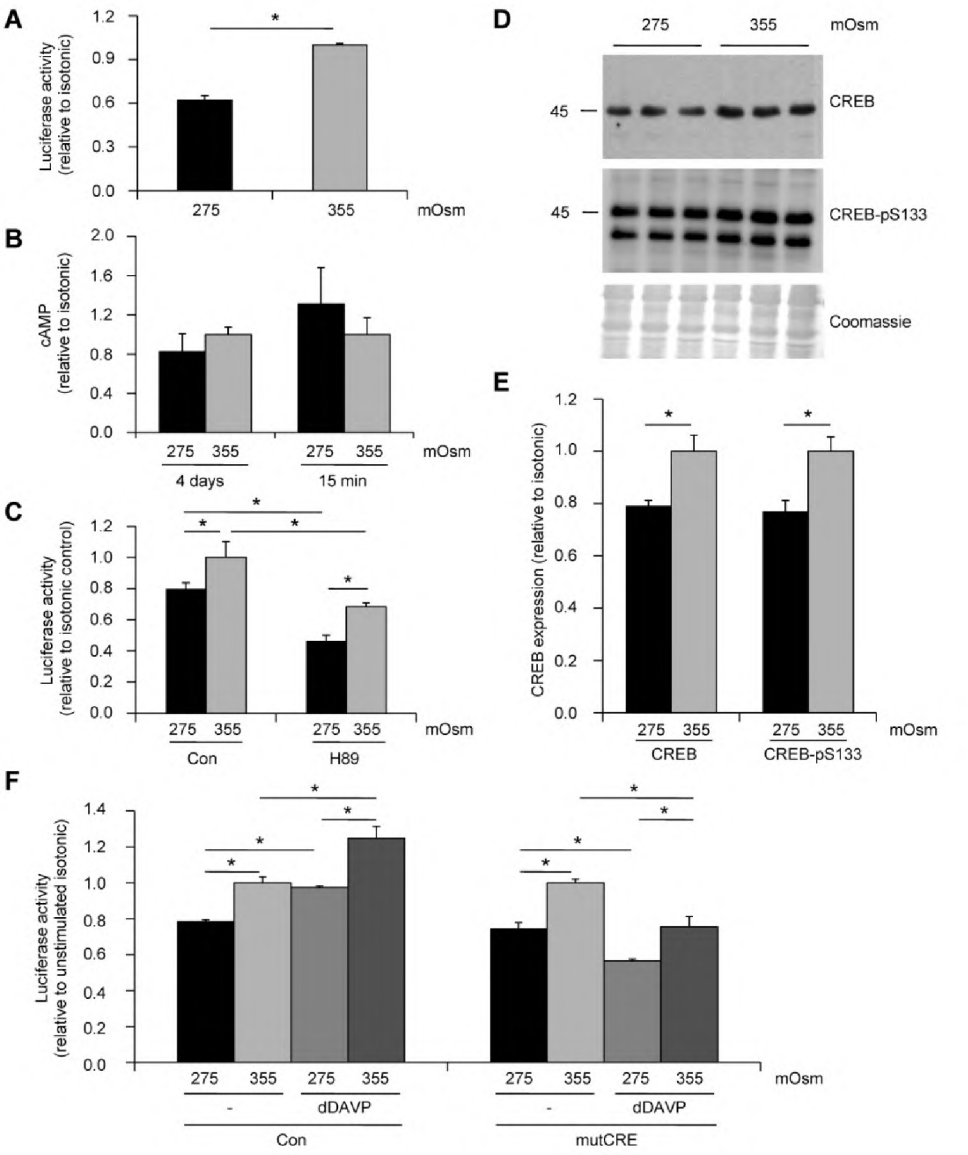

Figure 6. Role of CREB in tonicity-induced AQP2 transcription. A) mpkCCD cells, stably-transfected with a luciferase gene driven by a multi-CRE promoter, were grown as described above and grown for the last day in hypotonic or isotonic medium in the presence of dDAVP, lysed and analysed for luciferase activity. **B)** mpkCCD cells were grown as above and either incubated with dDAVP for 4 days or only 15 minutes before harvesting. In both conditions, cells were cultured in hypotonic or isotonic medium during the last 24 hours. After harvesting, cAMP levels were measured. **C)** mpkCCD cells, stably-transfected with a luciferase reporter construct containing 0.4 kb of the AQP2 promoter. were grown as above. The last 24 hours, cells were treated with dDAVP and the PKA-inhibitor H89 (10 µM) and grown in hypotonic or isotonic medium, lysed and analyzed for luciferase activity. **D)** mpkCCD cells were grown as described under A. Cells were lysed and subjected to immunoblotting for CREB and S133-phosphorylated CREB (indicated). In the CREB-pS133-blot, the upper band represents phosphorylated CREB, whereas the lower band represents ATF-1 which is also recognized by this antibody. Molecular masses (in kDa) are indicated on the left. **E)** Relative abundance of total and S133-phosphorylated CREB derived from fig. D and normalized for their expression in isotonic medium. **F)** mpkCCD cells stably-transfected with constructs encoding luciferase preceded by the -0.4 kb AQP2 promoter (Con) or the same promoter, containing a mutated CRE sequence (mutCRE), were grown as above, with or without dDAVP and the last 24 hours in hypotonic or isotonic medium. After harvesting, luciferase activity was determined. Mean values (+/- SEM) were determined from three independent filters per condition. Significant differences ($p<0.05$) are indicated by an asterisk.

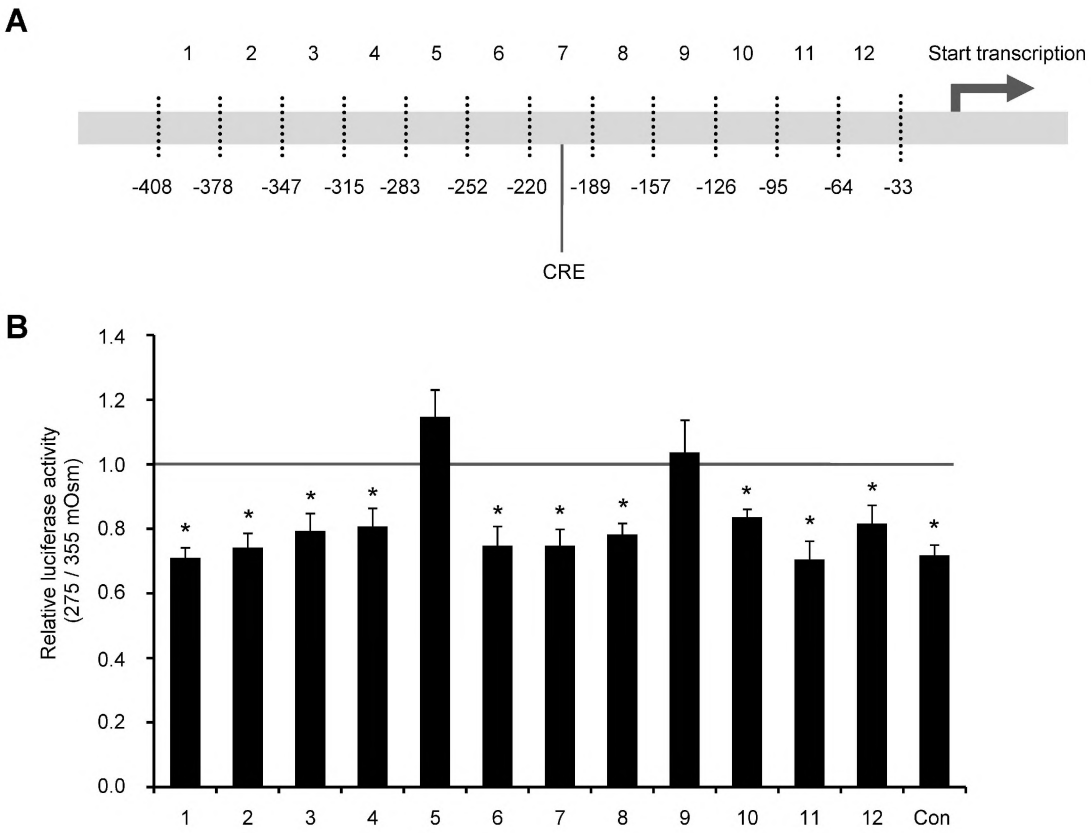

Figure 7. AQP2 promoter segments involved in hypotonicity-response. A) Position of the 31-bp segments deleted from the AQP2 promoter in relation to the transcription start site. **B)** mpkCCD cells stably-transfected with a -0.4 kb AQP2 promoter construct, containing deletions as indicated in A, were grown as above. The last 24 hours, cells were grown in hypotonic or isotonic medium. After harvesting, luciferase activity was determined. The relative luciferase activity is normalized for differences in transfection efficiencies by dividing the values obtained in the hypotonic medium by the values obtained in the isotonic medium.

Discussion

mpkCCD cells are a proper model to study tonicity-induced regulation of AQP2 expression

Our data reveal that mpkCCD cells are a proper model to study hyper- and hypotonicity-induced changes in AQP2 expression and transcription. First, following transfection of a luciferase reporter construct preceded by a 3.0 kb AQP2 promoter, we showed that dDAVP induced an increase in AQP2 protein expression as well as transcription in mpkCCD cells (Fig 1A,B). This illustrated that the 3.0 kb promoter of AQP2 is sufficient for the response to dDAVP, and is consistent with data of others using the human AQP2 promoter of a similar length in other cells (2; 3; 30; 31). Secondly, both AQP2 protein expression and transcription were increased with hypertonicity (Fig. 1C,D), which is in line with the hypertonicity-induced increased AQP2 protein expression seen *in vivo* (9) and *in vitro* (10).

We also observed a down-regulation of AQP2 in response to *hypo*tonicity *in vitro*. This down-regulation of AQP2 was prevented when re-adjusting osmolality with sorbitol, sucrose, or NaCl, but not urea (Fig. 1E). It is therefore tonicity, and not osmolality, that affects AQP2 expression, which is in agreement with previous results (10). Besides, we showed that the final tonicity and not the change in tonicity determined the extent of AQP2 transcription (Fig. 2).

Hypotonicity-induced AQP2 down-regulation is independent of dDAVP, prostaglandins and nitric oxide production

Similar to *in vivo* and other *in vitro* studies with hypertonicity (7; 9), we show that the hypotonicity-induced decrease in AQP2 expression is independent of dDAVP, as the relative effects of hypotonicity were similar in the absence or presence of dDAVP (Fig. 4).

We investigated this further. Hypotonicity did not affect the cAMP-PKA pathway, as cAMP levels were not changed by hypotonicity and PKA blockade with H89 had no effect on the hypotonicity-induced down-regulation of AQP2. The decrease in luciferase activity in cells treated with H89 shows that this drug was still active (Fig. 6C).

Interestingly, hypotonicity significantly decreased luciferase activity in cells possessing several CRE-elements coupled to the luciferase cDNA (Fig. 6A), indicating that tonicity does affect CRE-mediated transcription. This decrease can be attributed to a decrease in total CREB protein levels, which was similar to the fractional decrease in CREB phosphorylation on serine-133 (Fig.6D,E). CRE, however, appeared not essential for the tonicity response of AQP2 expression, as mutating or deleting the CRE-sequence in the AQP2 promoter had no effect on the tonicity-mediated AQP2 transcription (Fig 6F,7) Considering the observed reduced expression of luciferase with hypotonicity from the CRE-luciferase construct, it may be that the 21 tandemly-placed CREs in this construct makes it more sensitive to detect CRE-mediated transcription than the AQP2 promoter. Alternatively, hypotonicity-regulated expression of AQP2 gene transcription is dominated by transcription factor binding sites other than its CRE. In either case, our data indicate that the CRE element in the AQP2 promoter is irrelevant for the reduced transcription of the AQP2 gene with hypotonicity, which is in line with our data that cAMP levels and PKA activity are not changed with hypotonicity (Fig.6A-C) and with the finding of us and others that the effect of tonicity is AVP independent.

A candidate involved in the cellular signaling leading to the hypotonicity-mediated decrease in AQP2 transcription is nitric oxide (NO), as it has been shown that NO decreases AQP2 expression (32; 33) and renal NO synthase expression correlates inversely with medullary tonicity (34; 35). Similarly, Murase *et al.* found that endogenous NO synthesis was

significantly increased in the vasopressin escape animals and that treatment with L-NAME decreased urine volume and partially increased AQP2 expression (24). In agreement with this, blocking NO synthesis by L-NAME resulted in increased AQP2 protein abundance in mpkCCD cells (Fig. 3A). L-NAME did however not affect the tonicity-mediated change in AQP2 abundance (Fig. 3B). Moreover, the tonicity-induced changes in AQP2 transcription were not changed with L-NAME (Fig 3C). These data reveal that, although NO decreases AQP2 abundance in mpkCCD cells, NO does not affect AQP2 gene transcription and is not involved in the tonicity-regulated change in AQP2 expression.

Another mechanism involved might be an increased prostaglandin production. Prostaglandin E_2 is known to reduce AVP-stimulated water reabsorption in the collecting duct (25; 26). Moreover, Gross *et al*. showed an increased urinary prostaglandin E_2 excretion in vasopressin escape and preventing this increase with indomethacin resulted in a delay in the onset of escape (27). In agreement with these data, blocking prostaglandin production with indomethacin resulted in an increased AQP2 protein expression in our mpkCCD cells (Fig. 3A). No effect was seen on AQP2 promoter-driven luciferase activity, however, indicating that the decrease of AQP2 abundance with prostaglandins occurs at the protein or mRNA stability level, rather than AQP2 gene transcription. In addition and similar to the effect of L-NAME, indomethacin treatment did not affect the tonicity-induced AQP2 expression, suggesting that prostaglandin production is not affected by tonicity, and is not involved in the tonicity-regulated expression of AQP2.

In the context of the *in vivo* data, our data indicate that paracrine-produced increased levels of prostaglandins and/or NO in the kidney may indeed contribute to the reduced AQP2 abundance observed in vasopressin escape animals, but that they cannot explain the effect of tonicity on AQP2 gene transcription.

Tonicity-induced AQP2 transcription is TonE independent

The role of TonE in tonicity-regulated AQP2 expression is controversial. In our experiments, the TonE in the AQP2 promoter was not important for AQP2 transcription regulation by tonicity, because (1) introduction of a TonE-mutation identical to the mutation introduced by Hasler *et al*. in a -3.0 kb AQP2 promoter had no effect on tonicity-induced AQP2-transcription (Fig. 5A) and (2) luciferase activity obtained from the -0.4 kb AQP2 promoter, which lacks TonE, and the -3.0 kb promoter were similarly influenced by the different tonicities used to culture the cells in (Fig. 5B).

Our data are in contrast to those obtained by Hasler *et al.,* who were using the same cell model, but under different conditions (13). Growing the cells for only 2 days of which the last day in serum-free medium and without dDAVP stimulation, Hasler *et al*. found an absence of

the hypertonicity-induced AQP2-promoter driven luciferase activity in mpkCCD cells when the TonE was mutated. We tested whether this could be due to the different experimental conditions. Indeed, growing the cells in the same way, we saw a significant reduction, but not complete absence, in hypertonicity-induced luciferase activity in the TonE mutant cells compared to the mpkCCD-AQP2-3.0-luc cells (not shown). This difference was caused by the shorter growth period, as growing the cells for 8 days, the last 24 hours with hypertonic serum-free medium without dDAVP, did not show any difference in the hypertonic-response between the promoters with an intact or mutated TonE element (not shown). As in our experience, mpkCCD cells are only polarized after 4 days of culture (not shown), we believe that growing the cells for 8 days as used in our experiments, better mimics the *in vivo* situation.

Our data seem in contrast to *in vivo* data, as TonEBP$^{-/-}$ mice and mice transgenic for dominant-negative TonEBP showed decreased AQP2 expression (15; 16). However, the severe atrophy of the renal medulla in the TonEBP$^{-/-}$ and dominant-negative TonEBP overexpressing mice complicates the interpretation of the direct involvement of TonE in tonicity-regulation of AQP2 expression. To resolve this apparent discrepancy, investigation of the role of TonE and TonEBP in a conditional knockout mouse in which the structure of the renal medulla would be intact is needed.

The segments from -283 to -252 and -157 to -126 bp of the AQP2 promoter are involved the hypotonicity-induced AQP2 down-regulation

Using a -0.4 kb promoter, hypo- and hypertonicity showed qualitatively similar changes in AQP2 transcription as compared to the -3.0 promoter (Fig. 5B), indicating that a tonicity-responsive element is present in the 408 bp AQP2 promoter. With subsequent segment deletions, the location of this TonE element could be pinpointed to two 31 bp promoter segments (5 and 9) in the -0.4 kb AQP2 promoter, covering nucleotides -283 to -252 and -157 to -126 bp.

Li *et al.* reported that the effect of hypertonicity on AQP2 transcription depended on the NFATc-sites in the AQP2 promoter (14). The NFATc-sites tested by Li *et al.* are localized in segments 1, 6 and 7 of the -0.4 kb promoter. Two segments are located more upstream; one of them representing the TonE-sequence. In our experiments, the upstream segments, nor segments 1, 6 or 7 appeared important in the hypotonicity-response. One explanation for this difference could be the differences in experimental conditions, as Li *et al.* used unpolarized mpkCCD cells and looked at an effect of tonicity after 6 hours. This time period of subjection to a changed tonicity indeed seems of relevance, because Hasler *et al.* have shown that the response to a change in tonicity with 3 hours is opposite to that obtained after 24 hours (10).

Another explanation could be that in the experiments performed by Li *et al*. all the NFATc-sites were mutated simultaneously, while we only deleted one or two sites at once. The inactivation of multiple NFATc-sites could influence the tonicity-effect whereas deletion of only one or two sites could give too small a change to detect.

To possibly identify the transcription factors involved, segments 5 and 9 were analyzed for potential transcription factor binding sites using TRANSFAC databases (36). In segment 5, binding sites for nuclear factor kappa B (NF-kappaB) and SP1 were identified and in segment 9, a GATA binding site was found (Fig. 8).

Based on literature, these transcription factors could well mediate the change in AQP2 gene transcription with tonicity, as data in intestinal epithelial cells suggest activation of NF-kappaB and stimulation of NF-kappaB-mediated transcription by osmotic stress (37). Similarly, hypertonicity activates NF-kappaB in renal medullary interstitial cells and water deprivation increases renal NF-kappaB-driven reporter gene expression in transgenic mice (38). In addition, GATA may play a role in tonicity-regulated AQP2 transcription, because hypertonic stress induces transcription of GATA-2 in placental trophoblast stem cells (39). It remains to be established whether these transcription factors and their promoter elements are responsible for the tonicity effect on AQP2 transcription.

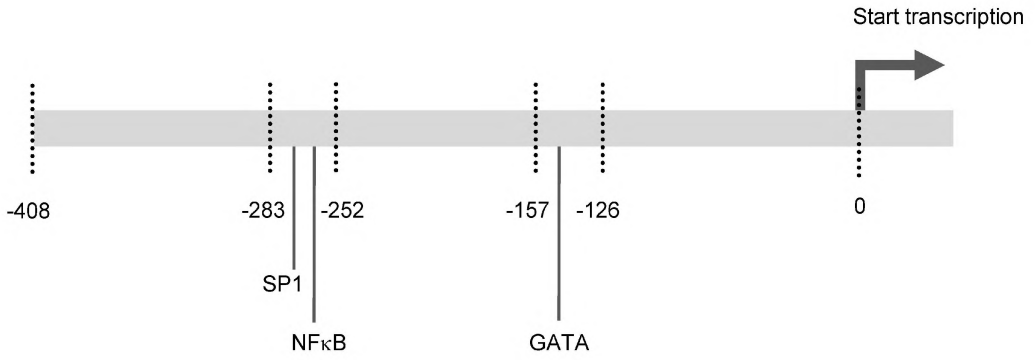

Figure 8. Transcription factor binding sites. Transcription factor binding sites identified in the -283 to -252 and -157 to -126 bp regions of the AQP2 promoter using TRANSFAC databases.

In conclusion, we showed that in mpkCCD cells, tonicity-induced AQP2 expression is mediated by transcription factors other than TonEBP or CREB and that it occurs in an AVP-independent manner. The tonicity responsiveness seems to involve AQP2 promoter segments covering nucleotides -283 to -252 and -157 to -126 bp of the AQP2 promoter. Identification of transcription factors and signaling proteins involved in hypotonicity-regulated AQP2 expression in follow-up experiments will provide us a better insight in physiological regulation of AQP2 expression and renal water reabsorption by tonicity and may lead to the discovery of targets for modulation of pathophysiological conditions of osmoregulation, such as with SIADH.

Acknowledgments

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CHAPTER 5

In mpkCCD cells, long-term regulation of aquaporin-2 by vasopressin occurs independent of protein kinase A and CREB, but may involve Epac

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Abstract

Urine concentration involves the hormone vasopressin (AVP), which stimulates cAMP production in renal principal cells, resulting in translocation and transcription of aquaporin-2 (AQP2) water channels, greatly increasing the water permeability, leading to a concentrated urine. As cAMP levels decrease shortly after AVP addition, whereas AQP2 levels still increase and are maintained for days, we investigated in the present study the mechanism responsible for the AQP2 increase after long-term dDAVP application using mouse collecting duct (mpkCCD) cells.

While 30 min dDAVP incubation strongly increased cAMP, cAMP was lower with 1 day, and was even further reduced with 4 days dDAVP, although still significantly higher than in control cells. 1 day dDAVP incubation increased AQP2 promoter dependent transcription, which was blocked by the protein kinase A (PKA) inhibitor H89. Moreover, phosphorylation of the cAMP responsive element binding protein (CREB) and CRE-dependent transcription was observed after short-term dDAVP stimulation. With 4 days of dDAVP, AQP2 transcription remained elevated, but this was not blocked by H89, and CRE-dependent transcription and CREB phosphorylation were not increased,

Exchange protein directly activated by cAMP (Epac) 1 and 2 were found to be endogenously expressed in mpkCCD cells. Application of dDAVP increased the expression of Epac1, while Epac2 was reduced. Incubation with a specific Epac activator after dDAVP pretreatment increased both AQP2 abundance and transcription compared to cells left unstimulated the last day.

In conclusion, the PKA-CRE pathway is involved in the initial rise in AQP2 levels after dDAVP stimulation, but not in the long-term effect of dDAVP. Instead, long-term regulation of AQP2 may involve the activation of Epac.

Introduction

The kidney plays a key role in regulating water balance. In states of hypernatremia or hypovolemia, the hormone arginine-vasopressin (AVP) is released from the pituitary. Binding of AVP to the vasopressin type-2 receptor stimulates a rise in intracellular cAMP in the renal collecting duct principal cells, leading to activation of protein kinase A (PKA), phosphorylation of aquaporin-2 (AQP2) water channels at Ser256 and subsequent redistribution of AQP2 from intracellular vesicles into the apical membrane (1-4). Driven by the transcellular osmotic gradient, water will enter principal cells through AQP2 and exit the cells through the basolaterally located AQP3 or AQP4 water channels, resulting in concentrated urine.

Besides this short-term regulation, AVP also increases transcription of AQP2 and hence increases AQP2 abundance (5; 6). Changes in expression of AQP2 have been attributed to PKA-induced phosphorylation of the cAMP responsive element binding protein (CREB) which stimulates transcription from the AQP2 promoter via the cAMP Responsive Element (CRE) (7-9).

Interestingly, cAMP levels peak immediately after addition of the stable AVP analogue dDAVP, but are reduced to low levels within 120 minutes following addition in AQP2-transfected MDCK cells (10). AQP2 levels, however, continue to rise during the first three days of dDAVP exposure in mouse collecting duct (mpkCCD) cells (11). Therefore, the purpose of this study was to delineate whether cAMP and CREB are involved in the long-term maintenance of increased AQP2 levels with dDAVP.

As cAMP not only activates PKA, but also the exchange protein directly activated by cAMP (Epac), and both Epac1 and Epac2 are expressed in renal principal cells (12), Epac might have a role in AQP2 regulation as well. This is further suggested by Yip, showing that Epac might have a role in the vasopressin-stimulated apical targeting of AQP2 in perfused inner medullary collecting ducts (13).

Here, we investigated the involvement of the PKA-CREB pathway as well as Epac in the long-term AQP2 regulation in the mpkCCD cell line.

Materials and Methods

Cell culture

MpkCCD cells were cultured as described previously (6). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. For the last 24 or 96 hrs, the cells were incubated with 1 nM dDAVP to the basolateral side, to induce an increase in AQP2 expression. During this 96 hrs incubation period, the medium was daily refreshed. The PKA blocker H89 (10 μM, Calbiochem, San Diego, CA) or the Epac activator 007-AM (8-pCPT-2'-O-Me-cAMP-AM, kind gift of Dr. H. Rehmann, UMC Utrecht, Utrecht, The Netherlands, 0.1 μM) were added to both sides of the filters for the last 24 hours.

Transfection and generation of stable mpkCCD cell lines with a 3.0 kb AQP2 promoter-luciferase reporter construct was described previously (14). Transfection and generation of a stable mpkCCD cell line with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly-placed CREs, was described previously as well (14).

Immunoblotting

MpkCCD cells from 1.13 cm^2 filters were lysed in 200 µl Laemmli buffer, sonicated and heated for 30 min at 37°C. PAGE, blotting and blocking of the PVDF membranes were done as described (15). Membranes were incubated for 16 hrs at 4°C with 1:3000-diluted affinity-purified rabbit R7 AQP2 antibodies (16), 1:2000 anti-cAMP responsive element binding protein (CREB) antibody (Sigma, St. Louis, MO, USA), 1:2000 anti-phosphorylated CREB antibody (Sigma, St. Louis, MO, USA), 1:1000 diluted mouse anti-Epac1 5D3 (17) or mouse anti-Epac2 3C12 antibodies (raised against the C-terminus of Epac2 protein, kind gift of Dr. Johannes L. Bos, University of Utrecht, The Netherlands) in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% non-fat dried milk.

Blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's or 1:2000-diluted goat anti-mouse IgG's (Sigma, St. Louis, MO) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

Luciferase assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence was measured for 10 seconds using an EG&G Berthold Lumat LB9507 luminometer. To verify that equal amounts of protein per sample were used for the luciferase assay, protein concentration was determined using the BioRad protein assay (München, Germany), according to manufacturer's instructions.

cAMP measurement

MpkCCD cells were seeded on filters for 8 days, the last 4 days with or without 1 nM dDAVP. The last day, the cells were incubated for 30 min with 0.5 mM phosphodiesterase inhibitor 3-isobutyl 1 methylxanthine (IBMX; Sigma, St. Louis, MO, USA). For short-term cAMP measurement, cells were treated for 30 minutes with IBMX with or without dDAVP. cAMP was measured using the cAMP enzyme immunoassay kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. Results were related to a standard curve based on the measurement of defined cAMP solutions done in triplicates.

Statistics

Students unpaired t-test was used when two groups with Gaussian distribution were compared. For multiple comparisons, Bonferroni correction was applied. A p value of less than 0.05 is considered significant. Data are presented as mean and bars are standard error of the mean (SEM).

Results

Short and long-term effects of dDAVP on cAMP levels and AQP2 transcription

At long-term incubation, dDAVP-induced AQP2 expression and plasma membrane accumulation is sustained, but cAMP levels drop to low levels within 120 minutes in MDCK cells (10). To investigate if cAMP levels are still increased after long-term dDAVP stimulation or return to basal levels, mpkCCD cells were grown to confluence for 4 days after which they were incubated with or without 1 nM dDAVP for the last 4 days, of which the last 30 minutes in the presence of the phosphodiesterase inhibitor IBMX. As a positive control, mpkCCD cells were grown similarly, but treated with dDAVP and IBMX only for the last 30 minutes. Analysis revealed that the cAMP levels were around 4 times higher in long-term dDAVP-stimulated cells as compared to control cells, whereas cells stimulated with dDAVP for only 30 minutes had cAMP levels approximately 40 times higher than control cells (Fig. 1A).

Allthough cAMP levels were lower after long-term dDAVP stimulation, Li *et al.* have shown that AQP2 abundance is still maximally stimulated after 4 days dDAVP in mpkCCD cells (11). Considering a half-life of 4 hours for AQP2 in mpkCCD cells (18), the sustained expression of AQP2 over a period of 4 days indicates a sustained AQP2 gene transcription.

Previously, we showed that dDAVP increases luciferase activity and AQP2 protein expression in pooled colonies of mpkCCD cells stably transfected with a 3.0 kb AQP2

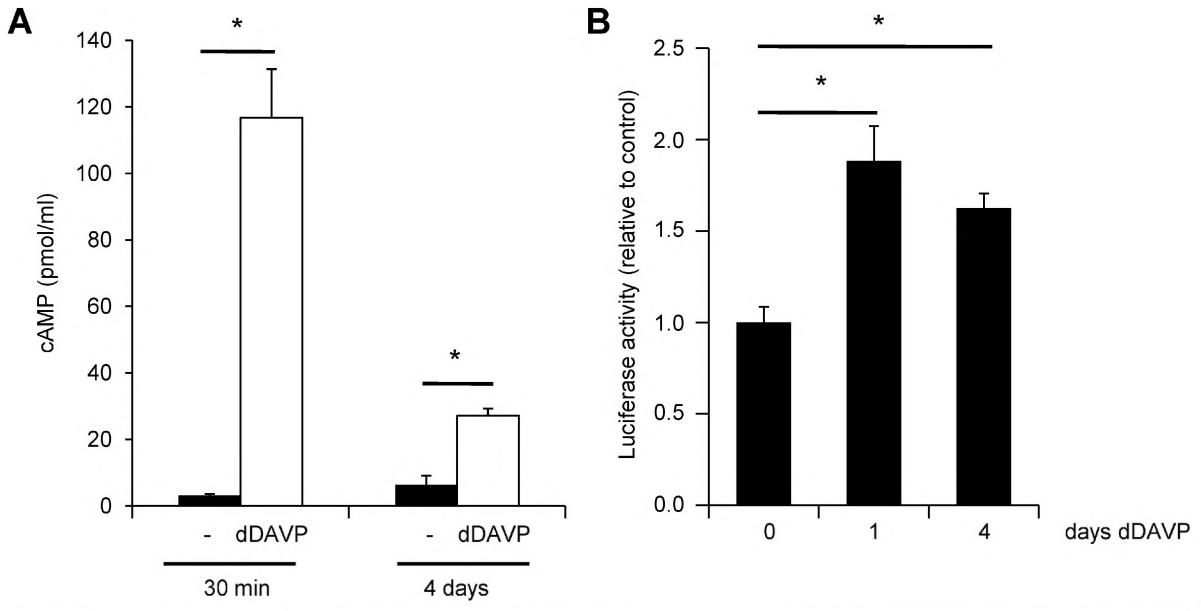

Figure 1. Effect of dDAVP on cAMP generation A) MpkCCD cells were grown for 8 days and incubated with dDAVP for the last 4 days or 30 minutes. During the last 30 minutes, IBMX was added. Cells were lysed and cAMP production was measured. **B)** mpkCCD-AQP2-3.0-luc cells were grown for 8 days, the last 1 or 4 days with or without 1 nM dDAVP. The cells were lysed and light emission was measured. For A-B: Data are the mean of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

promoter luciferase construct (mpkCCD-AQP2-3.0-luc; (14)). This dDAVP-induced luciferase activity in mpkCCD-AQP2-3.0-luc cells was due to AQP2 promoter-specific transcription, as in mpkCCD cells stably-transfected with the luciferase reporter construct without the AQP2 promoter, luciferase activity was hardly observed, and this was not increased by dDAVP (14). Therefore, to test the long and short-term dDAVP effects on AQP2 transcription, mpkCCD-AQP2-3.0-luc cells were treated as above, except that the short-term incubation lasted one day instead of 30 minutes, because 30 minutes was too short to observe changes in transcription (not shown). As shown in figure 1B, both short and long-term dDAVP application resulted in an increased luciferase activity compared to unstimulated cells, and there was no difference in the level of transcriptional activity after 1 or 4 days of dDAVP stimulation.

Role of PKA in the dDAVP-induced increase in AQP2 transcription

Although the observed sustained, but lower, cAMP production after 4 days stimulation with dDAVP may provide an explanation for the long-term increased AQP2 expression, we wanted to investigate whether the cAMP signaling cascade is really important during long-term dDAVP stimulation. To study this, mpkCCD-AQP2-3.0-luc cells were grown as described above, and incubated for the last 24 hours or 4 days with dDAVP and the last 24 hours with or without the PKA blocker H89. As luciferase has a half-life of 2-4 hours (19; 20), the obtained luciferase activity will reflect the AQP2-transcription during the day incubated with H89. Analysis of luciferase-activity revealed that addition of H89 after 3 days of dDAVP stimulation did not reduce AQP2 transcription. In contrast, and in line with an essential role of PKA in initiating AQP2 gene transcription with dDAVP, H89 reduced AQP2 transcription when added at initiation of dDAVP stimulation (Fig. 2). These data indicate that, in contrast to

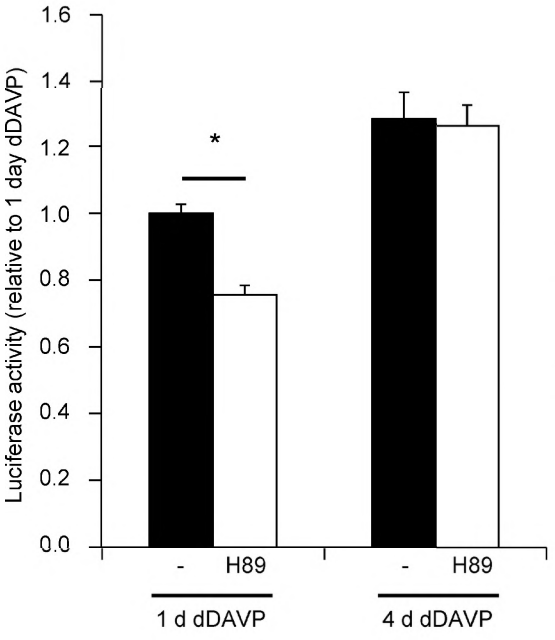

Figure 2. Role of PKA in the dDAVP-AQP2 pathway. MpkCCD cells containing an AQP2-promoter-luciferase construct were treated with dDAVP for the last 1 or 4 days, with or without the PKA blocker H89 for the last 24 hours. The cells were lysed and light emission was measured. Data are the mean of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

initial stimulation of AQP2 transcription, PKA activity is not essential for AQP2 transcription after prolonged activation by dDAVP.

Effect of dDAVP on CREB and CRE-mediated transcription

PKA is known to activate CREB through phosphorylation of its serine 133 (S133), which has been reported to increase AQP2 gene transcription via the CRE element in the AQP2 promoter (7; 8; 21).

To investigate CRE-mediated transcription after long-term dDAVP administration, we stably-transfected mpkCCD cells with pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly-placed CREs. As luciferase has a half-life of 2-4 hours (19; 20), luciferase activity derived from this promoter consisting only of intact CRE elements allows us to

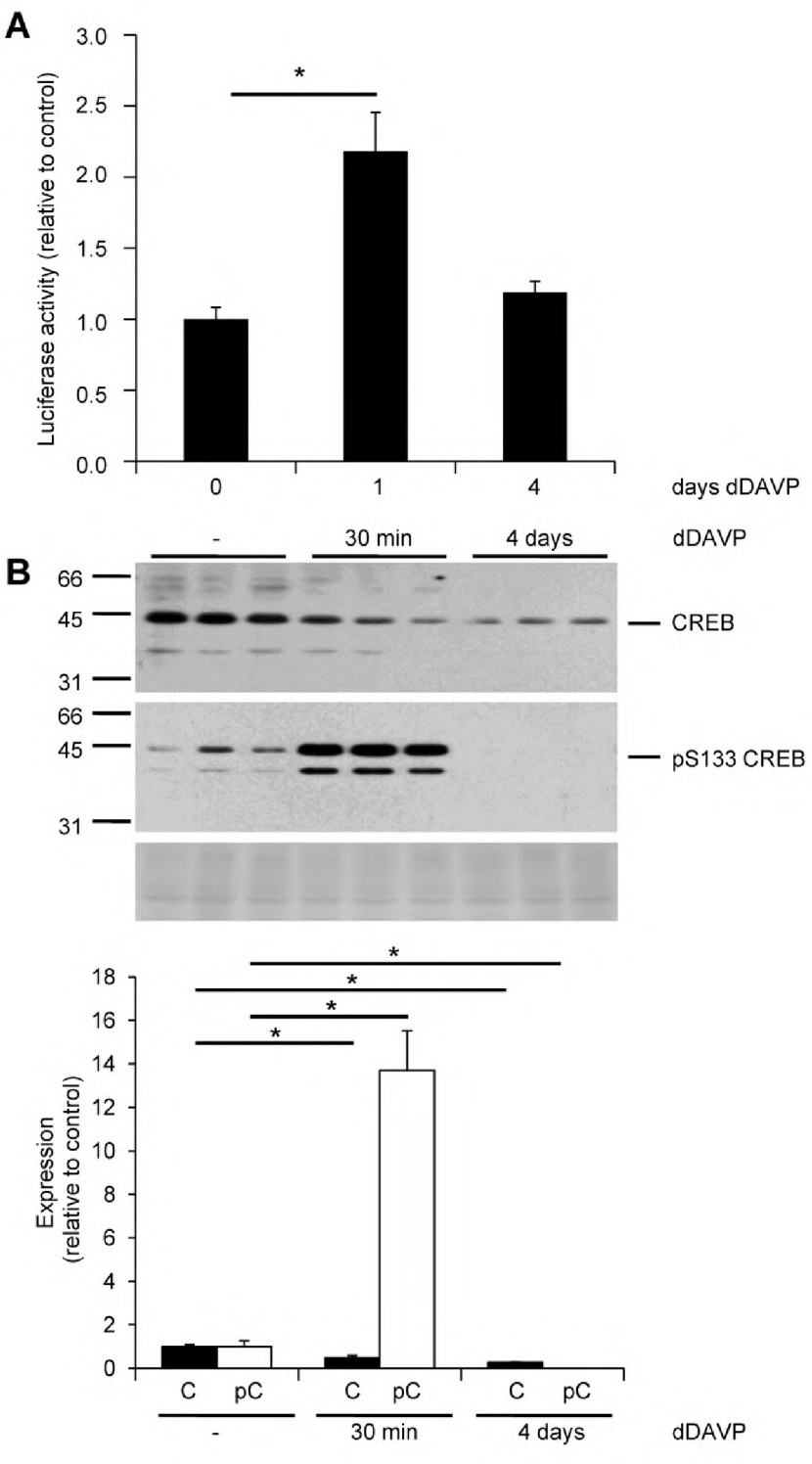

Figure 3. Role of CREB in dDAVP-induced AQP2 transcription. A) MpkCCD cells containing an CRE-luciferase construct were treated with or without dDAVP for 1 or 4 days. The cells were lysed and light emission was measured. **B)** MpkCCD cells were grown to confluence and incubated with or without dDAVP for the last 4 days or 30 minutes. Cells were lysed and subjected to immunoblotting for total CREB (C) and pS133-CREB (pC). For A-B: Data are the mean of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

evaluate the role of CRE-mediated transcription during dDAVP stimulation for 1 or 4 days. While incubation with dDAVP for 1 day yielded a more than two-fold increase in luciferase activity, luciferase activity was not different from untreated cells after 4 days of dDAVP stimulation (Fig. 3A). As CRE-mediated transcription is increased after 1 day dDAVP, but not after 4 days, these data suggest that CRE-mediated transcription is important to initiate, but not to maintain, dDAVP-induced transcription from the AQP2 promoter.

To test whether the difference in CRE-mediated transcription after long-term dDAVP stimulation could result from a change in the abundance and S133 phosphorylation of CREB, the effect of dDAVP on CREB was investigated by incubating mpkCCD cells with dDAVP for 30 minutes, to look at the initial response, and 4 days to investigate the effects of long-term dDAVP stimulation. Immunoblot analysis revealed that dDAVP application for 30 minutes decreased total abundance of CREB, but strongly increased the abundance of pS133 CREB (Fig. 3B). After 4 days of dDAVP treatment, total CREB was similarly diminished as after 30 minutes of dDAVP treatment, but now p133-CREB was undetectable. These data indicate that after an initial activation of CREB by phosphorylation at S133, the reduced abundance of total and S133-phosphorylated CREB may underlie the absence of CRE-mediated transcription upon long-term dDAVP treatment.

Expression of Epac1 and 2 in mpkCCD cells

As our data indicate that PKA and CREB are not involved in the long-term transcription of AQP2, and as cAMP levels were still increased after 4 days dDAVP stimulation, as compared to untreated cells, we wanted to test whether Epac1 and/or its close relative Epac2 could be involved.

To test whether mpkCCD cells do express the Epac proteins and whether their abundance is influenced by dDAVP, mpkCCD cells were grown on filters for 8 days of which the last 4 days with or without dDAVP. Immunoblotting for Epac1 and Epac2 revealed a band of the anticipated mass of 96 kDa for Epac1 and 116 kDa for Epac2 in untreated cells (Fig. 4). Upon incubation with dDAVP for 4 days, Epac1 levels increased significantly, while Epac2 levels were reduced. Epac1 and 2 abundance is thus dDAVP-sensitive.

Effect of Epac activity on AQP2 expression

To investigate whether Epac activation affects AQP2 promoter-dependent transcription and AQP2 abundance, mpkCCD-AQP2-3.0-luc cells were again grown 8 days, in the presence or absence of 0.1 μM of the Epac-specific activator 007-AM during the last 24 hrs. As positive controls, mpkCCD-AQP2-3.0-luc cells incubated with dDAVP for 1 or 4 days were taken along. Immunoblot analysis, however, revealed that, in contrast to dDAVP, 007-AM treatment alone did not stimulate AQP2 abundance (Fig. 5A), nor AQP2 promoter driven luciferase

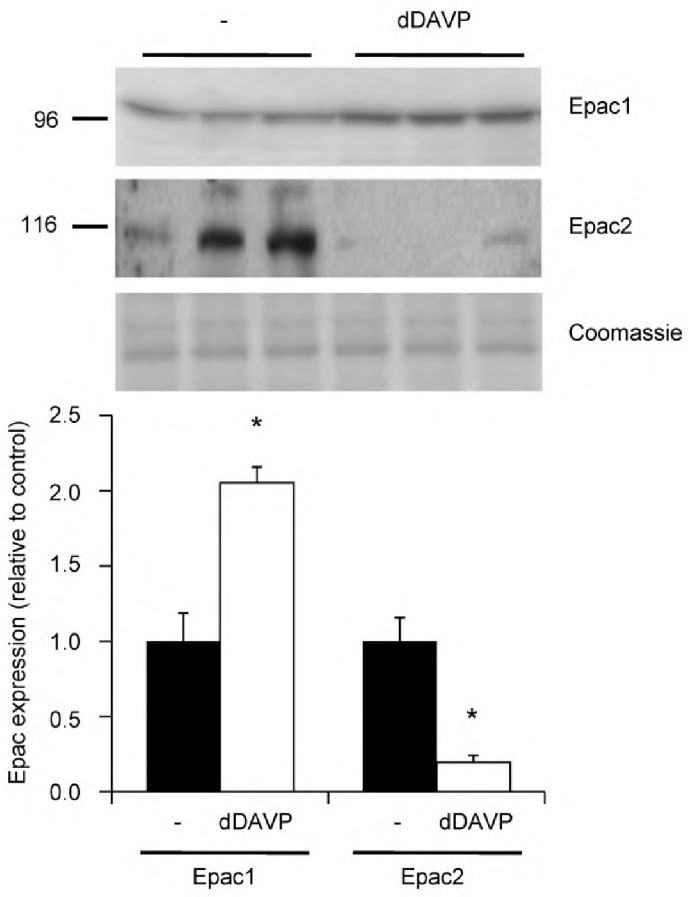

Figure 4. Epac expression in mpkCCD cells. mpkCCD cells were grown to confluence and incubated with or without dDAVP for 4 days. Cells were then lysed and subjected to immunoblotting for Epac1 and 2. The signals from immunobloting were densitometrically quantified and Epac signals were normalized for coomassie blue staining (indicated). Values are means +/- SEM. Significant differences ($p<0.05$) between control and experimental groups are indicated by an asterisk.

activity (Fig. 5B). These data indicate that Epac activation alone is not enough to stimulate AQP2 gene transcription.

To determine whether Epac activation could stabilize expression of AQP2 initially stimulated by dDAVP, mpkCCD-AQP2-3.0-luc cells grown as above were incubated with dDAVP for 3 days, followed by one day with or without dDAVP, or without dDAVP, but in the presence of 007-AM. Interestingly, luciferase activity analysis revealed that 007-AM treatment significantly increased AQP2 transcription compared to unstimulated cells (Fig. 5C), suggesting that Epac is involved in AQP2 transcription. To investigate whether the effects seen on AQP2 transcription are reflected in AQP2 protein abundance, the same samples were immunoblotted for AQP2 (Fig. 5D). 007-AM treatment significantly increased AQP2 abundance compared to cells left unstimulated, confirming the ability of Epac to stimulate AQP2 expression. The AQP2 abundance of 007-AM treated cells was however significantly lower than cells incubated with dDAVP during the last day.

Discussion

Short and long-term dDAVP-incubation increase AQP2 transcription by different pathways

It has previously been shown that AVP increases AQP2 transcription by the cAMP-PKA-CREB pathway (6-8). This pathway is also activated in mpkCCD cells after dDAVP stimulation, as shown by the dDAVP-stimulated increase in cAMP levels, the PKA dependence of AQP2 transcription and the dDAVP-mediated increase in CREB

phosphorylation as well as CRE dependent transcription (Fig. 1-3). However, our data indicate that there is a different pathway involved in the long-term regulation of dDAVP-induced AQP2 transcription. Although both short- and long-term dDAVP administration increased intracellular cAMP levels, this increase was clearly lower after long-term stimulation (Fig. 1). Long-term regulation of AQP2 transcription seemed to be independent of PKA, as H89 did not reduce AQP2 promoter-mediated transcription on the long term, whereas it did strongly reduce initial stimulation of AQP2 expression (Fig. 2). This cannot be explained by a lack of dDAVP stimulation after 4 days, as removal of dDAVP during the last day decreased AQP2 transcription (Fig. 5C) and likely also leads to decreased cAMP levels. The initial increase in AQP2 transcription observed after 1 day of dDAVP incubation was dependent on PKA, although the effect of the PKA blocker did not fully reduce luciferase activity (compare Fig. 2 and 5B). As the *in vitro* IC50 of H89 for PKA is approximately 50 nM (22), the 10 μM concentration used in our experiments is anticipated to block PKA completely. This suggests that PKA is not the only factor involved in the increased AQP2 transcription or H89 is not stable over the 24 hours incubation as used in our experiments. Another difference between the long and short-term regulation of AQP2 is the involvement of CREB. While acute dDAVP treatment increased CREB phosphorylation and CRE-dependent transcription, CREB expression and phosphorylation were reduced to nearly undetectable levels with long-term dDAVP treatment. In agreement with this, long-term dDAVP incubation did not increased CRE-mediated transcription. These changes may be the consequence of a cellular desensitization to dDAVP. Continuous stimulation of CREB is known to lead to the synthesis of ICER (inducible cyclic AMP response element repressor), reducing CRE-dependent transcription (23). Furthermore, cAMP stimulation has been shown to desensitize PKA (24), potentially explaining the absence of CREB phosphorylation and role of PKA in long-term AQP2 regulation.

The temporary increase in CREB phosphorylation as seen in our experiments, is in agreement with previous studies, where forskolin treatment leads to a rapid phosphorylation of CREB within 15 minutes after treatment which starts to decay already after 30 minutes, returning to baseline levels within 8 hours (25-27). In agreement with our results, Zhang *et al.* also observed a decrease in total CREB levels already after 30 minutes forskolin treatment in HEK293 cells (26). dDAVP treatment for 96 hours has also previously been shown to decrease total CREB levels in mpkCCD cells (11).

Long-term dDAVP induces a small cAMP response compared to short-term dDAVP stimulation

In our study, both short- and long-term dDAVP administration increases intracellular cAMP

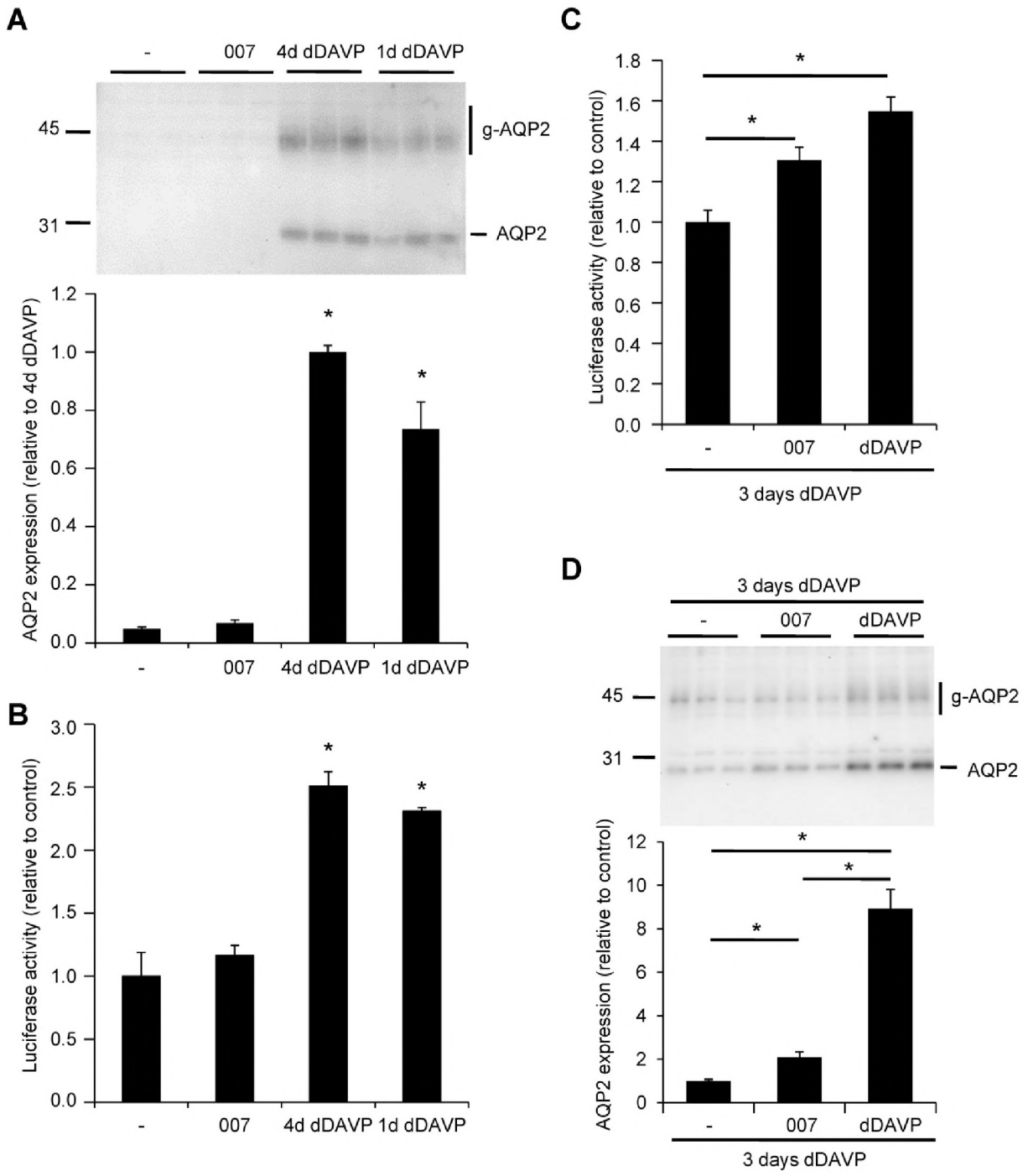

Figure 5. Effect of the Epac activation on AQP2 expression. A) MpkCCD cells were grown for 8 days and were treated with 007-AM or dDAVP during the last 24 hours, or with dDAVP for 4 days. Cells were then lysed and subjected to immunoblotting for AQP2. **B)** MpkCCD cells containing an AQP2-promoter-luciferase construct were treated as in A. The cells were lysed and light emission was measured. **C)** MpkCCD cells containing an AQP2-promoter-luciferase construct were incubated with dDAVP for 3 days, followed by 24 hours unstimulated (-), 007-AM or dDAVP treatment. The cells were lysed and light emission was measured. **D)** MpkCCD cells were incubated with dDAVP for 3 days, followed by 24 hours unstimulated (-), 007-AM or dDAVP treatment. The cells were lysed and subjected to immunoblotting for AQP2. For A-D: Data are the mean (+/- SEM) of three samples. Significant differences ($p<0.05$) are indicated by an asterisk.

levels, although this increase was clearly lower after long-term stimulation (Fig. 1). Previously, it has been shown that cAMP levels peak immediately after addition of dDAVP, but are reduced to very low levels within 120 minutes following addition in AQP2-transfected MDCK cells (10).

An increased cAMP level after long-term dDAVP as found in our study is in agreement with a previous study, showing that *in vivo*, in rats subjected to intramuscular injections with dDAVP for 3 days, cAMP was still increased compared to untreated rats (28). Later, Dublineau *et al.* also showed that in isolated collecting ducts of similarly treated rats, addition of 1 nM AVP evoked a smaller cAMP response than in untreated rats (29). Both our studies and those of Dublineau point to desensitization of the cellular system to vasopressin.

A possible explanation for the lower cAMP production after 4 days dDAVP is a lower receptor expression on the cell surface, in line with earlier observations that V2R receptor activation leads to its internalization (30). Adenylate cyclase 6, which is expressed in the collecting duct, has also been shown to be desensitized upon continued activation of PKA (31; 32). Long-term dDAVP treatment also results in an increased cAMP breakdown *in vivo*, as cAMP stimulation leads to an upregulation of cAMP phosphodiesterases (33). However, as our cAMP measurements were done in the presence of the phosphodiesterase inhibitor IBMX, an increased breakdown by increased phosphodiesterase activity cannot explain the difference in cAMP levels in our experiments

Epac activation increases AQP2 transcription and abundance after pretreatment with dDAVP

Our results indicate that long-term regulation of AQP2 by dDAVP may involve Epac. In agreement with *in vivo* studies, where both Epac1 and Epac2 proteins were found in the collecting duct, mpkCCD cells endogenously express Epac1 and Epac2 proteins (Fig. 4). Epac1 is mainly expressed in intercalated cells, although it is also found in principal cells in the outer medulla. Epac2 is highly expressed in all principal cells along the entire collecting duct (12). Yip showed that Epac activation increases vasopressin-stimulated translocation of AQP2 to the apical membrane (13).

In mpkCCD cells, Epac1 expression was stimulated by dDAVP, while Epac2 expression was reduced (Fig. 4), suggesting that dDAVP regulates Epac protein abundance. Moreover, Epac activity increases both AQP2 transcription and AQP2 protein abundance after sustained dDAVP stimulation (Fig. 5), although AQP2 abundance was not maintained to the same level as with dDAVP. As after prolonged dDAVP incubation cAMP levels are still increased compared to control cells, Epac activation by the increased cAMP levels may form part of the PKA independent pathway to maintain high AQP2 levels at long-term dDAVP incubation. The reduced level of maintaining AQP2 abundance may be due to instability of the Epac stimulator over the 24 hour period used, or may indicate that Epac is not the only factor

involved in stimulating long-term AQP2 expression. It remains to be established whether Epac has a similar role *in vivo*.

In conclusion, our study shows that the PKA-CRE pathway is involved in the initial increase in AQP2 abundance after dDAVP stimulation, but not in the long-term effect of dDAVP. Instead, long-term regulation of AQP2 may involve the activation of Epac.

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CHAPTER 6

Reduced adenylate cyclase activity leading to reduced aquaporin-2 expression explains the diuretic action of demeclocycline

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Abstract

Demeclocycline and minocycline are antibiotics of the tetracycline group, which display aquaretic effects. Because of the effect on water diuresis, demeclocycline is currently used to treat sustained hyponatremia in patients with the syndrome of inappropriate antidiuretic hormone secretion. Though these compounds are thought to act in the distal part of the nephron, their exact mechanism of action has not yet been described. We show that demeclocycline and minocycline decrease the protein level of the water channel aquaporin-2 in the mpkCCD mouse collecting duct cell line in a time and concentration dependent manner. This effect is abolished by inhibiting protein synthesis. Moreover, demeclocycline decreased luciferase activity using a 3.0 kb AQP2 promoter reporter construct. These observations suggest that the effect of demeclocycline on aquaporin-2 is mediated via decreased transcription. Our data further indicate that demeclocycline decreases AQP2 gene transcription by decreasing adenylate cyclase expression and cAMP generation.

Introduction

The kidney is the main organ for regulating water homeostasis. In states of hypernatremia or hypovolemia, the hormone arginine-vasopressin (AVP) is released from the pituitary. Subsequent binding of AVP to the vasopressin type-2 receptor in the basolateral membrane of renal collecting duct principal cells induces a signaling cascade (1). This includes Gs protein mediated activation of adenylate cyclase, a rise in intracellular cAMP, activation of protein kinase A (PKA), and subsequent phosphorylation of aquaporin-2 (AQP2) water channels at Ser256. This results in the redistribution of AQP2 from intracellular vesicles to the apical membrane (2-4). Driven by the transcellular osmotic gradient of sodium and urea, water will enter principal cells through AQP2 and will exit the cells through AQP3 or AQP4 located in the basolateral membrane, resulting in concentrated urine. Besides this short-term regulation, AVP regulates AQP2 on the long term by increasing its transcription, as activated PKA mediates phosphorylation of the cAMP responsive element binding protein (CREB) (5; 6). CREB stimulates AQP2 transcription via the cAMP Responsive Element (CRE) in the AQP2 promoter (7; 8).

High levels of AVP leading to hyponatremia with or without hypervolemia occur in several diseases, like the syndrome of inappropriate antidiuretic hormone secretion (SIADH), congestive heart failure and severe liver cirrhosis (9-11). In these diseases, AQP2 abundance is increased, indicating that AQP2 plays an important role in the water retention in these pathological states (12-14).

Demeclocycline is a bacteriostatic antibiotic of the tetracycline group, which has been shown to cause water diuresis and nephrogenic diabetes insipidus (15; 16). Because of the effect on water diuresis, demeclocycline is currently used to treat sustained hyponatremia in patients with SIADH (17). Demeclocycline has been shown to restore the sodium plasma concentration in SIADH patients to normal levels, permitting unrestricted water intake in these patients (18).

Wilson *et al*. showed that the aquaretic effect of demeclocycline is exerted by selective inhibition of the water reabsorption in the distal part of the nephron (19). Others have shown that demeclocycine inhibits the AVP-induced osmotic water flow in the toad urinary bladder (15; 20; 21), a model system of the mammalian collecting duct. Just like demeclocycline, the tetracycline antibiotics minocycline, doxycycline and tetracycline have been shown to reduce the water flow in toad bladders (20), and tetracycline has been reported to decrease urinary concentrating ability in men (19), suggesting that other tetracycline antibiotics affect collecting duct function and urinary concentrating ability as well.

The exact mechanism of action of these tetracycline antibiotics, however, is unknown, which is investigated here.

Materials and methods

Cell culture

MpkCCD cells were cultured as described previously (22). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were exposed to 1 nM of the stable AVP analogue dDAVP at the basolateral side for the last 96 hrs, to induce AQP2 expression. Tetracycline hydrochloride (Sigma T3383), minocycline hydrochloride (Sigma M9511), or demeclocycline hydrochloride (Sigma 30910) were added to the apical and basolateral side of the filters for the last 2-24 hours with or without 50 μM cycloheximide or 10 μM forskolin. At the end of the experiment transcellular electrical resistance was measured using a Millicell-ERS meter (Millipore corp., Bedford, MA, USA).

Transfection and generation of a stable mpkCCD cell line with a 3.0 AQP2 promoter-luciferase reporter construct was previously described (23).

Immunoblotting

MpkCCD cells from 1.13 cm^2 filter were lysed in 200 μl Laemmli buffer, sonicated and heated for 30 min at 37°C. PAGE, blotting and blocking of the PVDF membranes were done as described (24). Membranes were incubated for 16 hrs at 4°C with 1:3000-diluted affinity-

purified rabbit R7 AQP2 antibodies (25), 1:200 anti-adenylate cyclase 3 or 1:200 anti-adenylate cyclase 5/6 (both gifts from C. Serradeil-Le Gal, Sanofi Recherche, Toulouse, France), all in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% non-fat dried milk.

Blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's (Sigma, St. Louis, MO) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Densitrometric analyses were performed using Biorad quantification equipment (Bio-Rad 690c densitometer, Chemidoc XRS) and software (QuantityOne). Equal loading of the samples was confirmed by subsequent staining of the blots with coomassie blue.

PNGaseF treatment

To 15 µl cell lysate, 30 µl Milli-Q water, 5 µl G7 buffer, 5 µl NP-40 and 1 µl PNGaseF were added before 1 hr incubation at 37ºC. Subsequently 7.5 µl Laemmli buffer was added before proceeding to immunoblotting.

cAMP measurement.

MpkCCD cells were seeded on filters for 8 days, the last 4 days with or without 1 nM dDAVP. The last day, the cells were exposed overnight to 50 µM demeclocycline, followed by 30 min of treatment with 0.5 mM phosphodiesterase inhibitor 3-isobutyl 1 methylxanthine (IBMX; Sigma, St. Louis, MO, USA). cAMP was measured using the cAMP-Glo assay (Promega, Madison, WI, USA) or cAMP enzyme immunoassay kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. Results were related to a standard curve based on the measurement of defined cAMP solutions done in triplicates.

Luciferase assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence was measured for 10 seconds using an EG&G Berthold Lumat LB9507 luminometer.

To verify that equal amounts of protein per sample were used for the luciferase assay, protein concentration was determined using the BioRad protein assay (München, Germany), according to manufacturer's instructions.

Statistics

Students unpaired t-test was used when two groups with Gaussian distribution were compared. For multiple comparisons, Bonferroni correction was applied. A p value of less

than 0.05 is considered significant. Data are presented as mean and bars are standard error of the mean (SEM).

Results

The effect of tetracycline antibiotics on AQP2 expression

The structures of the tetracycline antibiotics demeclocycline, minocycline and tetracycline are shown in Fig. 1.

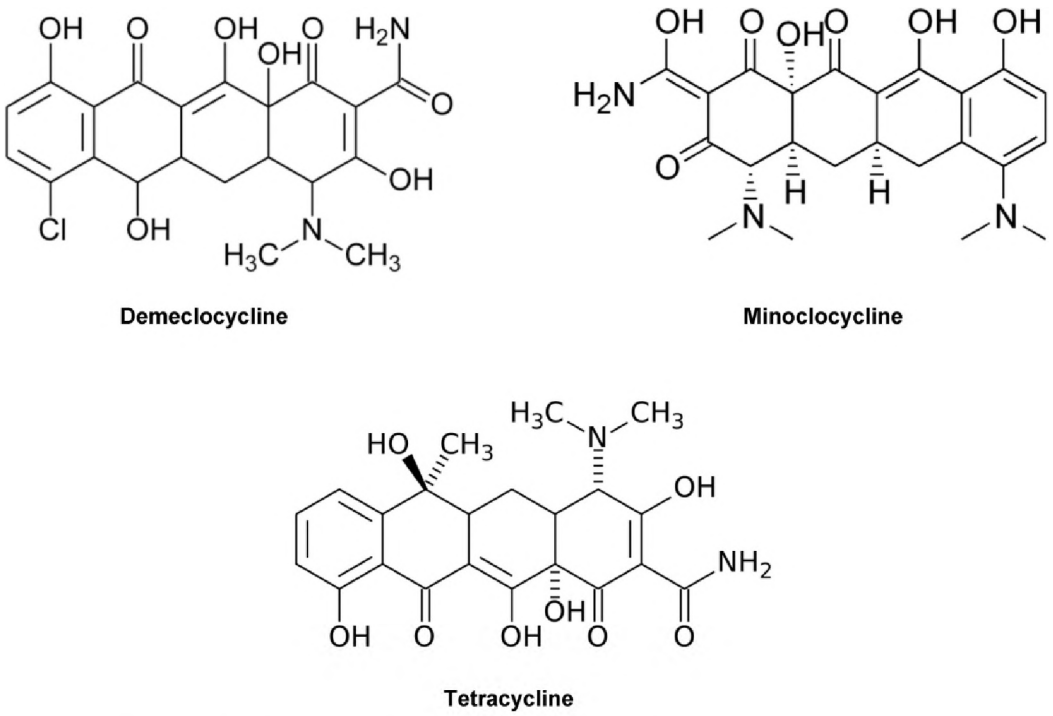

Figure 1. Structures of demeclocycline, minocycline and tetracycline

To study the effect of demeclocycline on AQP2 expression, mpkCCD cells were grown for 8 days, the last 4 days in the presence of 1 nM dDAVP to induce maximal endogenous AQP2 expression (26). During the last 24 hours, the cells were exposed to different concentrations of demeclocycline. Demeclocycline dose-dependently decreased the AQP2 protein abundance in mpkCCD cells, setting in at 50 µM (Fig. 2A). Analysis of the transcellular resistance revealed that this varied between 2-3 kOhm until 50 µM, but that this was significantly decreased at 100 µM (Fig. 2B), indicating that at demeclocycline concentrations higher than 50 µM the cell monolayer was affected.

To investigate whether minocycline and tetracycline also reduce AQP2 expression, mpkCCD cells were exposed to different concentrations of these compounds during the last 24 hrs.

Minocycline dose-dependently decreased AQP2, effects setting in at 25 µM (Fig. 2C). At this concentration, the transcellular resistance was not different from lower concentrations, but this was again affected at higher concentrations (not shown).
Tetracycline displayed a non-significant (p=0.15) tendency to decrease AQP2 expression at 100 µM (Fig. 2D). The transcellular resistance was not affected (not shown).
As demeclocycline is clinically used for its aquaretic action, we used 50 µM demeclocycline concentrations in all following experiments. We investigated the time frame for AQP2 down-regulation by demeclocycline and found that the effect was already present after 8 hrs (Fig 2E). Coomassie labeling secured that loading and overall protein amount were equal among all samples.

Effect of demeclocycline on AQP2 transcription

The effect of demeclocycline on AQP2 expression could be caused by a decrease in AQP2 protein/mRNA synthesis, and/or an increase in degradation. To investigate the involvement of protein degradation, cells were incubated with demeclocycline with or without co-incubation with the protein synthesis inhibitor cycloheximide. In the presence of cycloheximide, demeclocycline did not affect AQP2 abundance, whereas demeclocycline still decreased AQP2 abundance in control cells (Fig. 3A). These data suggested that demeclocycline does not increase AQP2 degradation but affects AQP2 transcription or RNA stability.
To further investigate whether demeclocycline reduces AQP2 transcription, pooled colonies of mpkCCD cells stably-transfected with a 3.0kb-AQP2 promoter followed by luciferase cDNA (pGL3-AQP2-3.0-luc) were used. As described before (23), dDAVP increased luciferase activity compared to unstimulated cells. Luciferase activity was reduced with demeclocycline, indicating that demeclocycline decreases AQP2 transcription (Fig. 3B).

Effect of demeclocycline on cAMP production

Because AQP2 transcription is upregulated by AVP-induced cAMP levels, it was investigated whether demeclocycline affects cAMP production. For this, cells were stimulated with dDAVP as above, the last 24 hours with or without demeclocycline, and IBMX was added for the last 30 minutes. As anticipated, dDAVP increased the amount of cAMP, which was about three-fold (Fig. 4). Co-incubation with demeclocycline decreased the amount of cAMP both in unstimulated cells and dDAVP-stimulated cells.

Effect of demeclocycline on adenylate cyclases

cAMP is produced by adenylate cyclases, which are stimulated by AVP-mediated activation

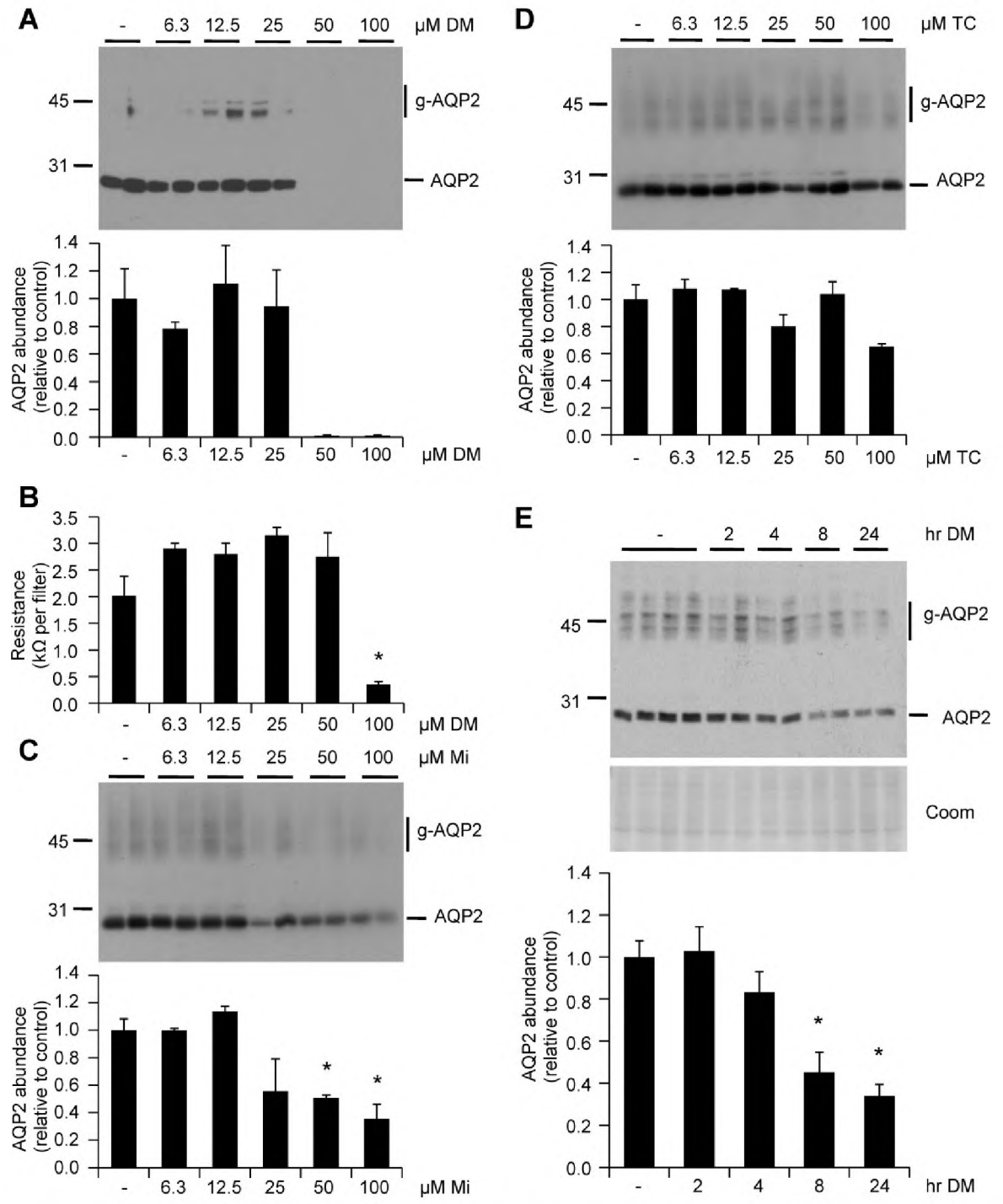

Figure 2. Effects of tetracycline antibiotic concentrations on the AQP2 protein level in mpkCCD cells. A) MpkCCD cells were grown to confluence and exposed to 1 nM dDAVP for 4 days. For the last 24 hrs the cells were incubated with various concentrations demeclocycline (DM). Cells were lysed and subjected to AQP2 immunoblotting. **B)** Transcellular resistance of cells as described in A. **C)** Cells were grown as described in A. During the last 24 hrs, cells were incubated with various concentrations minocycline (Mi). Cells were lysed and subjected to AQP2 immunoblotting. **D)** Cells were grown as described in A. During the last 24 hrs, cells were incubated with various concentrations tetracycline (TC). Cells were lysed and subjected to AQP2 immunoblotting. **E)** Cells were grown as described in A. During the last 2-24 hrs cells were incubated with 50 µM demeclocycline. A-E: Molecular masses (in kDa) are indicated on the left. Concentrations are in µM. The signals for non-glycosylated and complex-glycosylated AQP2 were densitometrically quantified. Mean values of normalized AQP2 expression per condition are relative to control (+/- SEM). Significant differences ($p<0.05$) from control (-) are indicated by an asterisk.

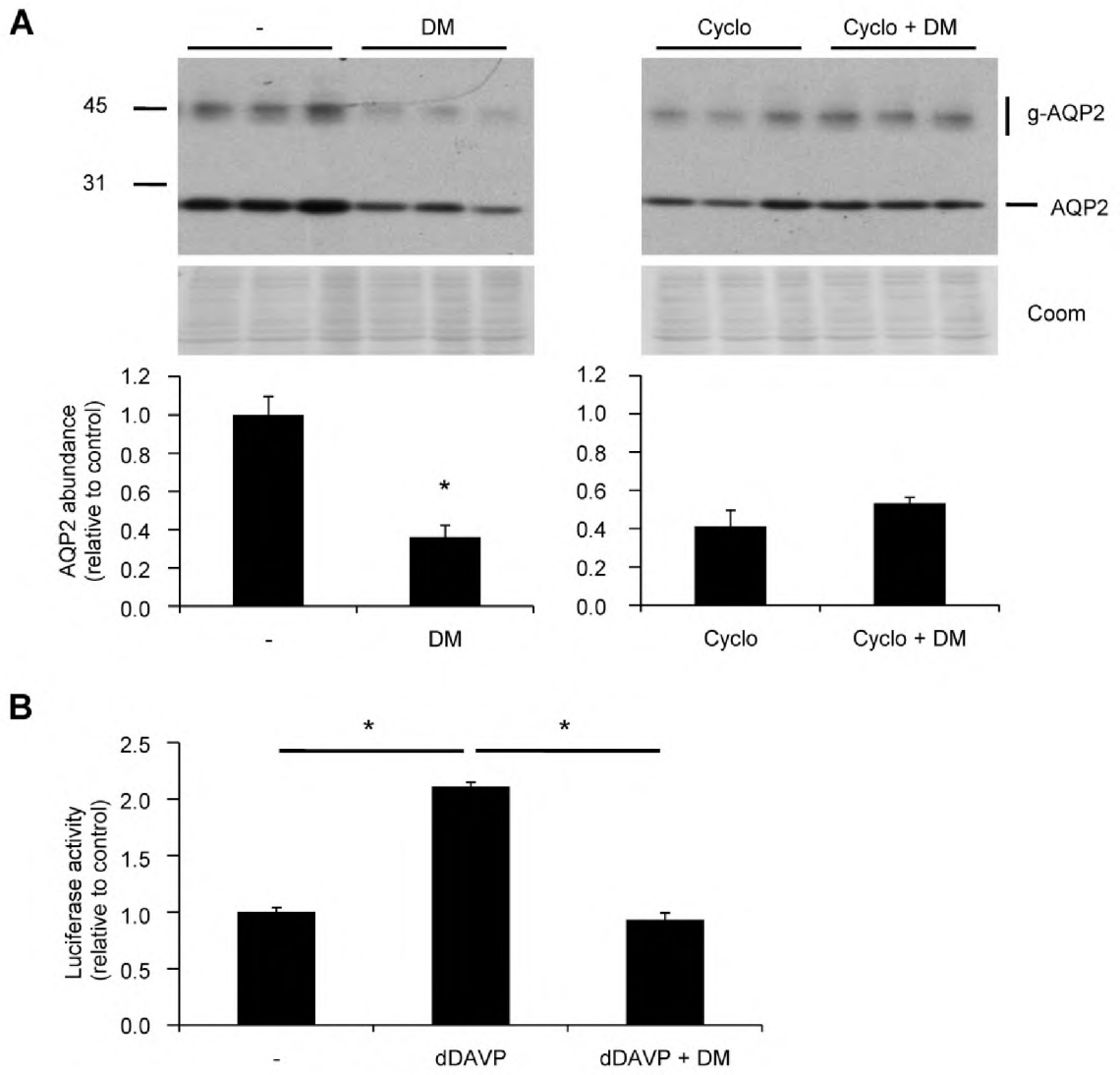

Figure 3. Effect of demeclocycline on AQP2 transcription A) MpkCCD cells were grown to confluence and exposed to 1 nM dDAVP for 4 days. For the last 8 hrs the cells were incubated with with 50 µM demeclocycline (DM) with or without cycloheximide. Cells were lysed and subjected to AQP2 immunoblotting. Mean values of normalized AQP2 expression per condition are relative to control (+/- SEM). **B)** MpkCCD cells containing a 3.0 kb AQP2-promoter-luciferase construct were grown to confluence and exposed to 1 nM dDAVP for 4 days, with or without 50 µM demeclocycline during the last 24 hrs. The cells were lysed and light emission was measured. Data are the mean of three samples and relative to control (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

of the V2R. To determine whether the decreased cAMP levels are due to effects of demeclocycline at steps before or directly at adenylate cyclase, mpkCCD-AQP2-3.0-luc cells were incubated with or without demeclocycline in the presence or absence of the adenylate cyclase activator forskolin for 1 day or dDAVP for 4 days before harvesting. As anticipated, both dDAVP and forskolin significantly increased AQP2-promoter driven luciferase activity (Fig. 5). While cells exposed to demeclocycline still showed an increased luciferase activity with forskolin and dDAVP as compared to unstimulated controls, demeclocycline caused a similar decrease in the luciferase activity in all conditions. These results indicate that

demeclocycline directly affects adenylate cyclase activity instead of affecting V2R or Gs stimulation.

We next investigated the effect of demeclocycline on the expression of adenylate cyclase (AC) 3 and 6, which have been shown to be expressed in collecting duct principal cells (27-29). Following exposure to dDAVP for 4 days, demeclocycline application resulted in a reduction of AC3 abundance to less than 40% of control levels after 24 hrs, while at 8 hrs a slight, but insignificant reduction is already observed (Fig. 6A). Demeclocycline incubation also resulted in a small but significant decrease in abundance of AC5/6 after 8 hours, and resulted in a decrease to 25% of control levels after 24 hours. Please note that an antibody recognizing both AC5 and AC6 was used and that for quantification, samples were treated with PNGaseF to remove glycosylation of AC5/6 (Fig. 6B, lower panel).

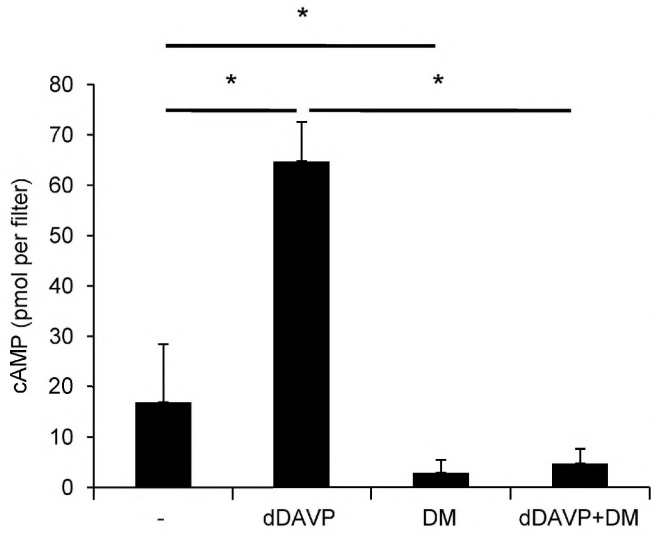

Figure 4. Effect of demeclocycline on cAMP production. MpkCCD cells were grown to confluence, incubated with or without dDAVP for 4 days and with or without demeclocycline (DM) for the last 24 hours. During the last 30 minutes, IBMX was added. Cells were lysed and cAMP production was measured. Bars are mean values of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

Discussion

Reduction of AQP2 abundance by tetracycline antibiotics

In this study we show that the tetracycline antibiotic demeclocycline down-regulates the AVP regulated water channel AQP2 in a time and concentration-dependent manner in mpkCCD cells. Besides demeclocycline, also minocycline decreased AQP2 expression, while only a small effect of tetracycline on AQP2 expression was seen.

The decrease in AQP2 expression explains the aquaretic effect of tetracycline antibiotics, which was previously shown to effect water reabsorption in the distal part of the nephron and the toad urinary bladder, a model system of the mammalian collecting duct (15; 19-21). Demeclocycline down-regulated AQP2 in mpkCCD cells at concentrations that are in line with those measured in urine from patients (30), indicating that the effect observed are at pharmacologically relevant doses. Resistance measurements and coomassie labeling

suggest that at 50 µM demeclocycline there is no decrease in cell viability but still AQP2 down-regulation. However, the loss of resistance at 100 µM suggests that the cells are seriously affected by demeclocycline at higher concentrations, which is also in line with reports on the risk of nephrotoxicity of demeclocycline (31; 32). Minocycline down-regulated AQP2 at similar concentrations as demeclocycline, which is in line with the effect of minocycline on the water flow seen in toad urinary bladders (20). Overt diabetes insipidus is not seen with this agent, probably because minocycline is clinically used in much lower doses than demeclocycline (20).

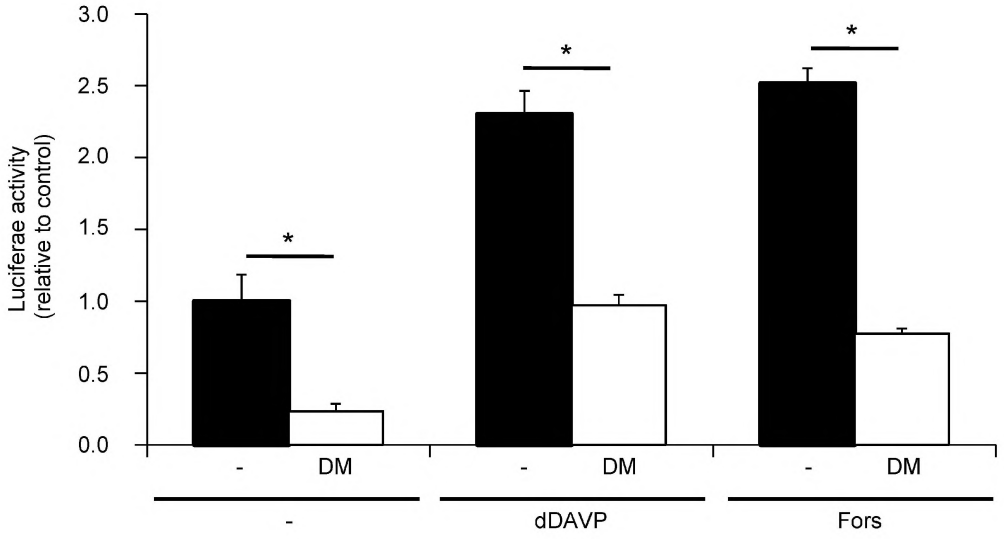

Figure 5. dDAVP-dependence of demeclocycline-induced AQP2 down-regulation. MpkCCD cells containing a 3.0 kb AQP2-promoter-luciferase construct were incubated with or without 4 days 1 nM dDAVP, 24 hrs 10 µM forskolin and/or 24 hrs 50 µM demeclocycline (DM). The cells were lysed and light emission was measured. Data are the mean of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

In this study we see a small effect of tetracycline on AQP2 expression. Tetracycline has been reported to decrease urinary concentrating ability in man as well as in toad urinary bladders, however, its effect were clearly smaller than that of demeclocycline (19; 20), which is in line with our data and indicating the link between protein levels of AQP2 and aquaretic ability.

Reduced adenylate cyclase abundance and activity explains the diuretic effect of demeclocycline

We show that the protein synthesis inhibitor cycloheximide abolishes the effect of demeclocycline, implying that a decrease in AQP2 production explains the decrease in AQP2 abundance. Using an AQP2 promoter-luciferase reporter construct, it was shown that demeclocycline decreases AQP2 transcription.

Our results show that demeclocycline down-regulates dDAVP-stimulated AQP2 expression, as well as basal and forskolin-stimulated AQP2. As demeclocycline affects forskolin-

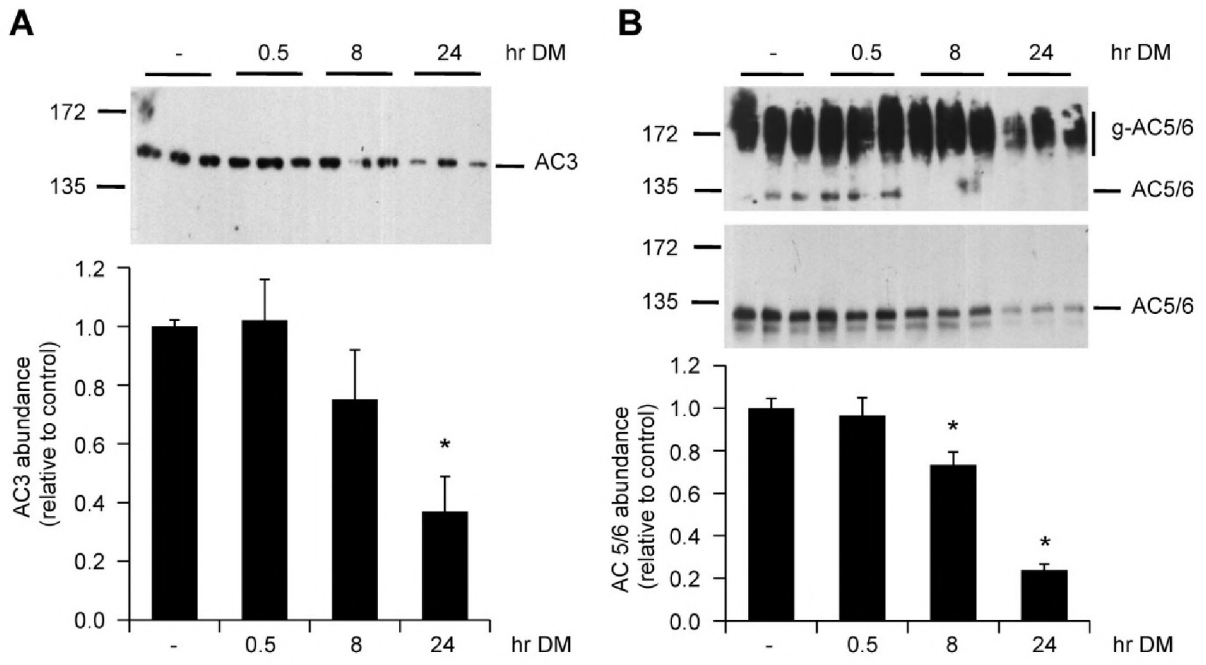

Figure 6. Effect of demeclocycline on AC expression. A) MpkCCD cells were grown to confluence and incubated with 1 nM dDAVP for 4 days, with or without 50 µM demeclocycline (DM) for various time periods. Cells were lysed and subjected to AC3 immunoblotting. **B)** Cells as described in A were subjected to AC5/6 immunoblotting (upper panel). Samples were treated with PNGaseF to remove glycosylation and again subjected to AC5/6 immunoblotting (lower panel). Mean values of normalized AQP2 expression per condition are relative to control (+/- SEM). Significant differences ($p<0.05$) from control (-) are indicated by an asterisk.

stimulated AQP2 expression, this indicates that the effect of demeclocycline is independent of V2R receptor or Gs stimulation and seems to be further in the signaling cascade.

A vasopressin independent effect was also suggested by a study of Horattas *et al*, who showed that in patients undergoing surgery, the plasma AVP concentration was increased after demeclocycline-treatment, but the effect of AVP on urinary concentrating ability decreased (33).

In regard to an effect on intracellular cell signaling, we found a blunting of dDAVP-induced cAMP production, which is in line with findings of Dousa and Wilson showing a decreased basal and AVP-induced cAMP generation in renal medulla tissue (34). Our results also show a decreased expression of both adenylate cyclase 3 and 6 after demeclocycline application, which explains the lower cAMP generation.

In conclusion, our data show that the aquaretic effect of tetracycline antibiotics such as demeclocycline and minocycline are mediated via down-regulation of the AVP regulated water channel AQP2. Demeclocycline decreases AQP2 gene transcription by decreasing adenylate cyclase expression and cAMP generation.

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CHAPTER 7

Lithium reduces AQP2 transcription independent of prostaglandins

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Abstract

Vasopressin (AVP) stimulated translocation and transcription of aquaporin 2 (AQP2) water channels in renal principal cells is essential for urine concentration. 20% of patients treated with lithium develop nephrogenic diabetes insipidus (NDI), a disorder in which the kidney is unable to concentrate urine. *In vivo* and in mouse collecting duct (mpkCCD) cells, lithium treatment coincides with decreased AQP2 abundance and inactivation of glycogen synthase kinase (Gsk) 3β. This is paralleled *in vivo* by an increased renal cyclooxygenase 2 (COX-2) expression and urinary prostaglandin PGE_2 excretion. PGE_2 reduces AVP-stimulated water reabsorption, but its precise role in lithium-induced down-regulation of AQP2 is unclear.

Using mpkCCD cells, we here investigated if prostaglandins contribute to lithium-induced down-regulation of AQP2. In these cells, lithium application reduced AQP2 abundance, which coincided with Gsk3β inactivation and increased COX-2 expression. Inhibition of COX by indomethacin, reducing PGE_2 and $PGF_{2\alpha}$, or dexamethasone-induced down-regulation of COX-2 increased AQP2 abundance, while PGE_2 addition reduced AQP2 abundance. However, lithium did not change the prostaglandin levels, and indomethacin and dexamethasone did not prevent lithium-induced AQP2 down-regulation. Further analysis revealed that lithium decreased AQP2 protein abundance, mRNA levels and transcription, while PGE_2 reduced AQP2 abundance by increasing its lysosomal degradation, but not by reducing AQP2 gene transcription.

In conclusion, our data reveal that in mpkCCD cells, prostaglandins decrease AQP2 protein stability by increasing its lysosomal degradation, indicating that *in vivo* paracrine produced prostaglandins might have a role in lithium-induced NDI via this mechanism. However, lithium affects also AQP2 gene transcription, which is prostaglandin independent.

Introduction

The hormone arginine vasopressin (AVP) is secreted from the pituitary gland during states of hypernatremia or hypovolemia. AVP binds to the vasopressin type-2 receptor in the basolateral membrane of renal connecting tubule and collecting duct principal cells, which initiates a signaling cascade that results in the redistribution of aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical membrane (1; 2). Driven by an osmotic gradient, water will enter principal cells through AQP2 and will exit through AQP3 or AQP4 in the basolateral membrane, resulting in concentrated urine. Besides this acute mechanism of regulation, chronic AVP stimulation also increases AQP2 expression via phosphorylation of

the cAMP responsive element binding protein (CREB), thereby stimulating transcription of the gene via the cAMP responsive element (CRE) in the AQP2 promoter (3-5).
Lithium is the drug of choice for the treatment of bipolar disorders. It is also used regularly to treat schizoaffective disorders as well as depression. In addition, lithium is also under consideration as a therapeutic for many diseases including Alzheimer's disease, AIDS and amyotrophic lateral sclerosis (6-8). Lithium is a frequently prescribed drug and is used by 1 in 1000 of the population (9). Approximately 20% of patients undergoing lithium treatment develop symptomatic nephrogenic diabetes insipidus (NDI), a disorder characterized by polyuria and polydipsia due to a urinary concentrating defect (10). This makes lithium-induced NDI the most common form of NDI.
Studies in rats have shown that lithium-induced NDI occurs in conjunction with AQP2 down-regulation (11; 12) and in line with this, lithium treatment reduces urinary AQP2 excretion in humans, indicating a decreased renal AQP2 expression (13). Lithium has been shown to inactivate glycogen synthase kinase (Gsk) 3β in mice. The development of NDI and inactivation of Gsk3β are temporally related to an increased cyclooxygenase-2 (COX-2) expression in the kidney, leading to an increased urinary prostaglandin E_2 (PGE_2) excretion (14). As PGE_2 reduces AVP-stimulated water reabsorption in the collecting duct (15; 16), this could suggest an important role for PGE_2 in lithium-NDI development. This is also suggested by studies showing that blocking prostaglandin production by indomethacin reduces the urine volume of lithium-treated rats (17) as well as of lithium-induced NDI patients (18; 19).
In vitro, mouse collecting duct (mpkCCD) cells are a good model system to study the effects of lithium on AVP-induced AQP2 expression. Following induction of endogenous AQP2 expression, addition of clinically-relevant concentrations of lithium to the apical side caused a reduction in AQP2 abundance and resulted in Gsk3β inactivation (20; 21). In the present study, we used the mpkCCD model system to gain additional insights into the mechanism whereby lithium reduces AQP2 abundance and the potential role of prostaglandins in this process.

Material and Methods

Cell Culture

Mouse $mpkCCD_{cl4}$ cells were essentially grown as described (22). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were treated for the last 96 hrs with 1 nM dDAVP to the basolateral side, to maximally induce AQP2 expression (20). Cells were incubated with 1 mM lithium chloride at the basolateral

side and 10 mM lithium chloride at the apical side for the last 24 or 48 hrs. Cells were incubated with 20 µM zinc chloride at both sides of the cells during the last 48 hrs. 1 µM dexamethasone, 10 µM indomethacin, 1 µM PGE_2 (all from Sigma, St. Louis, MO, USA) or 1 µM $PGF_{2\alpha}$ (Calbiochem, San Diego, CA) were administered at both sides of the cells during the last 48 hours.

Constructs

Transfection and generation of a stable mpkCCD cell line with a 3.0 AQP2 promoter-luciferase reporter construct was previously described (23). Transfection and generation of a stable mpkCCD cell line with an 0.4 kb AQP2 promoter-luciferase reporter construct as well as pGL3-CRE(21)-luc, a construct in which luciferase transcription is driven by a promoter existing of 21 tandemly-placed CREs, was described previously as well (23).

pGL3-AQP2-2.5-luc, containing -2.5 kb till +60 bp of the AQP2 promoter, was made by cloning the *Bgl*II - blunted *Ssp*I fragment of the AQP2 promoter into the *Bgl*II - blunted *Kpn*I sites of pGL3-AQP2-3.0-luc. pGL3-AQP2-1.7-luc (-1710 till +60) was made by digesting pGL3-AQP2-3.0-luc with *Kpn*I, followed by religation. For pGL3-AQP2-1.4-luc, possessing -1350 till +60 bp of the AQP2 promoter, a *Bgl*II - blunted *Sph*I fragment of the promoter was cloned into the *Bgl*II - blunted *Kpn*I sites of pGL3-AQP2-3.0-luc. For pGL3-AQP2-1.1-luc (-1110 till +60), pGL3-AQP2-3.0-luc was cut with *BamH*I and *Kpn*I, blunted and religated. For pGL3-AQP2-0.9-luc (-900 till +60), pGL3-AQP2-3.0-luc was cut with *Xho*I – *Kpn*I, blunted and religated. For pGL3-AQP2-0.7-luc (-650 till +60 bp) a 0.7 kb AQP2 *Hinc*II - *Bgl*II fragment from the AQP2 promoter was cloned into *Bgl*II - blunted *Kpn*I sites of pGL3-AQP2-3.0-luc. For pGL3-AQP2-0.2-luc (-220 till +60), pGL3-AQP2-3.0-luc was cut with *Aat*II – *Kpn*I, blunted and religated.

MpkCCD cells were stably transfected using the calcium-phosphate precipitation technique as described (24). Transfected colonies were selected with G418 (0.25 mg/ml) and pooled to level out differences between individual colonies.

Immunoblotting

$MpkCCD_{cl4}$ cells from 1.13 cm^2 filter were lysed in 200 µl Laemmli buffer and 15 µl samples were analyzed. PAGE, blotting and blocking of the PVDF membranes were done as described (25). Membranes were incubated for 16 hrs with 1:3000-diluted affinity-purified rabbit R7 AQP2 antibodies (26), 1:1000 diluted rabbit anti-Ser9-Gsk3β (Cell Signaling Technology Beverly, MA, USA), 1:5000 diluted mouse anti-Gsk3β (BD Transduction Laboratories; Lexington, KY, USA), 1:1000 diluted mouse anti-COX-2 (Cayman Chemicals, Ann Arbor, MI, USA) in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% w/v non-fat dried milk. Next, blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit

IgG's or 1:2000 goat anti-mouse IgG's (Sigma, St. Louis, MO) coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Films were scanned using a Bio-Rad 690c densitometer and signals were analyzed using Bio-Rad software. Two-fold dilution series of a control sample was blotted in parallel to allow semi-quantification. Equal loading of the samples was confirmed by subsequent staining of the blots with Coomassie Brilliant Blue G250 (Serva, Heidelberg, Germany)

Luciferase Assay

Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) following the manufacturer's instructions. Luminescence was measured for 10 seconds using an EG&G Berthold Lumat LB9507 luminometer.

RT-PCR

MpkCCD cells were grown on semi-permeable filters for 8 days as described above, and total RNA was isolated using TriZol extraction reagent (Gibco, Life Technologies, Rockville, MD), according to the manufacturer's instructions. To remove genomic DNA, total RNA was treated with DNase (Promega, Madison, WI) in DNase buffer, incubated for 1 hr at 37 °C, extracted with phenol/chloroform and precipitated. RNA was reverse-transcribed into cDNA using MMLV Reverse Transcriptase and random primers (Promega, Madison, WI). During cDNA production, a control reaction without the reverse transcriptase enzyme was conducted to exclude amplification of genomic DNA.

SYBR Green Real-time quantitative PCR was performed on an iQ5 Real-Time PCR Detection System from Bio-Rad by utilizing the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers for the prostaglandin receptors or the mouse AQP2 gene. To amplify AQP2 mRNA, primers binding to exon 2 (CTCCACAACAATGCAACAGC) and exon 3 (GAGCAGCCGGTGAAATAGAT) were used. To amplify AQP2 pre-mRNA, the exon 3 primer was used together with an intron 2 primer (GGCAGTCTCAGCTGTCCTGA). Primers for prostaglandin receptors were designed to be intron overlapping (see Table 1). Signals for the house-keeping gene, which was amplified in parallel were used to normalize for differences in the amount of starting cDNA, using either primers for β-actin or ribosomal 18S (Table 1).

Prostanoid analysis

Samples were prepared as described (27) with minor modifications. Briefly, cell culture supernatants were spiked with ~1 ng of deuterated internal standards, and the methoximes were obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer.

After acidification to pH 3.5, prostanoid derivatives were extracted, and the pentafluorobenzylesters were formed. Samples were purified by thin layer chromatography, and a broad zone with R_F 0.03-0.4 was eluted. After withdrawal of the organic layer, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis on a Finnigan MAT TSQ700 GC/MS/MS (Thermo Electron Corp., Dreieich, Germany) equipped with a Varian 3400 gas chromatograph (Palo Alto, CA) and a CTC A200S autosampler (CTC Analytics, Zwingen, Switzerland).

Table 1: Overview of primer sets

Protein	Forward primer (5´-3´)	Reverse primer (5´-3´)
EP1	GCACGGAGCCGAGGAGC	GCAGGGGCTCATATCAGTGG
EP4	TACGCCGCCTTCTCTTACAT	TTCACCACGTTTGGCTGATA
FP	CGTCACGGGAGTCACACTCT	TTCACAGGTCACTGGGGAAT
TP	GTGGGCATCATGGTGGTGG	CACACGCAGGTAGATGAGCAGC
β actin	GTATGCCTCTGGTCGTACCAC	ACGATTTCCCTCTCAGCTGTG
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Results

Effect of lithium on Gsk3 and COX-2

In vivo, lithium treatment has been reported to lead to the inhibition of Gsk3β, which is suggested to lead to increased COX-2 abundance and prostaglandin release (14; 28). COX-1 and COX-2 catalyze the production of prostaglandins (29) and the released prostaglandins activate prostaglandin receptors in a paracrine fashion, resulting in reduced AVP-induced water permeability in the collecting duct (15; 16; 30; 31).

To test whether a similar response to lithium is seen in mpkCCD cells, these cells were grown for 8 days, the last 4 days in the presence of dDAVP to induce AQP2 expression, and treated with lithium for the last 48 hrs. As in patients on lithium therapy, the concentration of lithium in serum is around 1 mM and around 10 mM in urine (9), cells were treated with 10 mM at the apical and 1 mM at the basolateral side. While AQP2 levels were decreased with lithium, Gsk3β activity was reduced as shown by the increased Ser9-phosphorylation of Gsk3β, while the total amount of Gsk3β was unchanged (Fig. 1A). Zinc also inhibits Gsk3β activity (32). Therefore we incubated mpkCCD cells with zinc to determine whether inhibition of Gsk3β by another metal ion also affects AQP2 abundance. 20 μM zinc reduced AQP2 protein levels and increased phosphorylation of Gsk3β in mpkCCD cells, again without

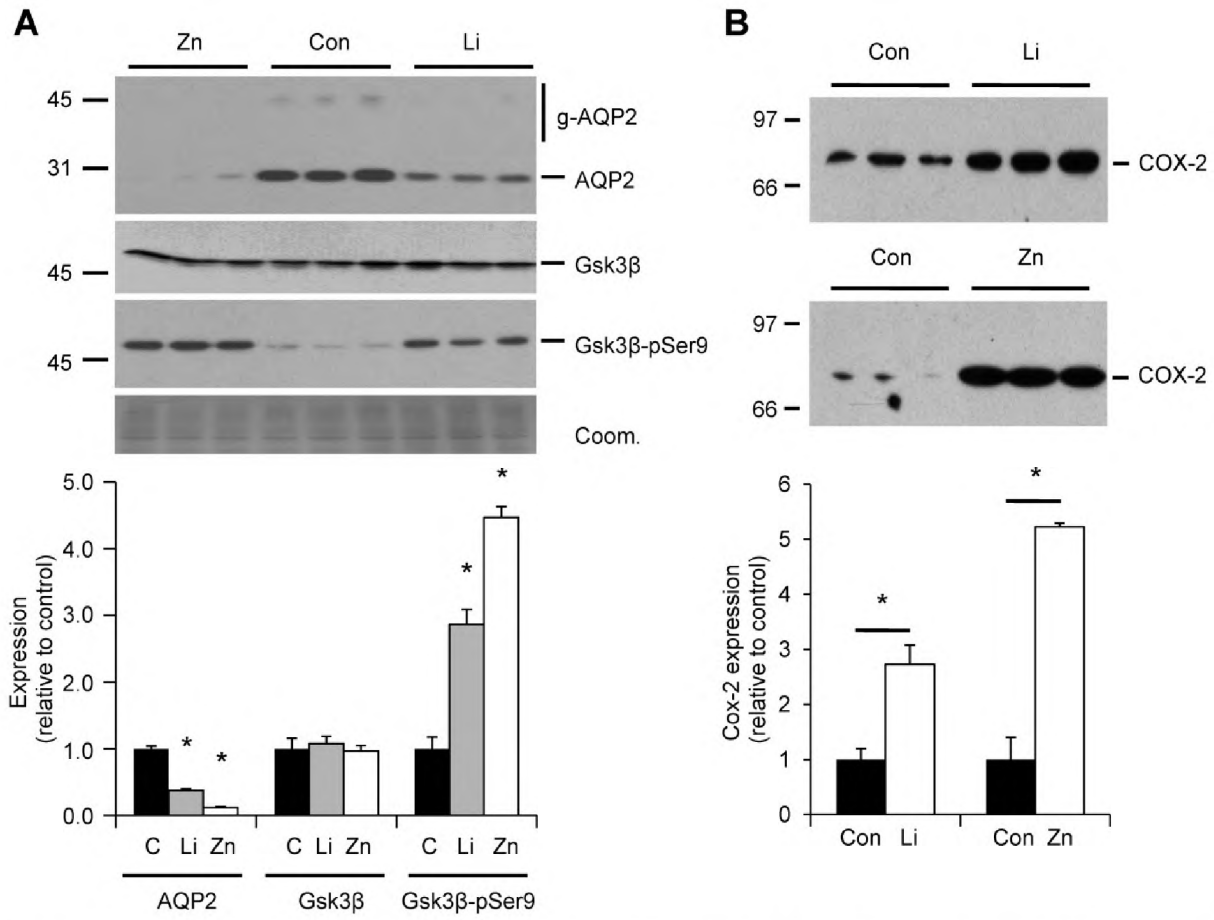

Figure 1. Effect of lithium on Gsk3 and COX-2 expression. A) MpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days. For the last 48 hrs the cells were treated with 20 μM zinc at both sides or 10 mM lithium at the apical side with 1 mM at the basolateral side. Cells were lysed and subjected to immunoblotting for AQP2, total Gsk3β and Ser9-phosphorylated Gsk3β. **B)** Samples as in A were subjected to COX-2 immunoblotting. Molecular masses (in kDa) are indicated on the left. The signals were densitometrically quantified. Mean values of normalized expression per condition are relative to control (+/- SEM). Significant differences ($p<0.05$) from control are indicated by an asterisk.

changing total Gsk3β expression levels (Fig. 1A). Both lithium and zinc significantly increased COX-2 abundance (Fig. 1B).

Effect of COX-2 on AQP2 expression in mpkCCD cells

Dexamethasone decreases COX-2 mRNA and protein abundance in various cell types (33; 34). Because previous studies have shown that prostaglandins inhibit AVP-induced water reabsorption *in vivo* (15; 16), we investigated whether dexamethasone-decreased COX-2 expression inversely correlates with AQP2 abundance in mpkCCD cells. Incubation with 1 μM dexamethasone caused a significant down-regulation of COX-2 and increase of AQP2 abundance (Fig. 2A). However, dexamethasone did not affect lithium-induced upregulation of COX-2 nor down-regulation of AQP2 (Fig. 2A).

To test if indomethacin, which inhibits both COX-1 and COX-2 activity, increases AQP2 abundance, cells were grown as described above, the last 48 hrs in the presence of 10 μM indomethacin. Even though COX-2 abundance was increased, immunoblotting revealed

increased AQP2-abundance with indomethacin (Fig. 2B), underscoring the correlation between COX activity, prostaglandin release and AQP2 abundance. Interestingly, even in the presence of indomethacin, lithium decreased AQP2 abundance and increased abundance of COX-2 (Fig. 2B), suggesting that the effect of lithium on AQP2 abundance may not be solely generated through prostaglandins, or that indomethacin did not completely block the effect of lithium on prostaglandin production.

Involvement of prostaglandins and their receptors in lithium-induced down-regulation of AQP2 in mpkCCD cells

COX-1 and 2 catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2). PGH_2 serves as a substrate for several prostaglandin and thromboxane synthases, which generate various prostaglandins and thromboxanes. These newly generated products bind their respective G-protein coupled receptors from the extracellular side, and activate their respective intracellular signaling pathways (Fig. 3A). Since lithium treatment increased COX-2 abundance, we questioned which prostaglandin could mediate the lithium-induced down-regulation of AQP2.

The prostaglandin receptors EP1, EP4, FP and TP could be detected by RT-PCR in mpkCCD cells, while results for the IP receptor were inconclusive. The same receptors were found after lithium treatment (data not shown). The release of the agonists of these receptors was investigated in cells treated with or without lithium, to determine the effect of lithium on prostanoid production. MpkCCD cells were grown as above, and incubated with lithium for the last 24 hours. The medium was collected and the amounts of prostaglandins were determined. Prostaglandin concentrations from fresh medium (before addition to cells) were subtracted. In dDAVP-treated cells, the amounts of PGE_2 and $PGF_{2\alpha}$ produced were clearly above the detection limit, while levels of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and TxB_2 (a stable metabolite of TxA_2) were low and close to or similar to the levels detected in fresh medium (Fig. 3B). There was no significant difference in the concentration of prostaglandins released into the apical or basolateral compartment (not shown). The concentrations of PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and TxB_2 were not significantly changed with lithium.

As co-incubation with indomethacin increased AQP2 abundance compared to lithium alone (Fig. 2B), we also analyzed prostaglandin levels from cells treated with lithium and indomethacin. Compared to lithium-treated cells, indomethacin significantly decreased PGE_2 and $PGF_{2\alpha}$ levels (Fig. 3B), suggesting that a reduction of these prostaglandins is involved in the attenuating effect of indomethacin on lithium-induced AQP2 down-regulation.

As lithium did not affect prostanoid production in mpkCCD cells, we investigated if a change in prostanoid receptor expression could explain the AQP2 down-regulation. The effect of

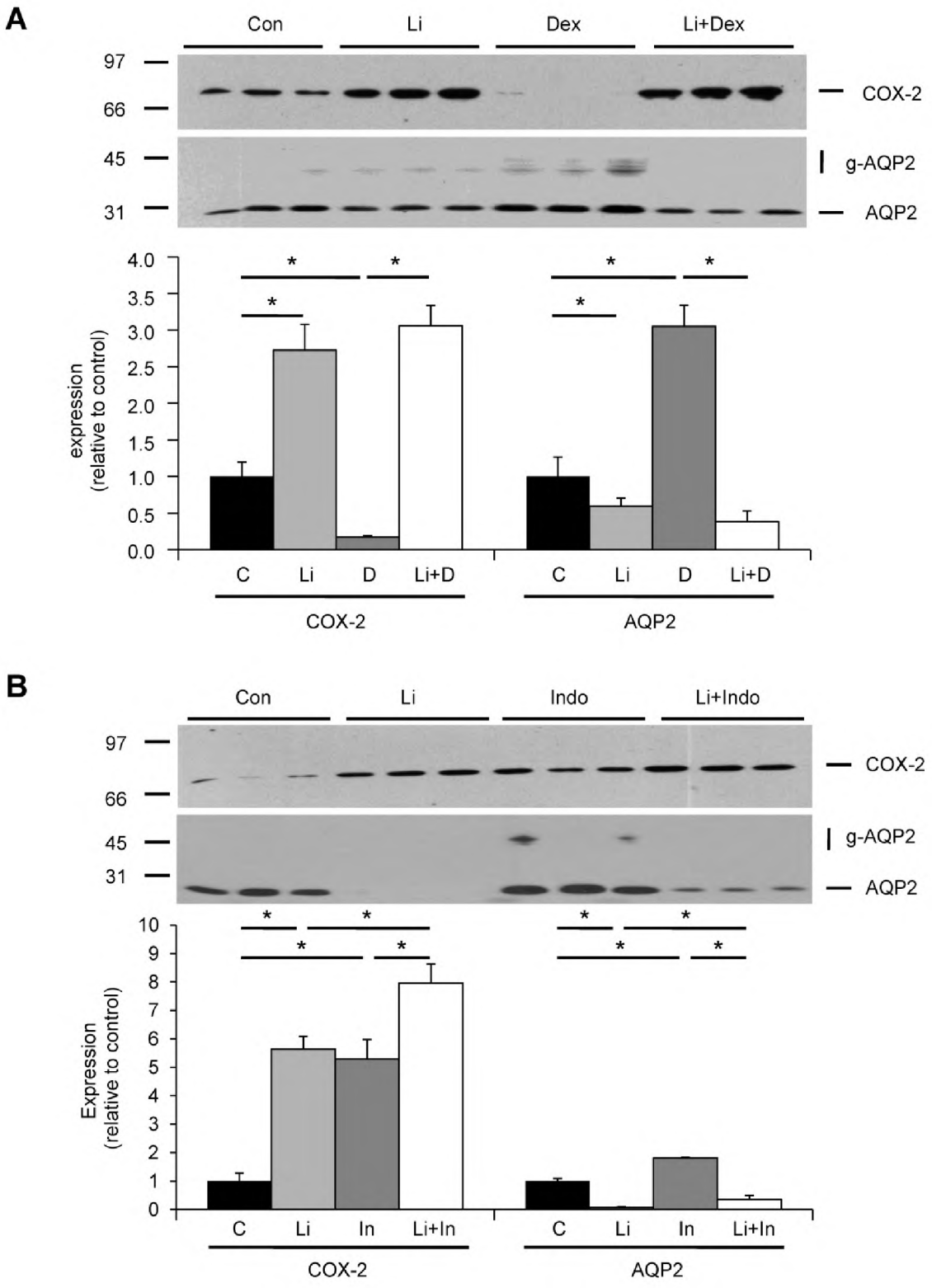

Figure 2. Effect of COX-2 on AQP2 expression. A) MpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days. For the last 48 hrs the cells were treated with 1 μM dexamethasone with or without lithium. **B)** MpkCCD cells were grown as in A. For the last 48 hrs the cells were treated with 10 μM indomethacin with or without lithium. For A-B: Cells were lysed and subjected to immunoblotting for AQP2 and COX-2. C=control, Li=lithium, D=dexamethasone, In=indomethacin.

lithium on the relative expression of the different prostanoid receptors was analyzed by Q-PCR and normalized for the amount of ribosomal 18S. As shown in figure 3C, the expression of FP and EP1 receptors was significantly decreased with lithium, while the expression of the TP or EP4 receptors was unchanged. As signalling via the FP and EP1 receptors is expected to inhibit water reabsorption (31; 35), a down-regulation of these receptors cannot explain the decrease in AQP2 expression conferred by lithium.

Effect of lithium on AQP2 transcription

Previously, we showed that lithium decreases AQP2 mRNA levels (20), suggesting an effect of lithium on AQP2 transcription and/or mRNA stability. Gene transcription results in the formation of a pre-mRNA transcript consisting of introns and exons, which is spliced to mRNA within minutes. While pre-mRNA levels are mainly determined by transcriptional rates, mRNA levels are regulated by both transcription/splicing and degradation (36). Therefore, when lithium affects transcription only, the AQP2 mRNA / pre-mRNA ratio should not decrease, but if lithium has an effect on mRNA stability, the relative amount of AQP2 mRNA would be decreased compared to AQP2 pre-mRNA levels. So, to investigate this, Q-RT-PCR was performed on mpkCCD cDNA using primers amplifying AQP2 mRNA (exon 2 and 3 primer) and pre-mRNA (intron 2 and exon 3 primer). Following 4 hours of lithium treatment, AQP2 pre-mRNA was decreased to 40% of control levels, whereas a non-significant decrease in AQP2 mRNA levels was observed (Fig 4A). After 6 hours, both AQP2 mRNA and pre-mRNA were decreased to 30-40%, which was sustained until at least 24 hours. Together, these data reveal that lithium reduces AQP2 mRNA levels by reducing AQP2 gene transcription and not by reducing AQP2 mRNA stability.

If lithium affects AQP2 transcription, one would also expect that the production of an AQP2 irrelevant transcript driven by the AQP2 promoter would show reduced expression after lithium treatment. To test this hypothesis, mpkCCD cells were stably transfected with pGL3-AQP2-3.0-luc, a construct containing luciferase cDNA under control of a 3 kb fragment of the mouse AQP2 promoter, generating mpkCCD-AQP2-luc cells. Moreover, mpkCCD-luc cells were generated as a negative control, by stably transfecting the luciferase construct lacking the AQP2 promoter (pGL3-luc). Pooled colonies were seeded on filters and grown with or without dDAVP for the last 4 days and treated with or without lithium during the last 48 hours. Basal luciferase activity was around 100 times lower in mpkCCD-luc cells compared to mpkCCD-AQP2-luc cells (Fig. 4B). Moreover, dDAVP application significantly increased luciferase activity in mpkCCD-AQP2-luc, but not mpkCCD-luc cells, revealing that dDAVP efficiently stimulates transcription from the 3.0 kb AQP2 promoter. Importantly, lithium significantly decreased the dDAVP-induced luciferase activity in mpkCCD-AQP2-luc cells to about 60%, which underscores our data above that lithium reduces AQP2 gene transcription.

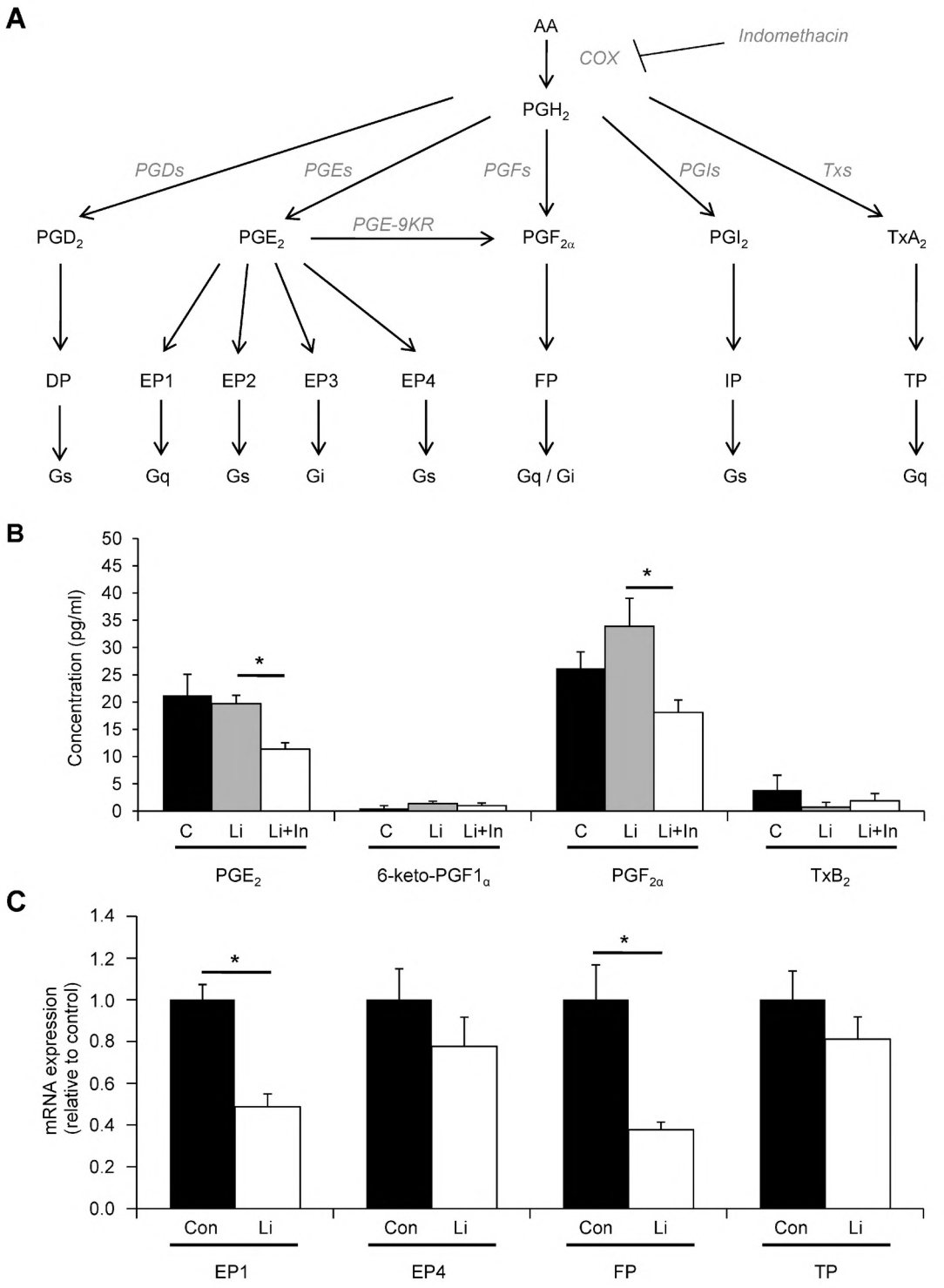

Figure 3. Effect of lithium on prostaglandin production in mpkCCD cells A) COX converts arachidonic acid to PGH_2. PGH_2 is subsequently metabolized to five major prostaglandins through their respective synthases. These prostaglandins act on their specific receptors: PGD_2 receptor (DP), PGE_2 receptor (EP) 1-4, $PGF_{2\alpha}$ receptor (FP), PGI_2 receptor (IP) and the thromboxane A_2 receptor (TP). **B)** mpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days. For the last 24 hrs the cells were treated with 10 mM lithium with or without 10 µM indomethacin. Medium was collected and prostaglandin concentrations were determined. **C)** mpkCCD cells were grown as in A, the last 24 hours with or without lithium. By quantitative RT-PCR the relative expression of the prostaglandin receptors was analyzed. B-C: Bars are mean values of at least three samples (+/- SEM). Significant differences ($p<0.05$) from control are indicated by an asterisk. C=control, Li=lithium, In=indomethacin.

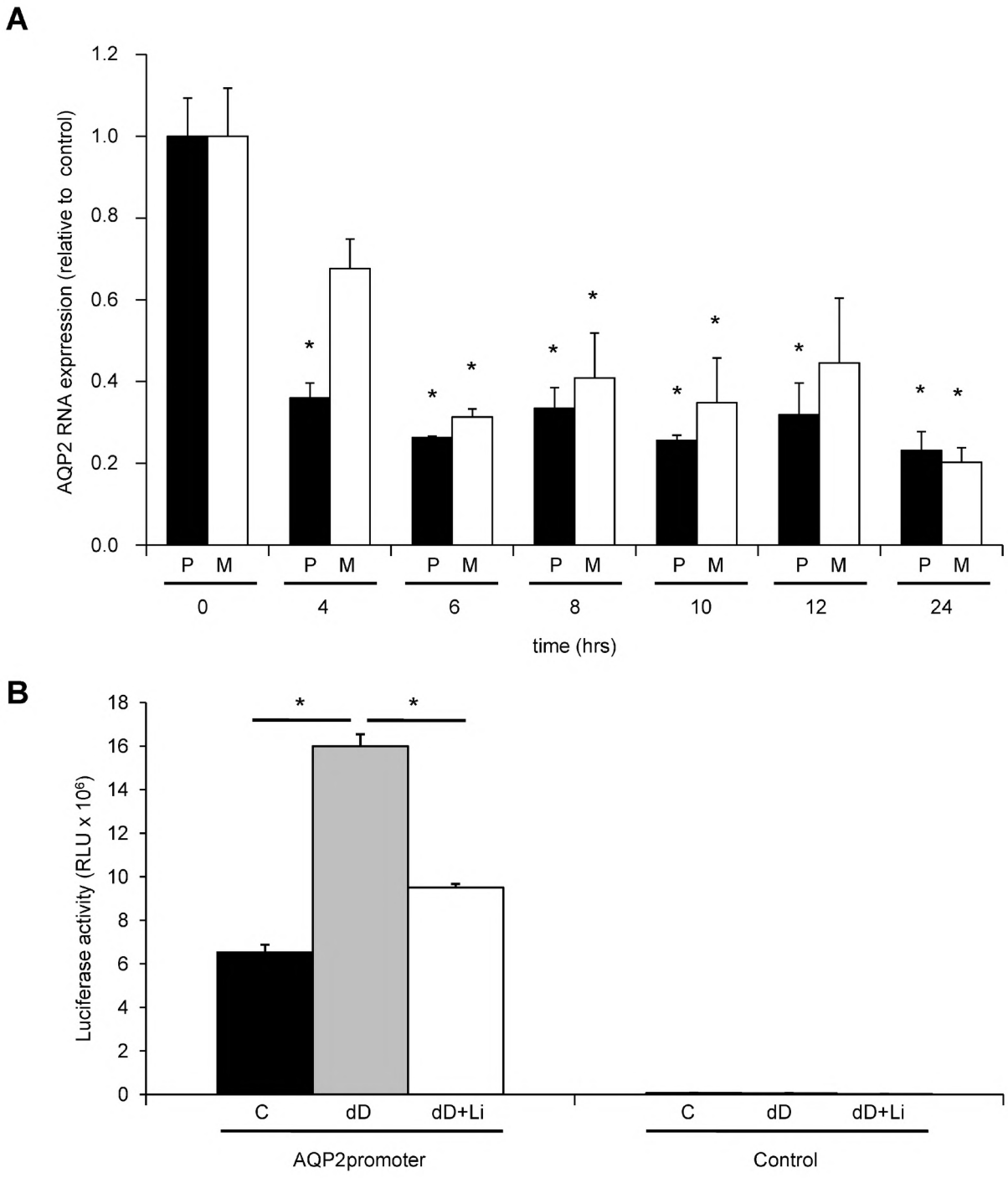

Figure 4. Effect of lithium on AQP2 transcription A) MpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days and with 10 mM lithium for various time periods. RNA was isolated and Q-PCR was performed, using exon-primers, detecting the AQP2 mRNA (M), and an intron and exon primer pair, to detect the unspliced pre-mRNA (P). The signals obtained from the house-keeping gene β-actin, were used to normalize for difference in the amount of starting cDNA. Mean values are relative to control (+/- SEM). **B)** MpkCCD cells containing a 3.0 kb AQP2-promoter-luciferase construct or a control construct without promoter were grown to confluence and treated with 1 nM dDAVP for 4 days, with or without lithium during the last 24 hrs. C=control, Li=lithium, dD=dDAVP.

Effect of prostaglandins on AQP2 transcription

As lithium reduces AQP2 transcripton of the 3 kb fragment of the promoter, this establishes a model system to further study the transcriptional mechanisms leading to AQP2 down-regulation by lithium. To test whether lithium-induced reductions in AQP2 transcription result from the release of prostaglandins, mpkCCD-AQP2-luc cells were grown without or with dDAVP for 4 days, and incubated for the last 48 hours with or without indomethacin, PGE_2 or $PGF_{2\alpha}$. dDAVP application again significantly increased luciferase activity (Fig. 5A). Indomethacin, however, did not further increase the luciferase activity, and PGE_2 or $PGF_{2\alpha}$ application did not reduce luciferase activity, which suggests that prostaglandins do not affect AQP2 transcription. In addition to these prostaglandins, incubation with PGD_2, the PGI_2 analogue carbacyclin, or the thromboxane analogue U4661 did not reduce luciferase activity (data not shown).

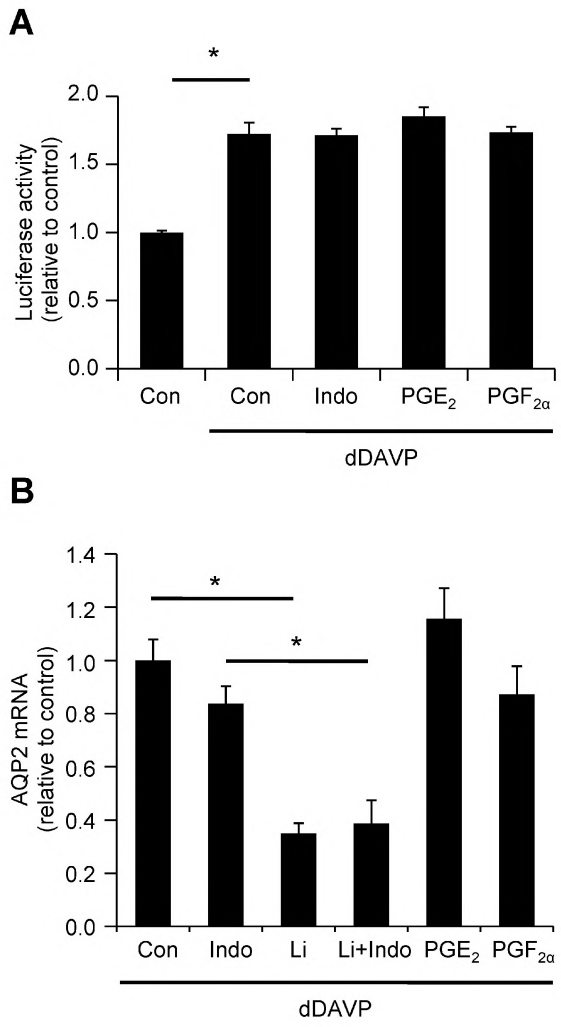

Figure 5. Effect of prostaglandins on AQP2 transcription A) MpkCCD cells containing a 3.0 kb AQP2-promoter-luciferase construct were grown to confluence and treated with or without 1 nM dDAVP for 4 days, with or without indomethacin, PGE_2 or $PGF_{2\alpha}$ during the last 48 hrs. Cells were lysed and light emission was measured. **B)** MpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days and with lithium, indomethacin, PGE_2 or $PGF_{2\alpha}$ during the last 48 hrs. RNA was isolated and Q-PCR was performed, using AQP2 mRNA primers. The signals obtained from the 18S were used to normalize for difference in the amount of starting cDNA. A-B: Data are the mean of three samples and relative to control (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

To establish whether the effect on luciferase expression mimics the effect on AQP2 mRNA levels, Q-RT-PCR assays were done. Consistent with the data above, AQP2 mRNA levels were not changed with PGE_2 or $PGF_{2\alpha}$ or indomethacin, while the lithium control showed a clear reduction in mRNA (Fig 5B).

The absence of any effect of prostaglandins on AQP2 mRNA levels indicated that they reduce AQP2 abundance by affecting AQP2 protein stability. In mpkCCD cells, chemical messengers like ATP and dopamine counteract vasopressin-induced AQP2 abundance by targeting it for lysosomal degradation, which can be blocked with the inhibitor chloroquine (37). Therefore, to test the effect of prostanoids on AQP2 stability

directly, cells were incubated with dDAVP for 4 days of which the last day with or without lithium or PGE_2, all in the presence or absence of the lysosome inhibitor chloroquine. Immunoblotting revealed that, in contrast to lithium, PGE_2 addition did not decrease AQP2 abundance in the presence of chloroquine (Fig. 6). Both lithium and PGE_2 reduced AQP2 abundance in the absence of chloroquine.

Together, these data reveal that prostaglandins reduce the dDAVP-induced AQP2 abundance solely by increasing lysosomal degradation of AQP2. This indicates that the effect of lithium on AQP2 transcription is mediated independently of prostaglandins.

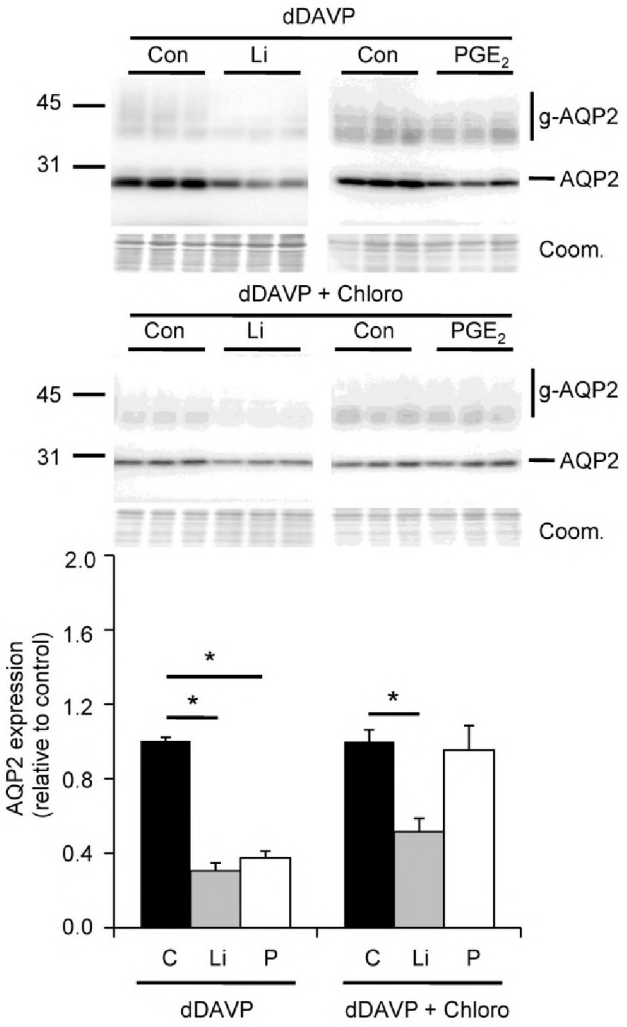

Figure 6. Effect of chloroquine on AQP2 abundance. mpkCCD cells were grown for 8 days and treated with 1 nM dDAVP for 4 days and with lithium or PGE_2 during the last 24 hrs, all with or without chloroquine. Cells were lysed and subjected to immunoblotting for AQP2. Molecular masses (in kDa) are indicated on the left. Data are the mean of three samples and relative to control (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk. C=control, Li=lithium, P= PGE_2.

Mapping of the AQP2 promoter segments involved in the inhibitory effect of lithium on AQP2 transcription

To further investigate how lithium affects AQP2 transcription, sites of interest were determined. It is well-known that the CRE, which localizes at -210 in the mouse AQP2 promoter, is essential for dDAVP-induced AQP2 transcription (4; 38). Moreover, Gsk3 has been shown to phosphorylate CREB at Ser129, increasing the transcriptional response to cAMP in PC12 and F9 cells (39). To directly analyse the effect of lithium on the CRE element in mpkCCD cells, pooled colonies were made of mpkCCD cells stably transfected with pGl3-CRE(21), a luciferase reporter construct, driven by a promoter consisting of 21 CREs in a row. dDAVP application resulted in a significant increase in CRE-mediated luciferase activity in mpkCCD cells, consistent with an increase in cAMP and CREB activity (Fig. 7). Co-incubation with lithium, however, did not affect this dDAVP-induced increase. As endogenous AQP2 abundance was reduced by lithium (not shown),

these data suggest that lithium reduces AQP2 transcription independently from the CRE pathway.

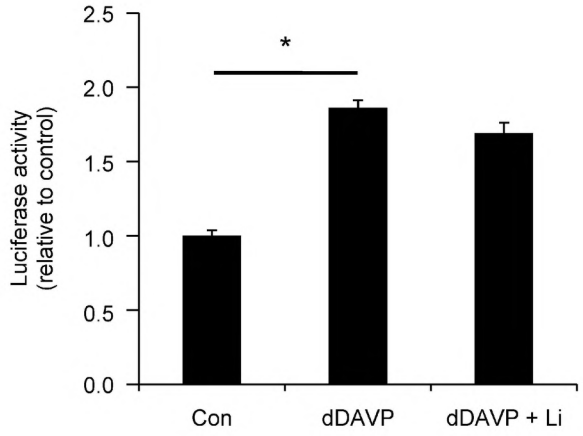

Figure 7. CRE-dependence of lithium-induced AQP2 down-regulation. MpkCCD cells containing a CRE-luciferase construct were treated with or without 1 nM dDAVP or 10 mM lithium for the last 24 hours. The cells were lysed and light emission was measured. Data are the mean of three samples (+/- SEM). Significant differences ($p<0.05$) are indicated by an asterisk.

To identify the segment(s) in the AQP2 promoter that are responsible for the reduced AQP2 transcription with lithium, mpkCCD cells were stably transfected with luciferase reporter constructs with AQP2 promoter segments ranging from 3.0 till 0.2 kb upstream from the transcription start site. dDAVP treatment resulted in a significant increase in luciferase activity in all cells, except cells transfected with a 0.2 kb promoter, which lacks the CRE (Fig. 8). Interestingly, lithium decreased the basal luciferase activity to about 70% of control in all constructs, and, in cells responsive to dDAVP stimulation, reduced dDAVP-stimulated activity to unstimulated levels (40 – 50% reduction). In cells transfected with the 0.2 kb promoter segment, dDAVP reduced AQP2 promoter-driven transcription by nearly 60%, which was further reduced to 70% with additional lithium. These results indicated that the putative lithium-sensitive promoter segments are within the most proximal 200 bp.

Discussion

mpkCCD cells as a model system to study lithium-induced regulation of AQP2 expression

Our data reveal that mpkCCD cells are a proper model to study lithium-induced changes in AQP2 expression. The 1 mM lithium used at the basolateral side in our experiments is similar to serum lithium levels found in patients, which are generally between 0.6 and 1.5 mM (9). From the cortex to the medulla of the collecting duct, lithium is concentrated as micropuncture studies in rats showed an increased lithium concentration in the early distal nephron and a more severe increase in the final urine (40). Also, in lithium-treated patients, lithium concentrations of 10-15 mM are found in their urine (41; 42). Collecting duct concentrations of lithium may thus vary between 1 mM in the cortex to 10-15 mM in the medulla. Based on these *in vivo* concentrations, we chose to use 10 mM lithium at the apical side in our experiments.

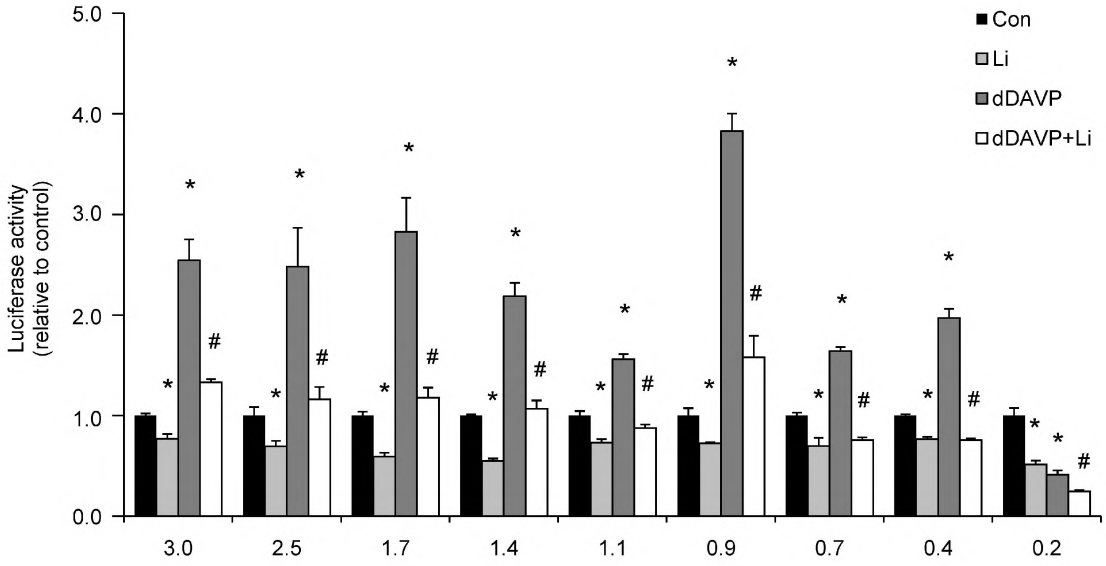

Figure 8. AQP2 promoter part involved in the lithium-response. MpkCCD cells containing an AQP2-promoter of various lengths (in kb) followed by luciferase cDNA were treated with 1 nM dDAVP for 4 days, with or without 10 mM lithium for the last 24 hours. After harvesting, luciferase activity was determined. Mean values of luciferase activity (+/- SEM) were determined from three independent filters per condition. Significant differences ($p<0.05$) from control are indicated by *, significant differences from dDAVP are indicated with #.

First, we show that incubation of the cells with lithium at these clinically relevant concentrations did lead to a down-regulation of AQP2. This is in agreement with our previous studies (20; 21) and consistent with AQP2 down-regulation observed *in vivo* in lithium-NDI rats (11; 12; 21). In this study, we further show that the AQP2 down-regulation coincides with inactivation of Gsk3β, in agreement with previous studies in lithium-NDI mice (14) and mpkCCD cells (21).

We furthermore show that Gsk3 inactivation coincides with an increased abundance of COX-2, which is consistent with previous *in vivo* effects of Gsk3 on COX-2 (28) as well as the observed increase in COX-2 abundance observed in lithium-NDI mice (14). In addition, we show that changes in COX-2 expression or activity influence AQP2 abundance in mpkCCD cells, as shown by the administration of both dexamethasone, leading to specific down-regulation of COX-2 (43), or indomethacin, blocking both COX-1 and COX-2 (44). Similarly, treatment with a COX-2 inhibitor increases AQP2 abundance in rats with lithium-induced NDI or bilateral ureteral obstruction (17; 45).

Although COX-2 is highly expressed in renal interstitial cells (14; 46) it is at present controversial whether COX-2 is also expressed in collecting duct cells. While immunohistochemistry reveals strong COX-2 expression in the collecting duct in three studies (47-49), absence of it was suggested in three other studies (50-52). Using collecting ducts isolated from AQP2-GFP transgenic mice, Ye *et al.* recently showed a 10 times lower expression of COX-2 mRNA in collecting duct cells compared to kidney cells not originating

from collecting ducts (53), suggesting constitutive COX-2 expression is low. Increased COX-2 levels, however, have been detected in collecting ducts of animals that were dehydrated or subjected to a chronic NaCl load (47; 49) indicating that COX-2 expression in collecting duct cells is increased under stress conditions. This suggests that COX-2 expression may also by induced in collecting duct cells during lithium treatment. Besides COX-2, COX-1 is highly expressed in the collecting duct (46).

The major prostaglandins produced in mpkCCD cells were PGE_2 and $PGF_{2\alpha}$. In line with PGE_2 and $PGF_{2\alpha}$ being produced in mpkCCD cells, PGE_2 and $PGF_{2\alpha}$ have been shown to be mainly produced in the collecting ducts, being highest in the medulla (54).

Lithium treatment does not increase prostaglandin production in mpkCCD cells

Since the abundance of COX-2 increased after treatment with lithium, we investigated which of the COX-derived compounds could mediate the lithium-induced down-regulation of AQP2. However, the lithium-induced increase in COX-2 abundance did not result in elevated prostaglandin levels in media of mpkCCD cells. This absence of increase in prostaglandins may be due to a lack of free arachidonic acid, which is rate-limiting in the production of prostaglandins (55-58).

Blocking COX by indomethacin increased AQP2 abundance in cells treated with or without lithium, suggesting that also in cells without lithium, AQP2 abundance is decreased by the action of endogenously produced prostaglandins. Indomethacin significantly reduced released PGE_2 and $PGF_{2\alpha}$ levels, but did not affect 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) or TxB_2 (a stable metabolite of TxA_2) levels, suggesting that a reduction of PGE_2 and/or $PGF_{2\alpha}$ is involved in the attenuating effect of indomethacin on lithium-induced AQP2 down-regulation.

However, as no effect of lithium on prostaglandin production was found in mpkCCD cells, this suggested that the effect of lithium on AQP2 expression in these cells occurs independently of prostaglandins. This is also suggested by the observation that, although indomethacin blocked COX activity leading to reduced prostaglandin production, it did not prevent the lithium-induced down-regulation of AQP2.

Dexamethasone reduces COX-2 abundance by decreasing COX-2 mRNA stability by inhibiting p38 (33). Interestingly, lithium did completely block the effect of dexamethasone on COX-2 expression, which suggests cross-talk between the p38 pathway and the Gsk3β-NFκB mediated effect on COX-2 transcription, or a direct effect of lithium on p38 activity.

The absence of a lithium-induced increase in PGE_2 production in our cells illuminates a difference from *in vivo*, because *in vivo* lithium leads to a large increase in PGE_2 production (14). This difference is likely due to the fact that *in vivo*, the lithium-induced increase in

prostaglandins is thought to be derived from the interstitial instead of principal cells (14). In line with the beneficial effects of COX-2 inhibition on AQP2 abundance in lithium-NDI rats (17), however, addition of PGE_2 to mpkCCD cells leads to AQP2 down-regulation.

Lithium decreases expression of the EP1 and FP receptors in mpkCCD cells

Expression of EP1, EP4, FP, IP and TP receptors was detected in mpkCCD cells, while no expression was seen of the DP, EP2 and EP3 receptors. Of the prostaglandin receptors found to be expressed the EP1, FP and TP receptors couple to pathways counteracting the vasopressin-induced Gs-cAMP pathway. TP receptors are unlikely to be involved in AQP2 down-regulation under normal circumstances, as TxA_2 levels produced are low and indomethacin, leading to increased AQP2 abundance, does not reduce TxA_2 production.
Application of lithium decreased the expression of FP and EP1 receptors. However, as signalling via these receptors is expected to inhibit water reabsorption (31; 35), a down-regulation of these receptors cannot explain the decrease in AQP2 expression conferred by lithium, and may instead be a compensatory mechanism.

In agreement with the expression in mpkCCD cells, collecting duct cells express EP1, EP4, FP and TP receptors and lack DP and EP2 receptors (35; 59-61). While expression of the IP receptor in mpkCCD cells is inconclusive, also this receptor is found in the collecting duct (62). In contrast to the mpkCCD cells, however, the EP3 receptor, which couples to the AVP-counteracting Gi pathway, is also expressed in collecting ducts, and may thus, besides EP1 and FP receptors, be involved in prostaglandin-dependent down-regulation of AQP2 *in vivo*. Conclusive evidence for the roles of particular prostaglandin receptors in mediating a prostaglandin-induced down-regulation of AQP2 in lithium-induced NDI awaits studies using collecting duct specific knockout of these receptors.

Lithium decreases AQP2 transcription independently of prostaglandins

PGE_2 application to mpkCCD cells reduced AQP2 abundance, in line with the inhibitory effect of PGE_2 on AVP-stimulated water absorption in the collecting duct (15; 16). This prostaglandin-induced AQP2 down-regulation could be prevented by co-incubation with the lysosome inhibitor chloroquine, showing that the effect of prostaglandins on AQP2 abundance is mediated by increasing AQP2 degradation. Interestingly, and in contrast to lithium, addition of PGE_2 or $PGF_{2\alpha}$ did not decrease AQP2 transcription or mRNA abundance. In line with this, indomethacin, blocking prostaglandin production, increased dDAVP-stimulated AQP2 protein abundance, but did not increase AQP2 transcription or mRNA levels. In agreement with previous results (20), we here found that lithium does not affect AQP2 degradation, but decreases AQP2 mRNA by decreasing AQP2 gene

transcription. Our results show that that the lithium-sensitive AQP2 promoter segment is within the most proximal 200 bp.Together, these data indicate that *in vivo* the reduction of AQP2 abundance by lithium consists of at least two parts, being an increased AQP2 degradation due to increased prostaglandin levels and a reduction of AQP2 gene transcription due to a prostaglandin-independent effect of lithium.

A limitation of our cell model is the absence of the EP3 receptor, which is found *in vivo* in the collecting duct. EP3 activation inhibits cAMP generation via Gi (35), and might therefore inhibit AVP-stimulated AQP2 transcription by decreasing CRE-mediated transcription. Although this might contribute to the AQP2 down-regulation, earlier studies show no decrease in cAMP after lithium treatment in *vivo* (20), suggesting that an *in vivo* effect of lithium on AQP2 transcription is EP3-independent.

Model of lithium-induced NDI

Based on the present and earlier data, we propose the following model (Fig. 9): lithium leads to AQP2 down-regulation in the principal cells, and, on the long term, to an increase in apoptosis as well as proliferation, resulting in a decreased fraction of principal cells (63-65).

Lithium reduces AQP2 abundance through two mechanisms: at first, lithium enters renal medullary interstitial cells and, possibly, renal principal cells resulting in increased production of PGE_2 (14; 66). Lithium entry in principal cells occurs through the epithelial sodium channel ENaC (21; 67), but the entry pathway of interstitial cells is unknown. In either cell type, this leads to inactivation of Gsk3β, which increases the abundance of COX-2 (14; 28) and release of prostaglandins. The increase in COX-2 expression has been reported to occur in the interstium *in vivo* (14), suggesting that the increased urinary PGE_2 excretion is predominantly due to the medullary interstitial cells. The released prostaglandins are detected by prostaglandin receptors on principal cells leading to lysosomal degradation of AQP2 and a decline in urine concentrating ability.

Secondly, lithium decreases AQP2 abundance by reducing AQP2 gene transcription. The ENaC-mediated entry of lithium in principal cells leads to inactivation of Gsk3β (14; 21). In line with an important role of Gsk3β in AQP2 expression, other Gsk3β inhibitors also lead to a down-regulation of AQP2 expression in mpkCCD cells (21). The effect on transcription is prostaglandin independent, and is likely a consequence of inhibition of one of multiple pathways steered by Gsk3β, like signaling involving the transcription factors Nuclear factor of activated T-cells (NFAT), β-catenin, or hypoxia-inducible factor (HIF). The effect of lithium on AQP2 transcription is independent of cAMP levels or CRE, and involves the most proximal 200 bp of the AQP2 promoter. This decreased AQP2 transcription will result in a further decline of the AQP2 abundance and of the urine concentrating ability.

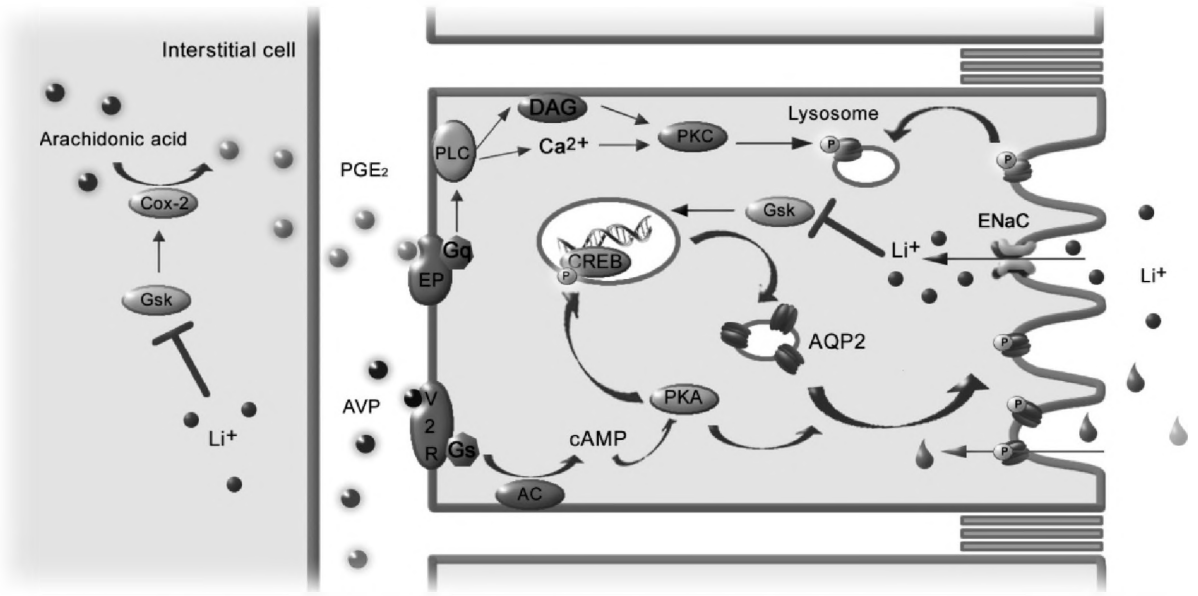

Figure 9. Model of the lithium-induced inhibition of AQP2-mediated water reabsorption. Indicated are adenylate cyclase *(AC)*, aquaporin-2 (AQP2), vasopressin *(AVP)*, cyclic adenosine monophosphate *(cAMP)*, cyclooxygenase-2 *(COX-2)*, cAMP responsive element binding protein *(CREB)*, Diacylglycerol *(DAG)*, epithelial sodium channel *(ENAC)*, prostaglandin E_2 (*PGE_2*), protein kinase A *(PKA)*, protein kinase C *(PKC)*, phospholipase C *(PLC)*, prostaglandin receptor *(PR)*, vasopressin V2 receptor *(V2R)*. For details, see text.

In conclusion, our data show that in mpkCCD cells, lithium decreases AQP2 protein abundance as well as AQP2 gene transcription. Lithium decreases AQP2 transcription independently of the endogenous prostaglandin production in mpkCCD cells. Our data furthermore show that in mpkCCD cells, released prostaglandins decrease AQP2 protein stability by increasing its lysosomal degradation. Based on these observations, *in vivo* paracrine produced prostaglandins might have an additional role in lithium-induced NDI by decreasing AQP2 protein stability, without affecting AQP2 transcription. Such an effect would currently be difficult to adequately separate from the 'direct' effect of lithium on AQP2 transcription and abundance in the intact animal. The two identified pathways leading to a decrease in AQP2 abundance might also have consequences for the clinical treatment of lithium-NDI. Both COX inhibitors and the ENaC blocker amiloride have been shown to individually reduce the NDI phenotype (18; 19; 68-70). However, since the effect of lithium on prostaglandin production in interstitial cells could be blocked by COX inhibitors, and the entry of lithium into the principal cells could be blocked by amiloride treatment, a beneficial effect of combining these two therapies for the treatment of lithium-NDI could potentially improve the treatment of lithium-NDI in the clinic. Future studies in humans addressing such therapeutical approaches are needed to definitively clarify this matter.

Acknowledgments

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CHAPTER 8

Amiloride attenuates lithium-induced nephrogenic diabetes insipidus by blocking cellular entry of lithium through the renal collecting duct epithelial sodium channel

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Abstract

Lithium therapy frequently induces Nephrogenic Diabetes Insipidus (NDI) which coincides with a decreased aquaporin-2 (AQP2) expression and principal/intercalated cell ratio. Amiloride reduces lithium-NDI in some clinical case studies, but the mechanism is poorly understood. As amiloride blocks the epithelial sodium channel (ENaC) located in the apical membrane of principal cells, we hypothesized that ENaC is the main entry site for lithium and that amiloride may be beneficial by blocking lithium entry. Indeed, in a mouse collecting duct cell line ($mCCD_{c11}$), the vasopressin-induced AQP2 expression was time- and dose-dependently reduced by clinically-relevant lithium concentrations, which could be prevented by co-incubation with amiloride or benzamil. Consistently, amiloride reduced transcellular lithium transport, the intracellular lithium concentration and lithium-induced inactivation of glycogen synthase kinase 3β. *In vivo*, simultaneous treatment of rats with amiloride attenuated the AQP2 down-regulation, reduction of principal/intercalated cell ratio and NDI phenotype compared to rats treated with lithium only.

In conclusion, we demonstrate *in vitro* and *in vivo* that ENaC is the major entry site for lithium in principal cells and that blocking lithium entry with amiloride attenuates lithium-NDI by preventing AQP2 down-regulation and the change in principal/intercalated cell ratio. As such, our data provide a rationale for the use of amiloride as a treatment for lithium-NDI.

Introduction

Lithium is regularly used to treat psychiatric diseases, such as bipolar disorders, schizoaffective disorders and depression. Lithium is prescribed to 0.1% of the population (1). Approximately 20% of patients develop nephrogenic diabetes insipidus (NDI), a disorder characterized by polyuria and polydipsia due to renal insensitivity to the antidiuretic hormone arginine vasopressin (AVP) (2). Therewith, lithium-NDI is the most common form of NDI. Lithium-NDI patients are at risk for dehydration-induced lithium toxicity, and prolonged lithium treatment might lead to end stage renal disease (1). However, since the symptoms of the underlying psychiatric disorder have a high impact on the quality of life, cessation of lithium therapy is not an option for most patients.

The kidney is the main organ for regulating water homeostasis. In states of hypernatremia or hypovolemia, AVP is released from the pituitary gland. Binding of AVP to the vasopressin type-2 receptor in the basolateral membrane of renal collecting duct principal cells results in the redistribution of aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical membrane. Driven by an osmotic gradient, water will enter principal cells through

AQP2 and will exit through AQP3 or AQP4 in the basolateral membrane, resulting in concentrated urine. Besides this short-term regulation, AVP also exerts a long-term regulation by increasing AQP2 expression (3).

From studies in rats, it became clear that lithium-NDI develops in two stages. At the short term (10 days), lithium-NDI coincides with AQP2 down-regulation and natriuresis, without gross changes in renal morphology (4-6). Chronic lithium treatment (4 weeks), however, also leads to a severe decrease in the fraction of principal cells. This is 'compensated' by an increase in the fraction of intercalated cells, which are involved in acid/base balance regulation (7; 8).

At present, it is unclear how lithium causes NDI and whether lithium-NDI can be attenuated. Some data, however, suggest that lithium may exert its effects by entering principal cells through the epithelial sodium channel, ENaC. First, NDI is due to an impaired water reabsorption in the connecting tubules and collecting ducts principal cells (9), where ENaC is expressed (10). Secondly, ENaC has a higher permeability for lithium than for sodium (11). Thirdly, lithium inhibits amiloride-sensitive sodium reabsorption in the toad urinary bladder and rat collecting duct, tissues known to express ENaC (12-14), and the ENaC-blocker triamterene increases lithium excretion (15). Moreover, it was shown in a limited number of lithium-NDI patients that blocking ENaC with amiloride significantly reduces urine volume and increases urine osmolality (16-18). The decreased urine volume is classically explained by an amiloride-induced hypovolemia followed by an increased proximal sodium and water retention. The increased urine osmolality was suggested to be caused by an amiloride-induced reduction of sodium reabsorption (16; 17).

Based on the above, we hypothesized that lithium enters principal cells through ENaC and that blocking ENaC by amiloride would reduce cellular entry of lithium, AQP2 down-regulation, alterations in the cellular composition of the collecting duct and the lithium-NDI phenotype.

Methods

Cell Culture

Mouse $mCCD_{c11}$ and $mpkCCD_{cl4}$ cells were essentially grown as described (19; 20). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell®, 0.4 μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were incubated with 1 nM dDAVP to the basolateral side for the last 96 hrs, to induce AQP2 expression. Lithium and zinc chloride were administered as indicated. 10 μM of amiloride or benzamil, concentrations specifically blocking ENaC (21; 22), were administered

to the apical side, unless indicated otherwise. BIO-Acetoxime (Calbiochem) was used as indicated. Medium with reduced sodium concentrations was made identical to the standard medium except that lower amounts of NaCl were added.

Lithium assays

For determination of transcellular lithium transport, $mCCD_{c11}$ cells were grown on 1.13 cm^2 filters. After 24 hrs incubation with 10 mM lithium in the presence or absence of amiloride at the apical side, the basolateral and apical media were collected and the lithium concentrations were determined with a flame photometer (Eppendorf 6341, Hamburg, Germany).

Determination of intracellular lithium concentrations was essentially as described (23). Shortly, $mCCD_{c11}$ cells were grown on 4.7 cm^2 filters. To determine the extent of lithium contamination from the extracellular side, fluorescein isothiocyanate dextran (FITC-dextran) was added to the lithium-containing medium to a final concentration of 10 μM just before harvesting, after which the medium was mixed. Then, the filters were washed three times with isoosmotic sucrose (pH 7.3) at 4°C and cells were lysed by sonication in 1 ml Milli-Q water. Of 800 μl sample, the amount of lithium was determined by flame photometry, from which the total amount of lithium in the sample was calculated.

Of 100 μl sample, the amount of FITC-dextran was measured using spectrofluorophotometry (Shimadzu RF-5301, Japan) at 492 nM (excitation) and 518 nm (emission) wavelengths. By comparing the obtained values with a two-fold FITC-dextran dilution series, the FITC-dextran concentration in each sample was determined, from which the extent of extracellular Li^+ contamination was calculated and subtracted from the total amount to obtain the intracellular lithium amount. With the used FITC-dextran concentration, a contamination above 1:5000 would be detected. To correct for differences in cellular yield, the intracellular lithium amounts were normalized for the protein amount in each sample, which was determined using the Biorad Protein Assay (Munchen, Germany). The intracellular lithium concentration (in mM) was estimated by calculating the cellular water content, based on the assumption that 20% of the cell weight consists of cellular proteins and the remaining 80% is water (24).

Experimental animals

Male Wistar rats, 200-300 g, were obtained from the Animal Facility of the RUNMC. Rats were treated with lithium as described (5).

Control rats received normal rodent diet (ssniff® R/M-H V1534, ssniff Spezialdiaten GmbH, Germany. n=6). For lithium therapy, lithium chloride was added to the chow to give a concentration of 40 mmol/kg for the first week and 60 mmol/kg dry food for the next 3 weeks.

Rats were then sacrificed (n=6). For amiloride treatment, amiloride was added to the lithium chow to a concentration of 200 mg/kg dry food for the entire 4 weeks (n=7). All rats had free access to water, food, and a sodium-chloride block (7). For the last 48 hrs of the experiment, the rats were housed in metabolic cages in order to measure water intake and urine output during the last 24 hours. All animal experiments were approved by the Animal Experiments Committee of the RUNMC.

Tissue preparation

Rats were anaesthetized with isofluorothane after which their blood was removed by heart puncture. Then, the rats were killed by cervical dislocation and the kidneys rapidly removed. One kidney was fixed for immunohistochemistry by immersion in 1% (wt/vol) periodate-lysine-paraformaldehyde for 2 hrs and 15% (wt/vol) sucrose in PBS overnight, while of the other kidney, the inner medulla, outer medulla and cortex were dissected for immunoblotting as described (25).

Blood and urine analyses

Blood serum was prepared by 16 hr incubation at 4°C, followed by centrifugation at 600 g for 2-3 min. Urine was centrifuged at 4000 g for 5 min to remove sediment. Both serum and urine samples were analyzed for osmolality, sodium and lithium concentrations by standard procedures of the General Clinical Chemical laboratory of the RUNMC.

Immunoblotting

$mCCD_{c11}$ cells from 1.13 cm^2 filter were lysed in 200 μl Laemmli buffer and 15 μl samples were analyzed, while 5-10 μg of kidney material was analyzed. PAGE, blotting and blocking of the PVDF membranes were done as described (26). Membranes were incubated for 16 hrs with 1:3000-diluted affinity-purified rabbit AQP2 antibodies (27), 1:2000–diluted affinity-purified rabbit anti-v1 H-ATPase antibodies (gift from Dr. S. Nielsen, Denmark), 1:1000 diluted rabbit anti-Ser9-Gsk3β (Cell Signaling Technology), 1:5000 diluted mouse anti-Gsk3β (BD Transduction Laboratories) or 1:100,000 diluted mouse anti-tubulin antibodies (gift from Dr. Kreis, Switzerland), in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% non-fat dried milk. ENaC detection was done as described (19). Blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's or 1:2000 goat anti-mouse IgG's (Sigma, St. Louis, MO) as secondary antibodies coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Films were scanned using a Bio-Rad 690c densitometer and signals were analyzed using Bio-Rad software. Two-fold dilution series of the respective proteins were blotted in parallel to allow semi-quantification.

Equal loading of the samples was confirmed by parallel immunoblotting for tubulin or staining of the blots with coomassie blue.

Immunohistochemistry

Immunohistochemical staining was performed on 7-µm sections of fixed frozen kidney samples, following antigen retrieval. The sections were blocked in goat serum dilution buffer (GSDB: 16% goat serum, 0.3% triton X-100, 0.3M NaCl in PBS) for 30 min and incubated for 16 hours at 4°C with rabbit anti-a4 H-ATPase antibodies (1:3000, provided by Dr. F Karet, United Kingdom). Following washes and incubation with Alexa 488–conjugated goat anti-rabbit antibodies, the sections were incubated with 1:50 diluted affinity-purified guinea pig AQP2 antibodies (28) for 1 hr at room temperature and Alexa 594–conjugated goat anti-guinea pig secondary antibodies (Molecular Probes, Leiden, the Netherlands). TOTO®-3 iodide (Invitrogen, Carlsbad, CA, USA) was used for counterstaining. Images were made using Bio-Rad MRC-1024 confocal laser scanning microscopy.

Quantification of principal and intercalated cells was performed on the confocal images of the fluorescent kidney sections. Tubules in >5 randomly selected areas of each kidney region of each animal were included. Only cells with distinct TOTO-3 staining were counted. Cells stained with H-ATPase/AQP2 staining were taken as intercalated/principal cells, respectively.

Data Analysis

Differences between groups were tested by the Student's *t*-test corrected by the Bonferroni multiple-comparisons procedure. Differences were considered statistically significant for $p < 0.05$.

Results

$mCCD_{c11}$ cells as a model for lithium-NDI.

To study the role of ENaC in lithium-NDI *in vitro*, a cell line needs to show (1) expression of all three ENaC subunits, (2) amiloride-inhibitable transcellular sodium transport, (3) deamino-8 D-arginine vasopressin (dDAVP)-induced expression of AQP2, and (4) lithium-induced down-regulation of AQP2.

$MpkCCD_{cl4}$ cells show a lithium-induced AQP2 down-regulation (25) and an amiloride-inhibitable transcellular voltage difference. However, immunocytochemistry did not reveal ENaC expression in these cells (not shown), in contrast to the novel mouse cortical collecting duct $mCCD_{c11}$ cell line (19). Therefore, we tested $mCCD_{cl1}$ cells as a model. To test whether dDAVP induces AQP2 expression in these cells, confluent cells were incubated for 1-4 days

with 1 nM dDAVP. Immunoblotting revealed the typical non-glycosylated 29 and complex-glycosylated 40-45 kDa AQP2 bands, besides a non-specific band of 35 kDa (Fig. 1A). Similar to mpkCCD$_{c14}$ cells (25), maximal expression of AQP2 was seen after 72-96 hrs. To have steady AQP2 expression, cells were therefore incubated with dDAVP for 96 hours in the following experiments.

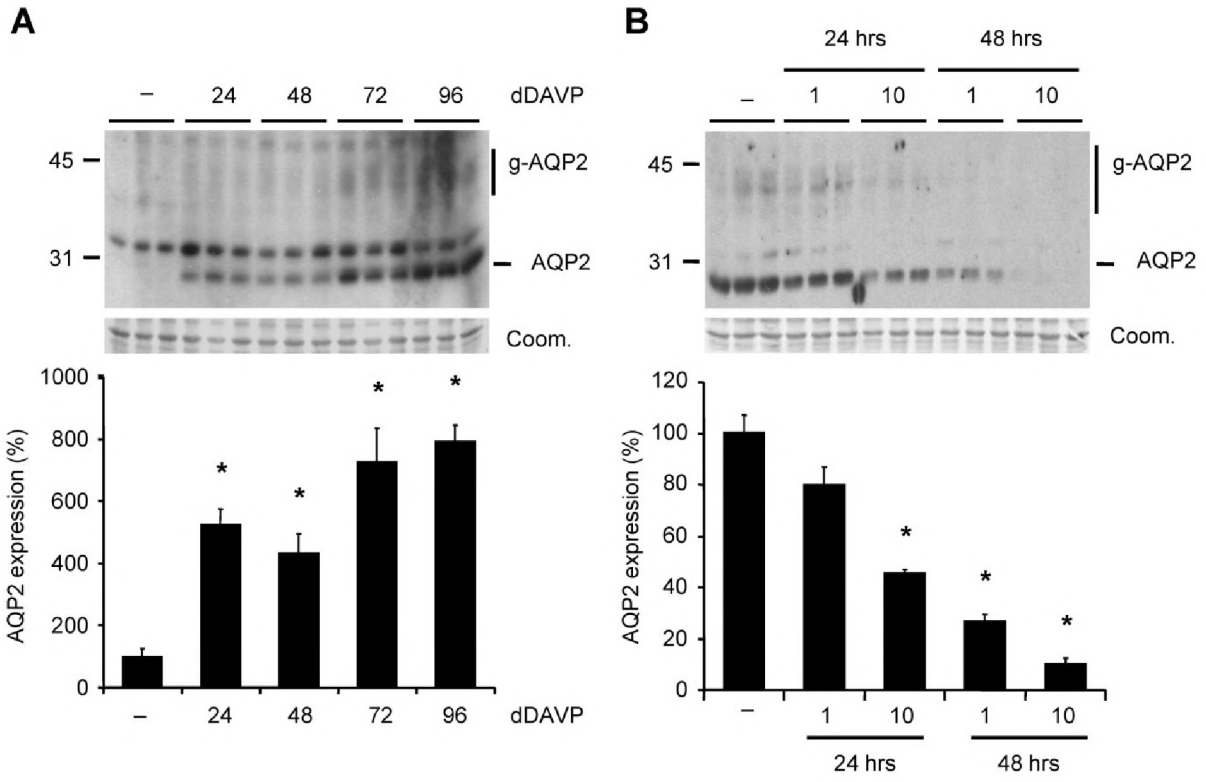

Figure 1. mCCD$_{c11}$ cells: a proper cell model to study lithium-NDI. A) mCCD$_{c11}$ cells were grown to confluence, treated for the indicated times (in hrs) with 1 nM dDAVP and subjected to AQP2 immunoblotting or, after blotting, stained with coomassie blue. Non-glycosylated (29 kDa) and complex-glycosylated (40-45 kDa) forms of AQP2, and an a-specific band of 35 kDa, are detected. **B)** mCCD$_{c11}$ cells, grown as above, were treated for 96 hrs with 1 nM dDAVP, and for the last 24 or 48 hrs, in the absence (-) or presence of 1 mM lithium at the basolateral side and 1 or 10 mM lithium at the apical side. Cells were lysed and immunoblotted for AQP2. Blots were also stained with coomassie blue. Molecular masses (in kDa) are indicated on the left. The signals for non-glycosylated and complex-glycosylated AQP2 were densitometrically quantified and normalized for coomassie blue staining. Mean values of normalized AQP2 expression per condition are given as percentage of control (+/- SEM) and were determined from three independent filters per condition. Significant differences ($p<0.05$) from control (-) are indicated by an asterisk.

In patients on lithium therapy, the concentration of lithium is around 1 mM in serum and ranges between 1 and 10 mM in urine (1). To test whether these concentrations affect AQP2 abundance, cells were incubated with 1 mM lithium at the basolateral side and with either 1 or 10 mM lithium at the apical side for 24 or 48 hrs. Immunoblotting revealed that lithium reduced the AQP2 expression in a time- and dose-dependent manner, with an almost complete absence of AQP2 after a 48 hrs incubation in 10 mM lithium (Fig. 1B). This concentration of lithium did not affect cell viability as indicated by similar coomassie-stained

protein levels (Fig. 1B) and a consistent transcellular resistance of >1000 Ohm/cm^2 (not shown). Together, these data reveal that mCCD$_{cl1}$ cells are a suitable model to investigate the role of ENaC in lithium-NDI.

The role of ENaC in AQP2 down-regulation and transcellular lithium transport

ENaC is located in the apical membrane of collecting duct and mCCD$_{c11}$ cells (19), and its activity is blocked by amiloride. To test whether ENaC mediates AQP2 down-regulation by lithium, mCCD$_{c11}$ cells were incubated with lithium at only the basolateral (1 mM) or the apical (10 mM) side with or without amiloride for 48 hrs. Application of lithium at the apical side reduced AQP2 abundance to about 30% of control values, which was prevented by amiloride (Fig. 2A). In contrast, addition of lithium with or without amiloride to the basolateral compartment did not change AQP2 levels. Similar results were obtained with another ENaC blocker, benzamil (Fig. 2B).

If ENaC is the main entry site for lithium, cellular uptake of lithium and the lithium-induced down-regulation of AQP2 should be influenced by the sodium concentration. In pilot experiments, mCCD$_{c11}$ cells were grown in medium containing lower concentrations of sodium at the apical side for variable time periods. Incubation of the cells for 12 hrs in medium containing 60, 90 or 150 mM sodium showed similar AQP2 expression, whereas lower concentrations of sodium or more prolonged incubations resulted in a reduction of AQP2 expression (data not shown). Therefore, we incubated the cells with lithium for 12 hrs in the presence of either 150, 90 or 60 mM sodium. Indeed, AQP2 expression negatively correlated to the sodium concentration (Fig. 2C), thus confirming competition between sodium and lithium and indicating that lithium enters the cells through a sodium-transporting protein.

If ENaC is the apical entry site for lithium, amiloride should reduce transcellular transport and the intracellular concentration of lithium. To test this, cells were incubated with 10 mM lithium at the apical side with or without amiloride. After 24 hrs, we observed a significant decrease of the lithium concentration at the apical side and an increase at the basolateral side, indicating that transcellular lithium transport occurred, which was inhibited by co-incubation with amiloride (Fig. 3A). We next determined intracellular lithium levels. A 24 hrs incubation of cells with 1 mM lithium at the basolateral side only resulted in an intracellular lithium concentration of 3±3 pmol lithium/μg protein, which corresponds to $[Li^+]_i$ of 0.7 mM (Fig 3B). This is well above that of control cells (below detection limit; not shown), indicating that some lithium enters the cells from the basolateral side. Intracellular lithium concentrations were markedly higher when 1 or 10 mM lithium was added to apical side as well, resulting in concentrations of 3.0 and 26.0 mM, respectively. In the presence of amiloride, intracellular

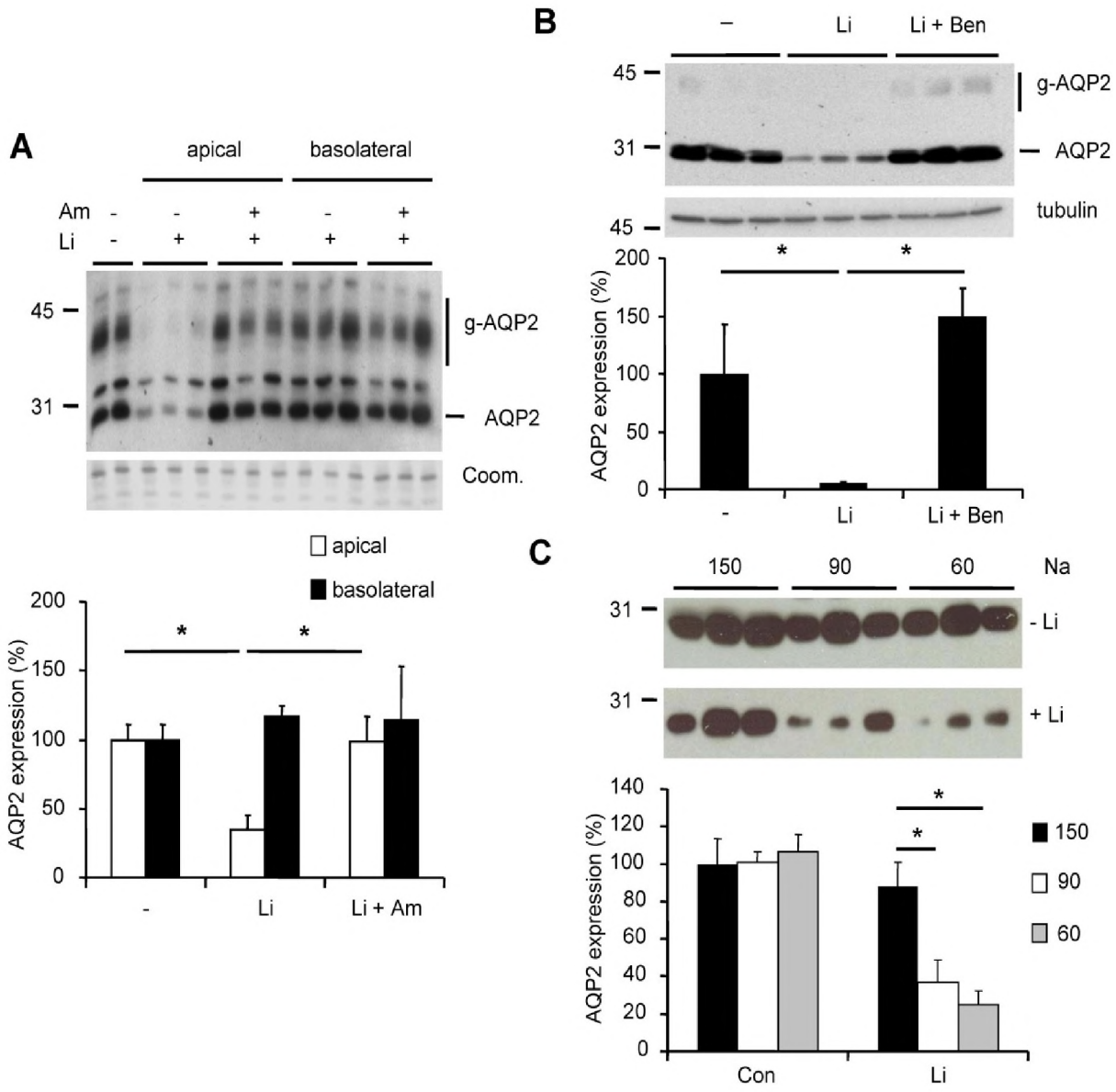

Figure 2. ENaC blockers reduce lithium-induced AQP2 down-regulation in mCCD$_{c11}$ cells. A) Confluent mCCD$_{c11}$ monolayers were treated for 96 hrs with 1 nM dDAVP and incubated for the last 48 hrs in the absence (-) or presence (+) of lithium and/or 10 µM amiloride as indicated. At the basolateral and apical side, 1 mM and 10 mM lithium was used, respectively. **B)** Confluent mCCD$_{c11}$ monolayers were treated as above with 10 mM lithium with/without 10 µM benzamil (Li + Ben) at the apical side for the last 24 hrs. **C.** mCCD$_{c11}$ cells were grown as above and treated for the last 12 hours in medium containing a lower sodium chloride concentration at the apical side only, with or without lithium (indicated). For A, B and C, cells were lysed and immunoblotted for AQP2. Molecular masses (in kDa) are indicated on the left. Semi-quantification of the AQP2 signals, normalization, and statistical analysis were done as described in the legend of Figure 1. Mean values were determined from three independent filters per condition.

lithium concentrations were reduced by more than 75%. To determine whether the obtained data were cell line specific, we repeated some of the above experiments in mpkCCD$_{cl4}$ cells. In these cells, amiloride also reduced the intracellular lithium concentration and prevented lithium-induced down-regulation of AQP2 (supplementary data Fig. S1).

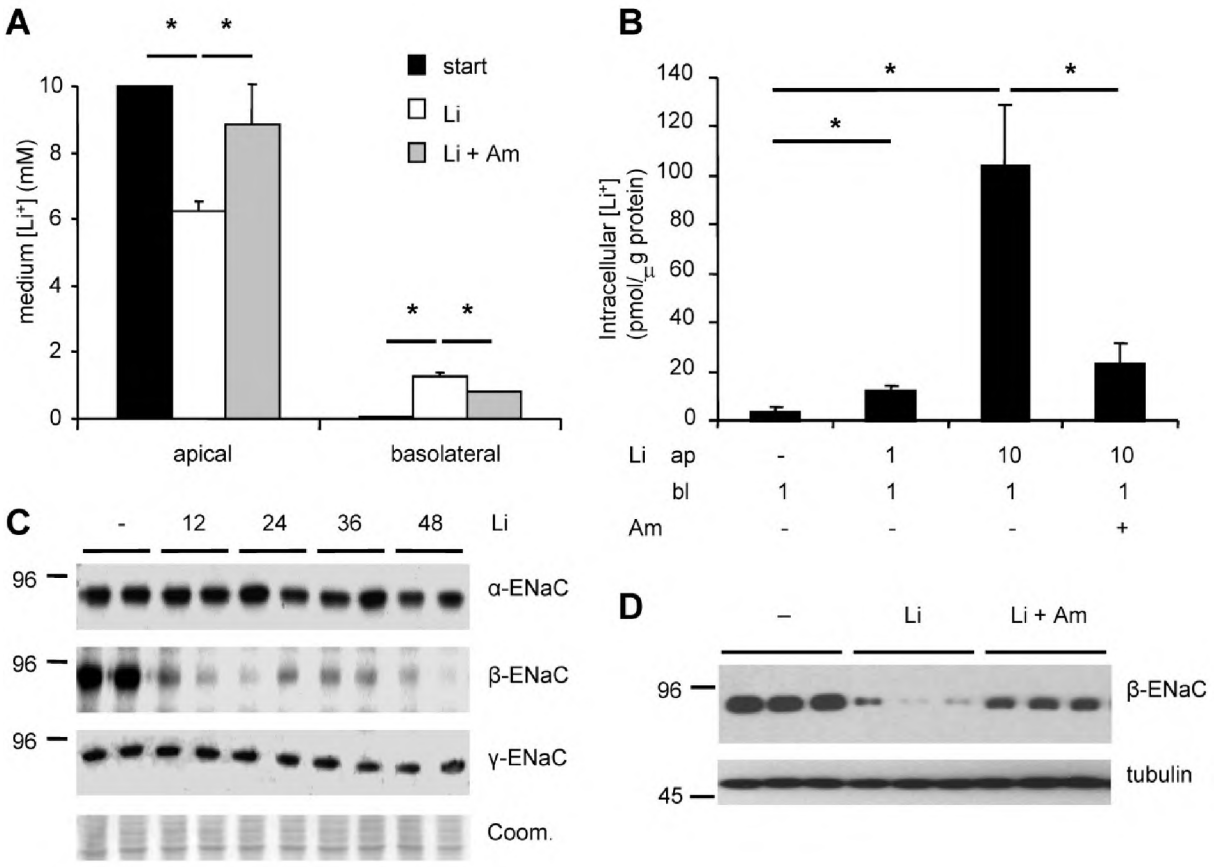

Figure 3. **Amiloride blocks lithium transport.** $mCCD_{c11}$ cells were grown as described above. **A)** After a 24 hrs treatment with 10 mM lithium with/without amiloride at the apical side, media from the apical and basolateral compartments were collected and lithium concentrations determined. **B)** $mCCD_{c11}$ cells were treated for the last 24 hrs with the indicated concentrations of lithium at the basolateral (bl) and/or apical (ap) side in the absence or presence of 10 µM amiloride. After 24 hrs, lithium concentrations were determined. Intracellular lithium concentrations were corrected for contamination with extracellular lithium and normalized for the amount of protein. For A and B, the mean lithium concentration ($[Li^+]$ +/- SEM in mM [A] or pmol/µg protein [B]) was determined from at least three independent filters per condition. Asterisks indicate a significant difference ($p < 0.05$). **C)** $mCCD_{c11}$ cells were treated with lithium on the apical (10 mM) and basolateral (1 mM) side for the indicated time periods (in hrs), lysed and analyzed for ENaC subunit expression. **D)** $mCCD_{c11}$ cells were treated as under C with or without amiloride for 24 hours and analyzed for β-ENaC. In C and D, molecular masses (in kDa) are indicated on the left.

Together, these data indicate that ENaC is the main cellular entry site for lithium in collecting duct cells and that blocking ENaC by amiloride prevents lithium-induced down-regulation of AQP2.

Effects of lithium and amiloride on ENaC subunit expression

In vivo, it has been shown that lithium treatment results in ENaC-downregulation (29). To see if similar effects occurred *in vitro*, $mCCD_{c11}$ cells were incubated with lithium for 12 to 48 hrs. Indeed, lithium reduced the expression of β-ENaC, but not of α or γ-ENaC (Fig. 3C). However, these cells showed an amiloride-dependent transcellular voltage (not shown),

indicating that ENaC was still functionally expressed. Amiloride partially prevented the down-regulation of β-ENaC (Fig. 3D).

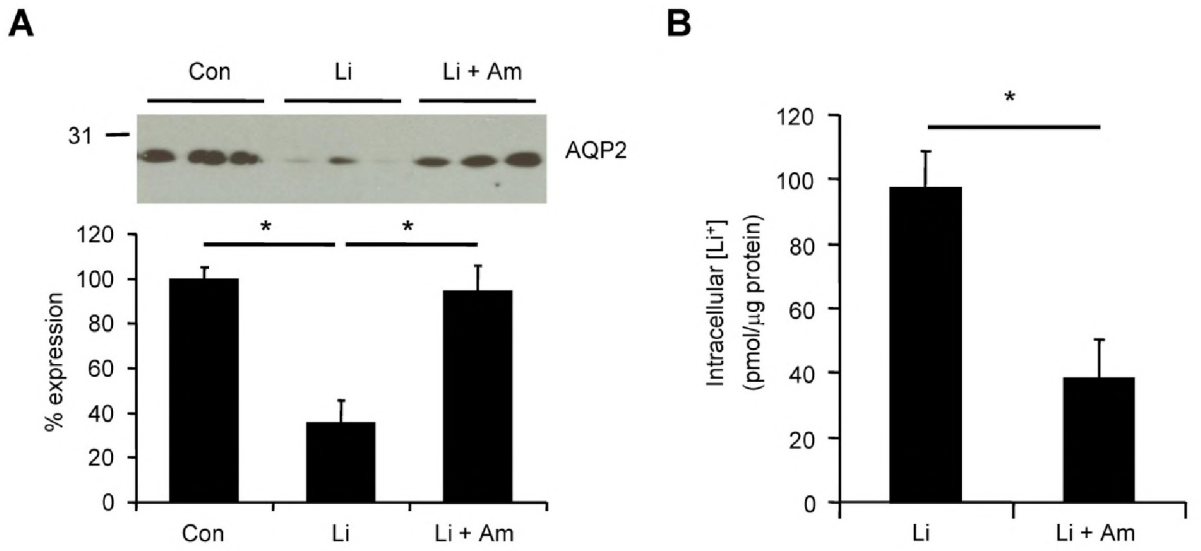

Figure S1. Effect of amiloride in mpkCCD$_{cl4}$ cells. A) Confluent mpkCCD$_{cl4}$ monolayers were treated for 96 hrs with 1 nM dDAVP and incubated for the last 24 hrs in the absence or presence of lithium and/or 10 μM amiloride as indicated. At the basolateral and apical side, 1 mM and 10 mM lithium was used, respectively. Proteins were immunoblotted for AQP2, of which the mass is indicated on the left (in kDa). Mean values of normalized AQP2 expression per condition are the mean of three independent experiments and are given as percentage of control (+/- SEM). **B)** Confluent mpkCCD$_{cl4}$ monolayers were treated as in **A**. 24 hrs after start of the lithium treatment, intracellular lithium concentrations were determined. These were corrected for contamination with extracellular lithium and normalized for the amount of protein. The mean lithium concentration ([Li^+] +/- SEM in pmol/μg protein) was determined from three independent filters per condition. For A and B, asterisks indicate a significant difference ($p < 0.05$).

Effects of lithium and amiloride on Gsk3β

Rao *et al.* showed a decreased glycogen synthase kinase (Gsk) 3β activity in lithium-treated rats, suggesting a possible involvement of Gsk3β in the pathway leading to lithium-NDI (30). To test whether Gsk3β is also involved in the lithium-induced down-regulation of AQP2, total Gsk3β expression and the extent of Gsk3β-inactivating phosphorylation at Ser9 was analyzed in conjunction to AQP2 expression. While lithium application for 48 hours again caused down-regulation of AQP2, it did not change total Gsk3β expression levels, but strongly increased the Ser9-phosphorylation of Gsk3β (Fig. 4A). Zinc also inhibits Gsk3β (31) and BIO-acetoxime is a specific Gsk3-inhibitor (32; 33). Indeed, similar to lithium, 20 μM, but not 1 μM, zinc reduced AQP2 levels and increased phosphorylated Gsk3β, but did not affect total Gsk3β levels (Fig. 4A). In line with an important role of Gsk3β in AQP2 expression, BIO-acetoxime also decreased the expression of AQP2 (Fig 4B).

Since amiloride prevents lithium-induced down-regulation of AQP2, we also tested whether amiloride reduced the effect of lithium on Gsk3β. Indeed, while amiloride again protected the

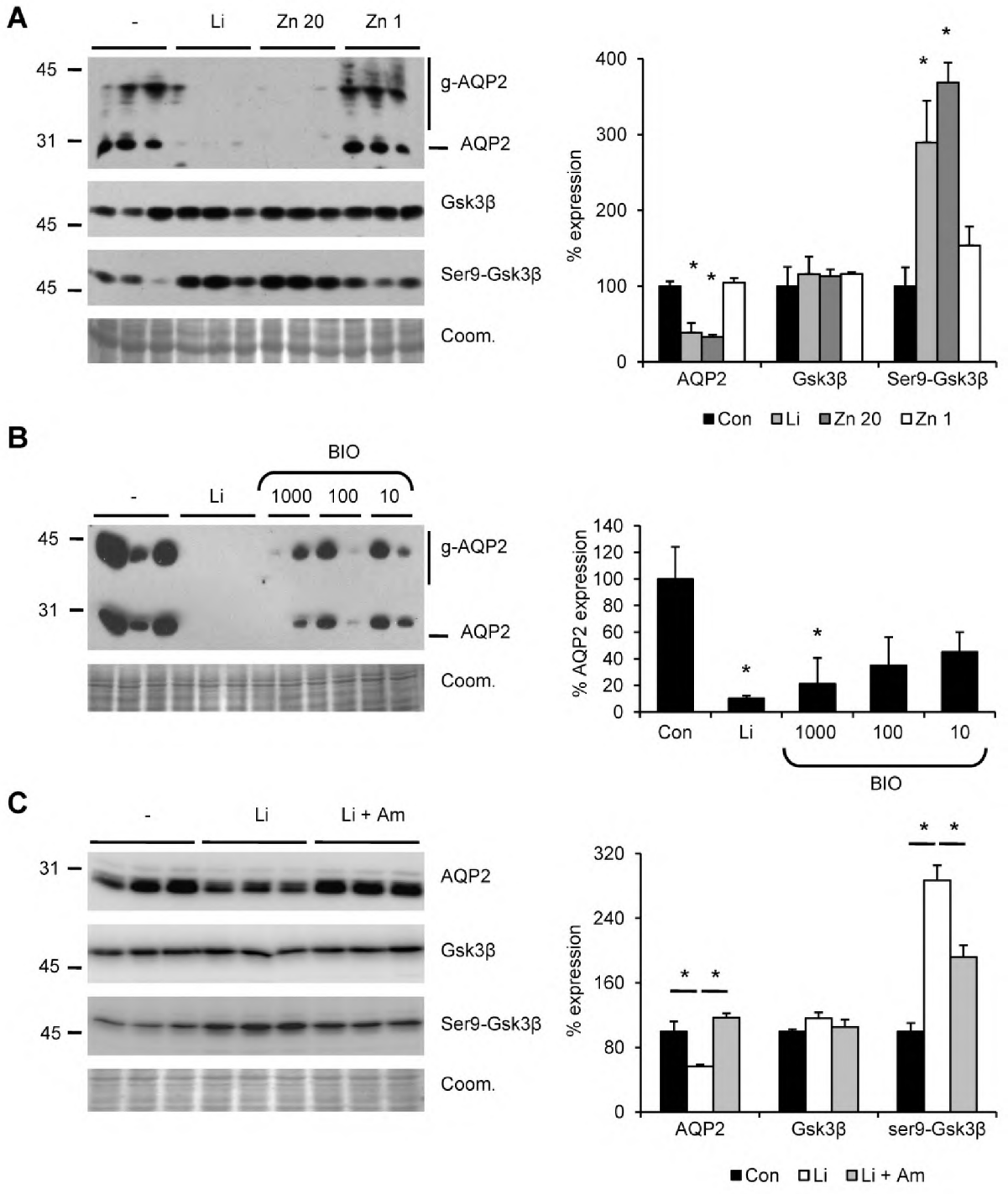

Figure 4. **Effects of lithium on Gsk3β.** Confluent mpkCCD$_{cl4}$ monolayers were treated for 96 hrs with 1 nM dDAVP. **A)** Cells were treated with 1 mM lithium at the basolateral side and 10 mM lithium at the apical side or 20 and 1 μM zinc at both sides for the last 48 hrs and subjected to AQP2, Gsk3β and Phospho-Gsk3β(Ser9) immunoblotting or, after blotting, stained with coomassie blue. **B)** mpkCCD$_{cl4}$ cells were treated with a specific Gsk3-inhibitor (BIO-Acetoxime) for the last 48 hrs and subjected to AQP2 immunoblotting. Concentrations are in nM. **C)** Cells were incubated for the last 48 hrs in the absence or presence of lithium with or without 10 μM amiloride as indicated. At the basolateral and apical side, 1 mM and 10 mM lithium was used, respectively. Molecular masses (in kDa) are indicated on the left. Semi-quantification, normalization, and statistical analysis were done as described in the legend of Figure 1.

lithium-induced down-regulation of AQP2, it did not affect total Gsk3β abundance, but significantly reduced the extent of lithium-induced phosphorylation of Gsk3β at Ser9 (Fig. 4C).

Together, these data strongly suggest that in mpkCCD$_{cl4}$ cells, lithium affects AQP2 expression by inactivating Gsk3β via phosphorylation at Ser9, which is attenuated by amiloride.

Table 1. Blood and urine parameters

	-	Li	Li + Am
Body weight (g)	287±11	212±7*	232±8*
Plasma osmolality (mOsm)	283±5	304±5*	288±3[a]
Plasma lithium (mmol/l)	/	0.69±0.08	0.57±0.08
Plasma sodium (mmol/l)	141±1	142±2	141±2
Plasma urea (mmol/l)	5.9±0.3	4.0±0.3*	5.6±0.7
Plasma creatinine (µmol/l)	43±3	41±1	41±2
Urine volume (ml/day)	13±2	189±15*	108±24*[a]
Urine osmolality (mOsm)	1537±158	98±25*	400±93*[a]
Total lithium excretion (mmol/24 hrs)	/	0.38±0.10	0.61±0.10
Total sodium excretion (mmol/24 hrs)	1.5±0.3	4.7±0.2*	11.4±1.9*[a]
Total potassium excretion (mmol/24 hrs)	3.2±0.5	2.4±0.4	3.0±0.3
Total urea excretion (mmol/24hrs)	10.9±0.2	6.8±0.5	8.6±1.3
Osmolar excretion (mOsm/24 hrs)	18.3±0.2	16.9±0.3	35.4±6.7
Creatinine clearance (ml/min)	0.93±0.05	0.42±0.09*	0.72±0.12
Urea clearance (ml/min)	1.31±0.25	1.20±0.07	1.15±0.17
Lithium clearance (ml/min)	/	0.65±0.10	0.98±0.25

Values are means +/- SEM. Significant differences between control (-) and other groups are indicated as *, significant differences between the lithium (Li) and lithium+amiloride group (Li + Am) are indicated as [a]. / indicates below detection limit.

The effect of amiloride on the development of lithium-NDI in rats

To analyze whether amiloride can prevent development of lithium-NDI *in vivo*, rats were kept on lithium chow for 4 weeks, with or without amiloride, while control rats received normal chow. All rats had free access to a salt block to compensate for the sodium losses that occur in lithium-NDI (34).

As anticipated (7; 8), the rats treated with lithium developed severe polyuria with reduced urine osmolality (Table 1). Amiloride treatment attenuated this polyuria and increased urine osmolality compared to lithium only. Serum lithium concentrations found in the lithium

(0.69±0.08 mM) and lithium-amiloride (0.57±0.08 mM) groups were similar and in the therapeutic range, whereas serum lithium concentrations in control rats were below the detection limit (0.05 mM; Table 1). As observed more often (7; 35-38), lithium treated rats had a slightly lower body weight. We observed no differences in serum sodium or serum creatinine concentrations. However, there were differences in the urinary excretion of the various solutes. Lithium treatment resulted in increased sodium losses, which was further exaggerated by amiloride, confirming the need for the salt block. To investigate if there were differences in food intake, the excretion of potassium and urea was measured. Urinary excretion of these solutes was numerically although not-significantly lower in rats treated with lithium only. In rats treated with lithium and amiloride values were similar to control. Also, creatinine clearance was lower in lithium treated rats than in control rats. This was not the case in the amiloride-lithium treated rats.

The effect of amiloride on the renal collecting duct in lithium-NDI rats

Lithium-NDI is characterized by a decreased fraction of AQP2-expressing principal cells with a parallel increase of H-ATPase-expressing intercalated cells (7). We evaluated the effect of amiloride treatment on AQP2 and H-ATPase expression and the cellular composition in different regions of the kidney. We observed a significantly reduced expression of AQP2 in the cortex of lithium-treated animals (Fig. 5; Table 2). Amiloride significantly attenuated this decrease, but did not restore the AQP2 expression to the level of untreated rats. Similar results were obtained for the outer and inner medulla (Table 2). With respect to the expression of H-ATPase, we noted an increased expression in the cortex of lithium-treated rats, which was prevented by amiloride (Fig. 5). Similar data were obtained for the outer and inner medulla (Table 2).

Table 2. Effect of amiloride on collecting duct marker protein expression

	AQP2			H-ATPase		
	-	Li	Li + Am	-	Li	Li + Am
cortex	100±6	4±2*	22±4*[a]	100±42	303±23*	150±19[a]
outer medulla	100±5	30±9*	53±6*[a]	100±30	179±17*	121±40
inner medulla	100±4	6±4*	42±4*[a]	100±29	129±13	65±20[a]

Mean values (+/- SEM) are expressed as percentages of the controls. Significant differences between control (-) and other groups are indicated as *, significant differences between the lithium (Li) and lithium+amiloride group (Li + Am) are indicated as [a].

To investigate whether amiloride treatment also affects the ratio of principal and intercalated cells, kidney sections were labeled with antibodies against AQP2, H-ATPase, and nuclear TOTO-3. In agreement with previous studies (7; 8) and the immunoblot data, lithium

treatment resulted in a decreased density of AQP2-expressing cells and increased density of H-ATPase-expressing cells (Fig. 6A). Amiloride treatment attenuated these changes.

We quantified the changes by calculating a principal/intercalated cell ratio. Lithium treatment significantly reduced the ratio in the cortex, an effect which was fully prevented by amiloride (control: 1.72±0.09; lithium: 0.83±0.06; lithium-amiloride: 1.78±0.15; Fig. 6B). Similar results were obtained for the outer medulla (control: 2.21±0.15; lithium: 1.02±0.07; lithium-amiloride: 1.98±0.13) and the inner medulla (control: 2.66±0.56; lithium: 1.16±0.12; lithium-amiloride: 1.91±0.07), indicating that amiloride prevents the change in the ratio of principal/intercalated cells.

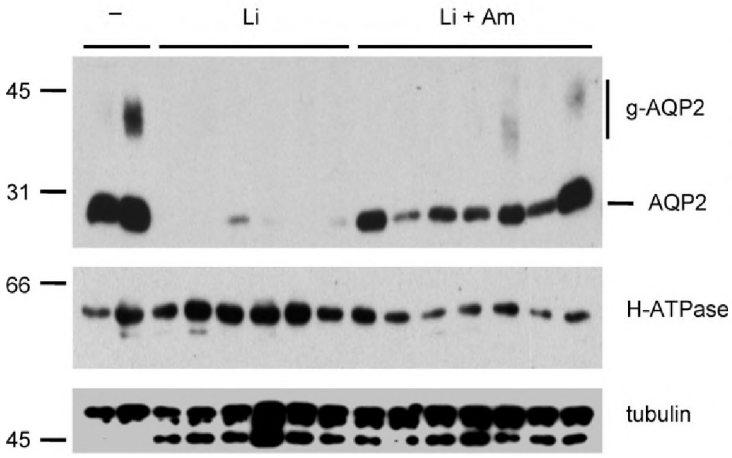

Figure 5. Amiloride prevents effect of lithium on AQP2 and H-ATPase expression in lithium-NDI rats. Wistar rats were fed a normal diet (-; n=6), a diet containing lithium (Li; n=6) or a diet containing lithium and amiloride (Li+Am; n=7). After 4 weeks, one kidney was divided in cortex, outer medulla and inner medulla segments and solubilized. An equal amount of protein of the cortex of each rat was immunoblotted for AQP2, H-ATPase or tubulin (indicated). Molecular masses (in kDa) are indicated on the left.

Discussion

$mCCD_{c11}$ cells are a proper model to study lithium-induced AQP2 down-regulation

A major handicap to study lithium-NDI at the cellular level was the lack of a suitable model. Our study indicates that $mCCD_{c11}$ cells are an appropriate model for the following reasons. First, $mCCD_{c11}$ cells express all three ENaC subunits in the apical membrane and demonstrate amiloride-inhibitable sodium transport (19). Secondly, and similar to collecting duct cells *in vivo*, $mCCD_{c11}$ cells show a dDAVP-dependent increase in expression of endogenous AQP2 (Fig. 1A). Thirdly, and in line with *in vivo* findings (5), $mCCD_{c11}$ cells show a time- and concentration-dependent decrease in AQP2 expression in response to therapeutically-relevant lithium concentrations (Fig. 1B).

ENaC is the main entry site for lithium and blocking ENaC prevents lithium-induced Gsk3β inactivation and AQP2 down-regulation in vitro

With 1 mM lithium added to the basolateral side of $mCCD_{c11}$ cells, a small but significant increase in the intracellular lithium concentration was observed (Fig. 3B). As the cells formed

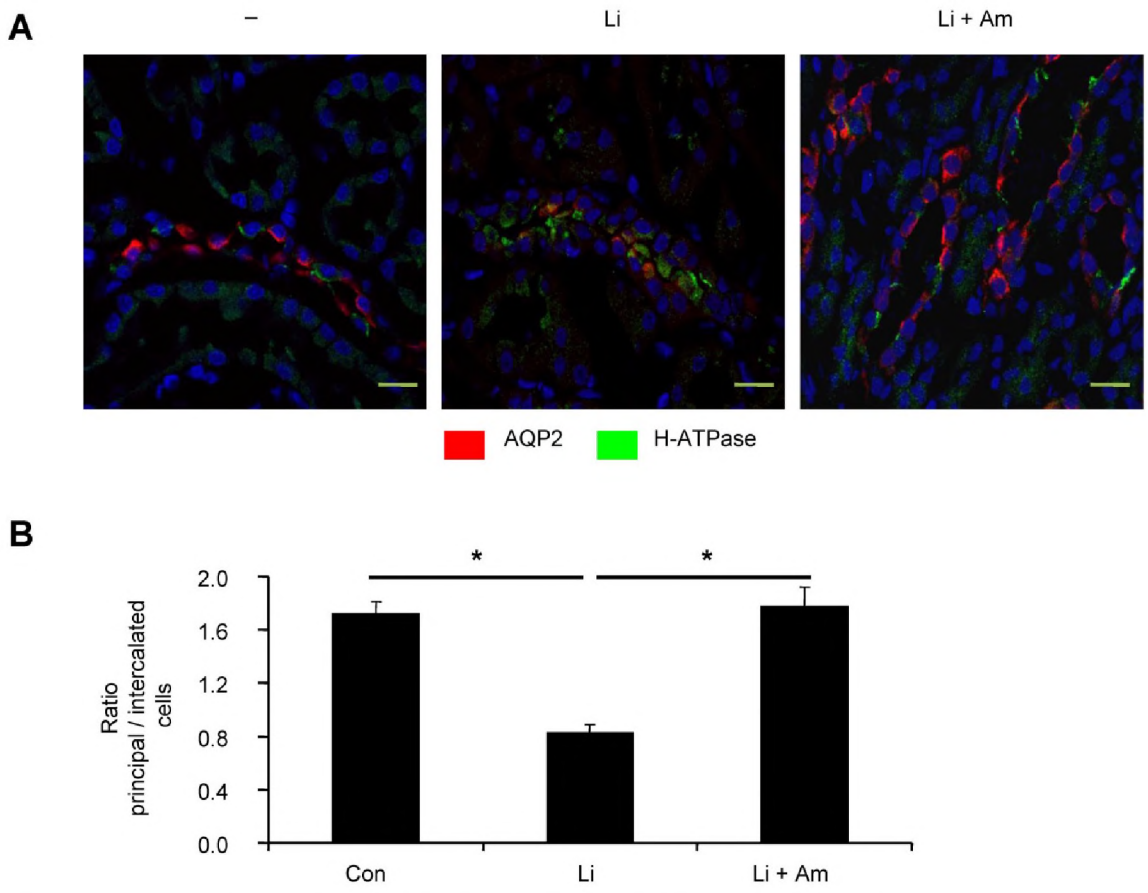

Figure 6. **Amiloride prevents cell conversion in lithium-NDI rats. A)** Of the rats described in the legend of Fig. 5, one kidney was removed and fixed. Cryosections were made and incubated with rabbit H-ATPase (green) and guinea pig AQP2 (red) antibodies, followed by Alexa-488-conjugated goat-anti-rabbit and Alexa-594-conjugated goat anti-guinea pig antibodies. TOTO-3 (blue) was used to counterstain the sections. Images were made with confocal laser scanning microscopy. Scale bars: 10 µm. **B)** Of >5 defined areas of the kidney cortex of each control (n=6), lithium (n=6) and lithium+amiloride (n=7) rat, cells positive for AQP2 or H-ATPase were counted and expressed as the ratio of principal / intercalated cells (+/- SEM) (total cells control [-]: 1244; Li: 1393; Li + Am: 2064). Significant differences (*: $p<0.05$) are indicated.

a tight monolayer, this indicated that lithium can enter through a basolateral site. While the identity of this entry pathway remains to be established, it did not reduce AQP2 expression (Fig. 2A).

Upon addition of 1 mM lithium to the apical compartment, intracellular lithium concentrations increased about 3.5-fold to 3.0 mM (Fig. 3B), which resulted in slightly, but not significantly, reduced AQP2 levels (Fig. 1B). Addition of 10 mM lithium together with amiloride to the apical compartment resulted in similar intracellular lithium concentrations (Fig. 3B) and normal AQP2 levels (Fig. 2A). In contrast, AQP2 expression was strongly reduced with 10 mM lithium at the apical side in the absence of amiloride (Fig. 1C), which coincided with an intracellular lithium concentration of 26 mM (Fig. 3B). The observed AQP2 reduction suggests that within 24 hours, AQP2 expression is down-regulated with a threshold of intracellular lithium concentrations between 3 and 26 mM, which are obtained with lithium

concentrations of 1 mM at the basolateral side and 10 mM at the apical side. However, at prolonged incubation times, AQP2 down-regulation may be induced with lower extracellular lithium concentrations, as illustrated by the reduced AQP2 levels upon our incubation with 1 mM lithium for 48 hours (Fig. 1B). In agreement with an important role of Gsk3β activity in lithium-NDI mice (30) and the protective effect of amiloride on lithium-induced down-regulation of AQP2, lithium, but also other Gsk3β inhibitors, increased inactivating Gsk3β phosphorylation and down-regulation of AQP2 expression in mpkCCD$_{cl4}$ cells, which was partially prevented by co-treatment with amiloride (Fig. 4).

The observed intracellular lithium concentration of 26 mM is higher than the concentration in the extracellular fluid, which has been observed before (23). Immunocytochemistry did not reveal a difference in cell size between lithium-treated versus control cells, and, in similar experiments, intracellular potassium levels were not different between control, lithium or lithium+amiloride treated cells, indicating that this is not due to an increased intracellular volume in the lithium-treated cells. More likely, the strong inwardly-directed electrochemical potential for lithium and the fact that lithium is a poor substitute for sodium with the Na-K-ATPase to be transported to the extracellular fluid (39) cause intracellular accumulation of lithium.
Overall, our study provides strong evidence that ENaC is the major cellular entry pathway for lithium and that blocking ENaC reduces lithium-induced Gsk3β inactivation and prevents AQP2 down-regulation.

Co-treatment with amiloride attenuates lithium-NDI
As reported (5; 7), lithium also induced NDI in our experiments, as indicated by the increased urine volume and decreased urine osmolality (Table 1). Our data furthermore reveal that amiloride treatment decreased urine volume and increased urine osmolality (Table 1). While these changes point to a better urine concentrating ability, the observed changes could theoretically be due to differences in respectively solute intake or extracellular volume: an increased solute intake increases urine volume due to osmotic diuresis, whereas hypovolemia will reduce urine volume and increase urine osmolality. However, our data clearly indicate that the effects of amiloride cannot be explained by these factors. First, the urinary excretion of osmolytes was higher in the amiloride-lithium group than in the group receiving lithium only. Second, if anything, volume depletion was more likely to be present in lithium only treated rats as suggested by the lower body weight, and the reduction in creatinine clearance.
Consistent with this and the protective effect of amiloride in mCCD$_{c11}$ cells, rats treated with amiloride and lithium had significantly increased AQP2 and decreased H-ATPase expression

compared to rats treated with lithium only (Figs. 5-6; Table 2) and the lithium-induced change in principal/intercalated cell ratio was completely prevented (Fig. 6). Taken together, our data indicate that amiloride has a protective effect on lithium-NDI development by preventing the lithium-induced change in cellular composition and partial protection of AQP2 down-regulation, leading to a preserved concentrating ability of the collecting duct.

The completely prevention of the change in cell ratio and the partial protection of AQP2 down-regulation by amiloride suggests that AQP2 expression is more sensitive to lithium than the integrity of the principal cells, which is in agreement with the fact that AQP2 down-regulation precedes the fractional decrease in principal cells in lithium-NDI development.(4; 8). It furthermore suggests that the used amiloride concentration did not completely prevent the deleterious effect of lithium and it is unclear whether a residual effect of lithium can explain the fact that the concentrating ability of amiloride-treated rats is still lower than in control rats. One explanation for the reduced concentrating ability is that blockage of sodium reabsorption by amiloride could lead to a decrease in osmolality in the interstitium. However, considering the small contribution of sodium transport through ENaC to the interstitial osmolality, which is even less when NaCl is given ad libitum as done here, this is rather unlikely.

Alternatively, this could be due to an incomplete block of ENaC by amiloride in the kidney or due to cellular lithium influx via (an)other transporter(s)/channel(s). Amiloride was added to the food of the rats at a concentration of 200 mg/kg dry food, which is equivalent with 5 mg of amiloride. Assuming a volume of distribution of 3.4 l/kg and a t½ of 21 hours, and a bioavailability of 10% (40; 41), this will lead to a plasma concentration of 3 µM. Amiloride clearance has been estimated to be about 3 times the creatinine clearance (42), leading to a amiloride excretion of 9 µmol per day and an end-urine concentration of 84 µM, which, as the IC50 of amiloride is 0.1-0.5 µM (22), very likely completely blocks ENaC. If these calculations hold for our rats, our data suggest that, in line with our *in vitro* data, some lithium may enter principal cells through another protein than ENaC.

In our lithium-induced NDI rats and as seen by others (8), we observed an increased expression of H-ATPase, which can be partly attributed to an increase in the number of intercalated cells (Figs. 5-6). It has been suggested that the observed reduction in AQP2 expression with lithium is partially due to a loss of principal cells, either by differentiation of principal cells to intercalated cells or by selective cell death of principal cells (7; 8). Occasionally, and as seen by others (7), we observed cells to stain positive for both AQP2 and H-ATPase, which may suggest that the change of principal/intercalated cell ratio in lithium-NDI is (partly) due to a transition of principal to intercalated cells. However, the numbers were too low to influence the counting of the principal/intercalated cell ratio. In line

with a loss of principal cells, Christensen *et al.* found an increased number of apoptosis-inducing factor (AIF)-labeled collecting duct cells in the IMCD after 10 and 15 days of lithium treatment, suggesting that apoptosis of principal cells may be involved, at least in part, in the changes in cellular composition in lithium-induced NDI (8). However, they also found an increased proliferation of not only intercalated cells but also principal cells and, therefore, the change in cell composition could be the result of an increased number of intercalated cells without a loss of principal cells. If so, the reduced expression of AQP2 could be the result of a decreased expression per cell only. The exact underlying mechanism awaits further experiments.
Lithium-NDI also coincides with reduced expression of urea transporters UT-A1 and UT-B and Bedford *et al*. recently showed that amiloride treatment also significantly increases UT-A1 expression and conserves the osmotic gradient compared to rats treated with lithium only (43; 44). Considering our data, the changes in UT-A1 expression and osmotic gradient with lithium and lithium-amiloride, respectively, could be due to a decreased/increased expression per cell, but are likely (partially) explained by the changes in principal/intercalated cell ratio.

In conclusion, our data reveal that $mCCD_{c11}$ cells form a proper model to study lithium-NDI. We demonstrate that ENaC forms the major entry pathway for lithium into principal cells and that blocking ENaC with amiloride reduces the lithium-induced AQP2 down-regulation, protects the cellular composition of the collecting duct and thereby attenuates lithium-NDI. As such, our data provide a rationale for the use of amiloride in treating lithium-NDI patients.

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CHAPTER 9

Summary and General Discussion

Introduction

Maintaining water homeostasis by controlling both the blood osmolality and blood volume is essential for most physiological processes in the human body. Body water homeostasis is tightly controlled by regulating both water intake and urinary water excretion.
In states of hypernatremia, specific osmoreceptors in the hypothalamus are activated, which results in the sensation of thirst and subsequent water intake (1; 2). Furthermore, the hormone arginine vasopressin (AVP) is released from the pituitary into the blood stream (3). Besides osmolality, AVP secretion is also influenced by blood volume and blood pressure changes, sensed by baroreceptors (2; 4). The released AVP regulates the body's retention of water, causing the kidneys to concentrate the urine and reduce urine volume (2).
As outlined in this thesis, the aquaporin-2 (AQP2) water channel is one of the key players involved in renal water excretion and is critical to osmoregulation and the maintenance of body water homeostasis. Binding of AVP to its type-2 receptor, present on the basolateral membrane of renal collecting duct principal cells and connecting tubule cells, induces a signaling cascade, resulting in the redistribution of AQP2 water channels from intracellular vesicles to the apical membrane, greatly increasing the osmotic water permeability, leading to a concentrated urine (5-7). In addition to its effect on AQP2 localization, AVP also increases AQP2 transcription (8; 9). Once the water balance is restored, AVP levels drop and AQP2 is internalized, leaving the apical membrane watertight again.
Considering its important role in water homeostasis, it is not surprising that AQP2 is involved in several diseases associated with disturbed water homeostasis, including the syndrome of inappropriate antidiuretic hormone secretion (SIADH) and nephrogenic diabetes insipidus (NDI). AQP2 is tightly regulated, not only by AVP, but also by other hormones, compounds and medications. However, the exact molecular mechanisms which regulate AQP2 in different conditions are still unknown. This thesis aimed to gain insight into the physiological and pharmacological regulation of renal water reabsorption and AQP2 expression.

Physiological regulation of AQP2

Regulation of AQP2 by dopamine

Besides AVP, several other hormones and chemical messengers regulate water balance by antagonizing AVP-induced water transport. Examples are extracellular purines, dopamine, endothelin, epidermal growth factor and prostaglandins (10). The underlying mechanisms of their actions are poorly understood.

Chapter 2 aimed to get more insight into the mechanism by which hormones and chemical messengers counteract the action of AVP on AQP2, by analyzing in detail the effect of ATP and dopamine on the AVP-induced AQP2 abundance, both in a collecting duct cell model (mpkCCD cells) as well is in kidney slices.

Dopamine has been shown to decrease AVP-induced water permeability and cAMP production in the cortical and inner medullary collecting duct of rats (11-13) and has been shown to cause AQP2 internalization from the plasma membrane into intracellular storage vesicles in Madin-Darby canine kidney (MDCK) cells (14). In line with these studies, in chapter 2 addition of dopamine to mpkCCD cells decreased total and plasma membrane abundance of AVP-induced AQP2, partly by increasing its internalization to vesicles. The reduction in AQP2 abundance could be blocked by co-incubation with chloroquine, a lysosomal degradation inhibitor, which indicates that dopamine induces lysosomal degradation of AQP2. Previously, it was demonstrated that AQP2 internalization and degradation is preceded by its monoubiquitination at Lys270 upon AVP removal or protein kinase C (PKC) activation (15). Similarly, dopamine increased AQP2 mono-ubiquitination. The AQP2 internalization was ubiquitin-dependent as the plasma membrane localization of AQP2-K270R, which cannot be mono-ubiquitinated, was unaffected by dopamine. A similar process occurs *in vivo*, as dopamine incubation of kidney slices, treated with the AVP analogue dDAVP, also resulted in the internalization of AQP2. Dopamine also reduced cAMP and AQP2 mRNA levels, suggesting an additional effect on AQP2 gene transcription. Interestingly, phorbol esters, activating PKC, only reduced AQP2 through the first pathway. Together, our results indicate that dopamine counteracts AVP-induced water permeability by increasing AQP2 degradation in lysosomes, preceded by ubiquitin-dependent internalization, and by decreasing AQP2 gene transcription by reducing the AVP-induced cAMP levels (Fig. 1).

Of the dopamine receptors, the D4 receptor is expressed in the collecting duct and has been shown to mediate the diuretic effect of dopamine in the cortical collecting duct (12; 16). However, in the inner medullary collecting duct, the inhibitory effect of dopamine is suggested to be mediated through α_2-adrenergic receptors, which can also be activated by high concentrations of dopamine (11). The receptor subtypes responsible for the observed AQP2 decrease in mpkCCD cells remain to be determined.

The major source of renal dopamine is its synthesis in proximal tubular cells and possibly in the inner medullary collecting duct (17; 18). Renal dopamine production is increased by expansion of the extracellular fluid volume with isotonic saline. Besides, the amount of sodium chloride in the diet has been shown to correlate with urinary dopamine excretion (18). Studies suggest that the chloride, and not the sodium, might be regulating renal dopamine production, as an increase in the intake of chloride, with or without sodium, increases urinary

dopamine (19; 20). Physiologically, the diuretic effects of dopamine could contribute to counteracting an extracellular fluid volume expansion.

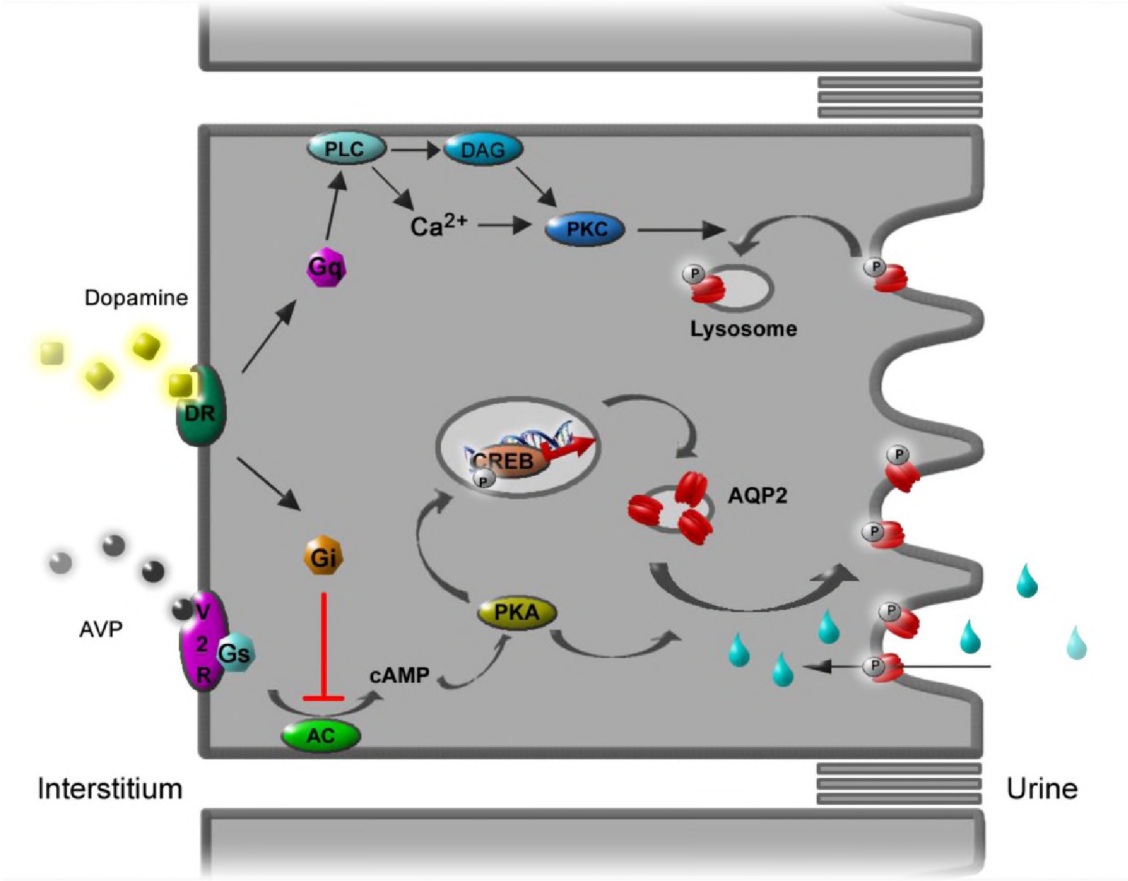

Figure 1. Dopamine inhibition of AQP2-mediated water reabsorption. Dopamine inhibits AVP-induced water transport by binding to its receptor, leading to an activation of PKC and internalization into lysosomes followed by degradation of AQP2. Dopamine also reduces cAMP levels, leading to a reduced AQP2 transcription. Indicated are adenylate cyclase (*AC*), aquaporin-2 (AQP2), vasopressin (*AVP*), cyclic adenosine monophosphate (*cAMP*), *DAG* Diacylglycerol (*DAG*), dopamine receptor (*DR*), protein kinase A (*PKA*), protein kinase C (*PKC*), phospholipase C (*PLC*) and vasopressin V2 receptor (*V2R*).

Regulation of AQP2 by ATP

Like dopamine, the extracellular purine ATP has been shown to decrease AVP-induced water permeability and cAMP production in isolated inner medullary collecting ducts of rats and has been shown to cause AQP2 internalization in mpkCCD cells (21-23).

In agreement with these studies, we show in chapter 2 that addition of ATP to mpkCCD cells decreased total and plasma membrane abundance of AVP-induced AQP2 and increased its internalization into vesicles. Co-incubation with the lysosomal degradation blocker chloroquine prevented the reduction in AQP2 abundance, indicating that ATP induces lysosomal degradation of AQP2. ATP increased AQP2 mono-ubiquitination and the AQP2 internalization was shown to be dependent on this ubiquitination as the plasma membrane localization of AQP2-K270R, which cannot be mono-ubiquitinated, was unaffected by ATP.

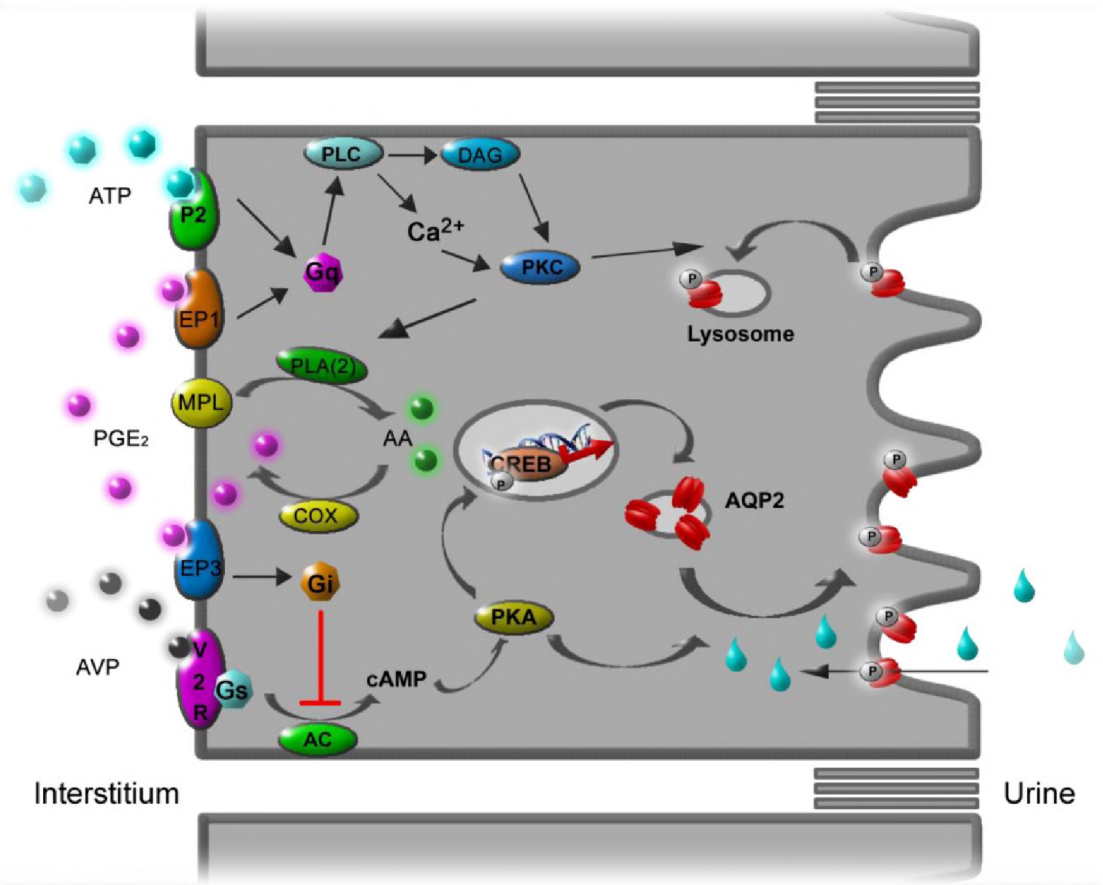

Figure 2. Inhibition of AQP2-mediated water reabsorption by ATP. ATP inhibits AVP-induced water transport by binding to its receptor, leading to an activation of protein kinase C (PKC) and internalization into lysosomes followed by degradation of AQP2. ATP also reduces cAMP levels, leading to a reduced AQP2 transcription. ATP may mediate this effect partly indirectly by stimulating prostaglandin E_2 (PGE_2) release by increasing arachidonic acid (AA) release. PGE_2 activates the AVP-counteracting EP receptors EP1 and EP3. Indicated are arachidonic acid (*AA*), adenylate cyclase (*AC*), aquaporin-2 (*AQP*), vasopressin (*AVP*), cyclic adenosine monophosphate (*cAMP*), cyclooxygenase (*COX*), diacylglycerol (*DAG*), membrane phospholipid (*MPL*), purinergic receptor (*P2*), protein kinase A (*PKA*), protein kinase C (*PKC*), phospholipase A (2) (*PLA(2)*), phospholipase C (*PLC*), and vasopressin V2 receptor (*V2R*).

Incubation of dDAVP-treated kidney slices with ATP also resulted in AQP2 internalization, indicating that a similar process occurs *in vivo*. ATP also reduced cAMP and AQP2 mRNA levels in mpkCCD cells, suggesting an additional reduction of AQP2 gene transcription.

In conclusion, our results indicate that ATP counteracts AVP-induced water permeability by increasing ubiquitination of AQP2, followed by internalization and AQP2 degradation in lysosomes, and by decreasing AQP2 gene transcription by reducing the AVP-induced cAMP levels (Fig. 2).

Various purinergic receptors have been identified in the collecting duct (24; 25). Of these, the basolaterally localized P2Y2 and apically localized P2X2 and P2Y4 have been suggested to be involved in the ATP-mediated reduction of AQP2 (21; 26). ATP is generally believed to be released locally in response to cell swelling or flow-induced shear stress (27). It could therefore be speculated that cell swelling under hypotonic conditions, for example in

hyponatremia, could lead to ATP release, which would help to maximize water excretion by inhibition of AQP2-mediated water reabsorption. Recently it was shown that also AVP increases ATP release in perfused cortical collecting ducts (28). AVP would thus both stimulate AQP2-mediated water reabsorption, and stimulate the release of ATP, which would oppose water transport via a local inhibitory feedback mechanism.
Previously it was shown that, besides ATP, UTP inhibits AVP-induced water permeability as well, which could be mediated via the P2Y2 or P2Y4 receptor (21-23). UTP release has been shown to be stimulated by mechanical stress (29). Whether UTP release is, like ATP, also stimulated by AVP needs further investigation.

AQP2 regulation by Ser261-phosphorylation

Recently, it has been shown that, besides phosphorylation at Ser256, which is important for the AVP-induced AQP2 translocation to the apical membrane (7; 30; 31), AQP2 can also be phosphorylated at Ser261, Ser264 and Ser269 (32). Phosphorylated Ser261-AQP2 is mainly found in intracellular vesicles and Ser261 phosphorylation decreases upon AVP incubation (33). Although protein kinase A (PKA) is responsible for the phosphorylation of AQP2 at Ser256, Hoffert *et al*. demonstrated that PKA is unable to phosphorylate Ser261 (34). *In vitro* experiments demonstrated that JNK, p38, CDK5 and CDK9 could potentially be involved in the phosphorylation of AQP2 at Ser261 (35). Interestingly, we show in chapter 2 that both dopamine and ATP increased AQP2 phosphorylation at Ser261, both in mpkCCD cells as well as in kidney slices. Ser261 phosphorylation seemed to be preceded by AQP2 ubiquitination, in agreement with recent results from Tamma *et al.* (36). However, this phosphorylation was not essential for the hormone-induced AQP2 internalization and degradation. The exact function of this phosphorylation site thus remains to be established.

Regulation of AQP2 by prostaglandins

Prostaglandins have a bifunctional role in water balance regulation. In particular, prostaglandin E_2 (PGE_2) has been shown to increase the osmotic water permeability of perfused collecting ducts in the absence of AVP (37; 38). Paradoxically, PGE_2 decreases water reabsorption in the presence of AVP (37-39).
In chapter 3 we describe that in the absence of AVP, AQP2 abundance in mpkCCD cells was increased by PGE_2, in line with the abovementioned effects of PGE_2 on water reabsorption. In the presence of the AVP analogue dDAVP, PGE_2 reduced AQP2 abundance. Besides PGE_2, $PGF_{2\alpha}$ application also reduced AQP2 abundance, which is in agreement with previous findings that $PGF_{2\alpha}$ inhibits AVP-stimulated water permeability in the collecting duct (40; 41). Blocking prostaglandin production by indomethacin in dDAVP-stimulated mpkCCD

cells increased AQP2 levels, showing that in cells without indomethacin, dDAVP-stimulated AQP2 abundance is decreased by the action of endogenously produced prostaglandins.

The major prostaglandins produced in mpkCCD cells were PGE_2 and $PGF_{2\alpha}$. In agreement with these findings, PGE_2 is the most abundant prostanoid in the kidney, followed by PGI_2 and $PGF_{2\alpha}$. (42). We showed that dDAVP application significantly increased PGE_2 production in mpkCCD cells, in agreement with studies showing that AVP stimulates PGE_2 synthesis in isolated collecting ducts (43; 44). $PGF_{2\alpha}$ levels were decreased by dDAVP in mpkCCD cells, and PGD_2 increased, although PGD_2 levels were still low compared PGE2 and $PGF_{2\alpha}$. Although no PGD_2 receptor (DP) was detected in mpkCCD cells, the increase in PGD_2 production could counteract dDAVP-induced AQP2 abundance via the prostaglandin $PGF_{2\alpha}$ receptor (FP), as PGD_2 has been shown to bind to the FP receptor with an affinity close to that for the DP receptor (45). The expression of the FP receptor was increased by dDAVP application to mpkCCD cells.

Incubation of mpkCCD cells with dDAVP resulted in an increased expression of the calcium mobilizing PGE_2 receptor EP1, but decreased the expression of the EP4 receptor, coupling to Gs-stimulated cAMP generation. As the inhibitory effects of PGE_2 on AVP-induced water reabsorption most likely occur through activation of EP1 and/or EP3 receptors (38; 46; 47), whereas the stimulatory effects of PGE_2 on basal water transport could be mediated via the EP4 receptor, this suggests that the differences in PGE_2-mediated actions on water permeability can most likely be attributed to the different relative expression of the E-prostanoid receptor subtypes (Fig. 3).

In contrast to *in vivo*, no expression of EP3 was found in the mpkCCD cells. EP3 activation is, besides EP1, also suggested to inhibit water reabsorption (48). Based on the observation above that the dDAVP signaling exerts a positive feedback on the receptor activating an opposite pathway, it would be expected that EP3 expression would also be increased *in vivo* after AVP release. However, this needs further investigation.

Altogether, our study shows that in mpkCCD cells, both PGE_2 and $PGF_{2\alpha}$ decrease dDAVP-stimulated AQP2 abundance, while in the absence of dDAVP, PGE_2 increases AQP2 levels, explaining the effects on water reabsorption *in vivo*. The different PGE_2 effects might be explained by the different receptor subtype expression induced by dDAVP. Based on our data, it is likely that *in vivo* AVP increases, besides AQP2, the expression of EP1 and EP3 and decreases expression of EP4. Consequently, upon conditions with increased PGE_2 production, such as ATP release (see below), AVP-induced AQP2 abundance would be reduced via activation of these EP1/3 receptors.

In addition to its role in ambient water homeostasis, PGE_2 has been suggested to play an important role in pathological conditions such as lithium-induced NDI and bilateral ureteral obstruction, associated with AQP2 down-regulation, increased abundance of the enzyme

cyclooxygenase 2 (COX-2), involved in prostaglandin production, and increased urinary PGE_2 levels (49; 50). Based on our data, these increased PGE_2 production will lead to a decrease in AVP-induced AQP2 abundance via activation of the EP1/3 receptors. This has to be established in future studies.

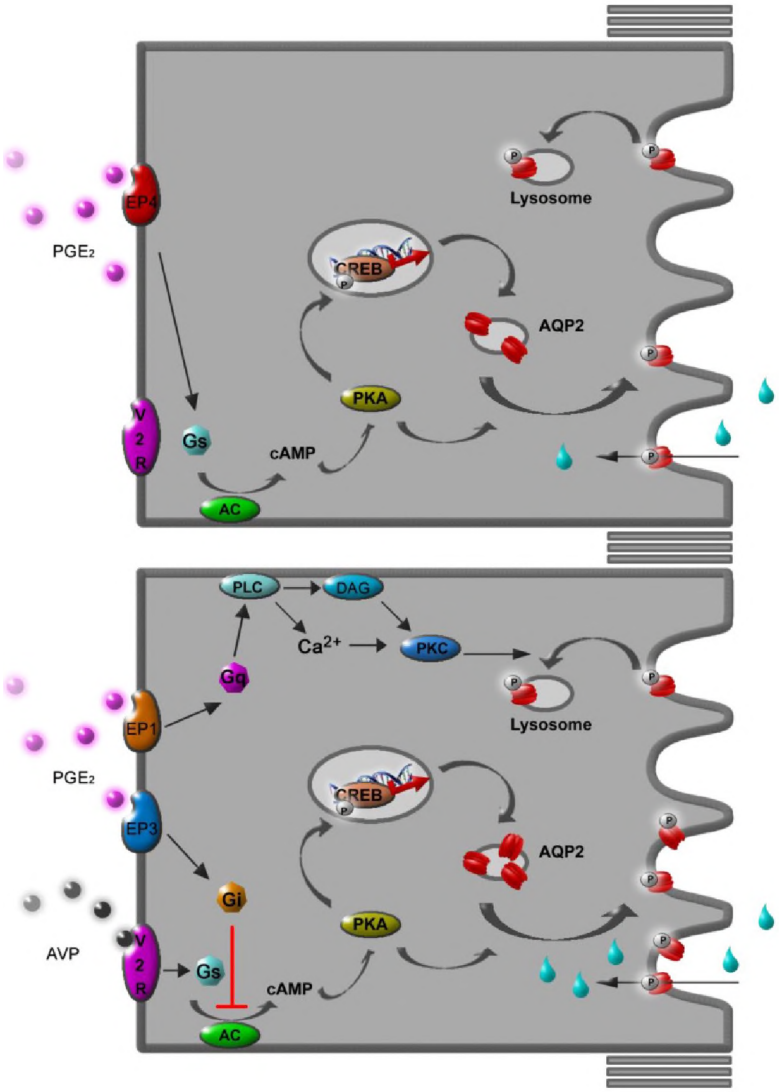

Figure 3. Model of PGE_2-mediated regulation of AQP2-mediated water reabsorption. In the absence of AVP, prostaglandin E2 (PGE_2) stimulates water reabsorption by binding to the EP4 receptor, coupling to the Gs protein, leading to cAMP generation, followed by AQP2 transcription and translocation. AVP induces the expression of the AVP-counteracting EP1 receptor, and possibly also EP3, and reduces EP4. Indicated are adenylate cyclase (*AC*), aquaporin-2 (*AQP2*), vasopressin (*AVP*), cyclic adenosine monophosphate (*cAMP*), Diacylglycerol (*DAG*), protein kinase A (*PKA*), protein kinase C (*PKC*), phospholipase C (*PLC*), and vasopressin V2 receptor (*V2R*).

Compensatory mechanisms of AQP2 regulation

It is interesting that, while dDAVP increases PGE_2 production and release, the mRNA expression of the EP4 receptor is reduced, whereas that of the EP1 receptor is increased. As both are bound and activated by PGE_2, these data suggest that it is not the agonist per se that determines the expression level of the receptors. Instead, our data indicate that the signaling cascade that is mainly activated exerts a negative feedback regulation on receptors stimulating the same pathway and a positive feedback on receptors activating an opposite pathway: dDAVP increases the cAMP-AQP2 pathway, which is also stimulated by EP4, whereas EP1 activates a pathway that leads to decreased AQP2 abundance and water permeability. Similarly, the increased FP receptor after dDAVP incubation of mpkCCD cells

might be a compensatory mechanism to counteract AVP-stimulation as well, as FP receptor activation inhibits water reabsorption (40).
The reverse is seen in chapter 7, were incubation with lithium leads to an AQP2 down-regulation and at the same time a decrease in the expression of the FP and EP1 receptors. As signaling via these receptors is expected to inhibit water reabsorption (40; 51), a down-regulation of these receptors may be a compensatory mechanism in response to the lithium-induced AQP2 down-regulation. In a similar way, dDAVP increases the mRNA levels of the purinergic receptor subunit $P2Y_2$ in mpkCCD cells, and targets the subunits $P2Y_2$ and $P2X_2$ to the plasma membrane, where activation of these receptors leads to AQP2 internalization and a decrease in water permeability (21). The same mechanism can be seen in the opposite response, where endothelin, counteracting AVP-mediated water permeability (52), leads to an increased abundance of the vasopressin V2 receptor in the inner medullary collecting duct of the rat (53). These feedback mechanisms might function to locally fine-tune the AQP2-mediated water reabsorption.

Regulation of AQP2 by integrative action of AVP counteracting hormones
As described above, several hormones or chemical messengers can counteract the AVP-stimulated water reabsorption. However, these hormones do not exert their effects completely independently, as for example the ATP-mediated AQP2 decrease may be mediated indirectly through prostaglandin receptors. Extracellular purines have been shown to stimulate P2Y2 receptor-mediated release of arachidonic acid leading to increased PGE_2 production (54), which activates the AVP-counteracting receptors EP1 and EP3 (Fig. 2). Like ATP, dopamine could potentially mediate AQP2 down-regulation indirectly via prostaglandins, as dopamine has been shown to stimulate prostaglandin production in isolated rabbit kidney and cultured inner medulla collecting duct cells (55; 56). However, the mechanism for this increased prostaglandin release is unclear.
Besides prostaglandins, dopamine might also exert its effect through increased nitric oxide (NO) release. NO is known to decrease AVP-induced AQP2 abundance as well (57; 58). Dopamine has been shown to increase both NO synthase activity in the medullary collecting duct and the urinary excretion of NO metabolites, and blocking NO production attenuated the diuresis and natriuresis caused by dopamine (59; 60), suggesting that part of the dopamine effect could be mediated by increased NO release.

Regulation of AQP2 by tonicity
Osmolality not only affects plasma AVP, but also appears to have direct effects on AQP2 expression and urinary concentrating ability. SIADH, for example, goes with excessive water uptake and hypotonicity. The extent of hypotonicity, however, is much less than anticipated

from the blood AVP concentrations (61), which is ascribed to a defense mechanism, the vasopressin escape, and has been shown to involve a down-regulation of AQP2 (62). Hypotonicity and/or volume expansion have been proposed to mediate this AVP-independent direct regulation of AQP2 expression (63).

Consistently, an AVP-independent increase of AQP2 expression has been suggested to occur with *hyper*tonicity. Water deprivation, hyperglycemia or increased blood NaCl levels of AVP-deficient Brattleboro rats leads to hypertonicity, increased papillary interstitial osmolality, increased AQP2 abundance, and concentration of the urine (64; 65).

In mpkCCD cells Hasler *et al.* showed that hypertonicity decreases the protein abundance of AQP2 on the short term, but increases its abundance on the long term (66). Here, hypertonicity did not affect the stability of AQP2 mRNA or protein, indicating that hypertonicity increases AQP2 abundance by increasing its transcription. Proteins suggested to be involved in the hypertonicity response are the tonicity-responsive enhancer binding protein (TonEBP) and the nuclear factor of activated T cells c (NFATc) (67; 68).

In chapter 4 we described that mpkCCD cells transfected with an AQP2-promoter luciferase construct showed a reduced and increased AQP2 abundance and transcription following culture in hypotonic and hypertonic medium, respectively. This depended on tonicity rather than osmolality.

It has been shown that production of both PGE_2 and NO, known to decrease AVP-induced AQP2 abundance (37; 39; 57; 58), are significantly increased in a vasopressin escape animal model. Although PGE_2 and NO reduced AQP2 protein abundance in mpkCCD cells, inhibition of their synthesis did not influence tonicity-induced AQP2 transcription, indicating that the tonicity-regulated AQP2 transcription is independent of NO or PGE_2. Increased prostaglandins and nitric oxide, as found *in vivo* in vasopressin escape (69; 70), may however contribute to reduced AQP2 levels, probably by increasing AQP2 degradation.

The effect of tonicity on AQP2 transcription occurred independent of the AVP analogue dDAVP, cAMP levels or PKA activity. Also, cells in which the cAMP responsive element (CRE) or the tonicity-responsive element (TonE) in the AQP2-promoter was mutated showed a similar response to hypotonicity. Instead, the tonicity-responsive elements were pin-pointed to nucleotides -283 to -252 and -157 to -126 bp of the AQP2 promoter. In these segments, binding sites for nuclear factor kappa B (NF-kappaB), SP1 and GATA were found. It remains to be established whether these transcription factors and their promoter elements are responsible for the tonicity effect on AQP2 transcription.

As we found in chapter 2 that ATP decreases AQP2 transcription and is released during hypotonicity-induced cell swelling, ATP might be a candidate involved in the signaling pathway involved in tonicity-regulated AQP2 transcription. This needs, however, further investigation.

Identification of the transcription factors and signaling proteins involved in hypotonicity-regulated AQP2 expression in follow-up experiments will provide a better insight into the physiological regulation of AQP2 expression and renal water reabsorption by tonicity and may lead to the discovery of targets for modulation of pathophysiological conditions of osmoregulation, such as SIADH.

Long-term regulation of AQP2 by vasopressin

AVP stimulation leads to phosphorylation of AQP2 at Ser256 and subsequent redistribution of AQP2 water channels from intracellular vesicles into the apical membrane (5; 7; 30). Besides, it increases transcription of AQP2 and hence increases AQP2 abundance (8; 9). Changes in expression of AQP2 have been attributed to PKA-induced phosphorylation of the cAMP responsive element binding protein (CREB), which stimulates transcription from the AQP2 promoter via the CRE (71-73). Interestingly, cAMP levels peak immediately after addition of the stable AVP analogue dDAVP, but are reduced to low levels within 120 minutes following addition in AQP2-transfected MDCK cells (74). AQP2 levels, however, continue to rise during the first three days of dDAVP exposure in mpkCCD cells (75). Therefore, the purpose of chapter 5 was to investigate the molecular mechanism responsible for the AVP-induced increase in AQP2 expression after long-term application of dDAVP using the mpkCCD cell line.

While short-term (30 min) dDAVP stimulation extensively increased cAMP levels, cAMP was strongly reduced with long-term (4 days) compared to short-term dDAVP stimulation, although it was still significantly higher than of control cells. dDAVP stimulation for 1 day increased AQP2 promoter dependent transcription, which could be blocked by H89, a specific inhibitor of PKA. Moreover, phosphorylation of CREB and CRE-dependent transcription were observed after short-term dDAVP application. With 4 days of dDAVP stimulation AQP2 abundance remained elevated, but this increase could no longer be blocked by H89. Moreover, CRE-dependent transcription and CREB phosphorylation were not increased with 4 days of dDAVP stimulation, indicating that the long-term regulation of AQP2 by dDAVP occurs independent of PKA and CREB.

The observed changes in the cAMP-PKA-CRE pathway may be the consequence of a desensitization to AVP. In agreement with our results, Dublineau *et al.* also showed that in isolated collecting ducts of rats treated with dDAVP for 3 days, addition of AVP evoked a smaller cAMP response than in untreated rats (76). However, Dublineau *et al.* also showed in similarly treated rats that, like in our mpkCCD cells, cAMP was still increased compared to untreated rats (77). A possible explanation for the lower cAMP production after 4 days dDAVP is a lower receptor expression on the cell surface, in line with earlier observations that V2R receptor activation leads to its internalization (78). The changes in the cAMP-PKA-

CRE pathway may also be the consequence of an intracellular desensitization to dDAVP, which can be caused by several factors, as continuous stimulation of the cAMP-PKA-CRE pathway has been shown to lead to the synthesis of ICER (inducible cyclic AMP response element repressor) which reduces CRE-dependent transcription (79), a desensitization of PKA (80), or a desensitization of adenylate cyclase 6, expressed in the collecting duct (81; 82). Although our experiments were done in the presence of a phosphodiesterase blocker, *in vivo* cAMP stimulation also leads to an upregulation of cAMP phosphodiesterases, which may further decrease cAMP levels (83).

In conclusion, our study shows that the PKA-CRE pathway is involved in the initial increase in AQP2 abundance after dDAVP stimulation, but the long-term regulation of AQP2 by dDAVP occurs independently of PKA and CREB.

Regulation of AQP2 by Epac

Our results in chapter 5 further indicate that long-term regulation of AQP2 by dDAVP may involve the exchange protein directly activated by cAMP (Epac). In agreement with *in vivo* studies, where both Epac1 and Epac2 proteins were found in the collecting duct, mpkCCD cells endogenously express Epac1 and Epac2 proteins. Epac1 is mainly expressed in intercalated cells, although it is also found in principal cells in the outer medulla. Epac2 is highly expressed in all principal cells all along the collecting duct (84).

In mpkCCD cells, Epac1 abundance was stimulated by dDAVP, while Epac2 abundance was reduced, suggesting that AVP regulates Epac protein expression. Epac activation alone, without dDAVP preincubation, did not increase the expression of AQP2. However, stimulation of Epac activity after sustained dDAVP stimulation increased both AQP2 transcription and AQP2 protein abundance, although AQP2 abundance was not maintained to the same level with the Epac activator as with dDAVP. As after prolonged dDAVP incubation, cAMP levels are still increased compared to control cells, Epac activation by the increased cAMP levels may form part of the PKA independent pathway to maintain high AQP2 abundance levels at long-term dDAVP incubation. The reduced level of maintaining AQP2 levels may be due to instability of the Epac stimulator over the 24-hour period used, or may indicate that Epac is not the only factor involved in stimulating long-term AQP2 expression. It remains to be established whether Epac has a similar role *in vivo*.

In conclusion, our study shows that long-term regulation of AQP2 may involve the activation of Epac. Besides an effect on AQP2 transcription, Epac activation also leads to Ca^{2+} mobilization, increasing AVP-stimulated translocation of AQP2 to the apical membrane, what suggests that both PKA and Epac are part of the signaling events in AVP-stimulated AQP2 trafficking (85). The distinct localization of Epac1 and Epac2 in the collecting duct would imply that Epac2 rather than Epac1 mediates the stimulatory effects on AQP2.

The pathway by which Epac activation increases AQP2 expression is not known. Epac selectively activates the Ras-like small GTPase Rap1 and Rap2. Epac has been shown to be involved in the effect of calcitonin on the H,K-ATPase in collecting duct intercalated cells by a cAMP/Epac/Rap-1/Raf-B/ERK cascade (86) and ERK-inhibition has been shown to prevent the insulin-mediated increase of AQP2 expression in mpkCCD cells, suggesting a role of ERK in AQP2 regulation (87). Epac has also been shown to stimulate integrin-mediated cell adhesion by influencing focal adhesions (88; 89), either by Rap1-mediated activation of integrins, or by increasing phosphorylated paxillin, involved in integrin-regulation (90). Recently, integrins have been suggested to influence AQP2 expression (91). Whether integrins or ERK are indeed involved in Epac-mediated AQP2 expression remains to be established.

Transcriptional control of AQP2

It is well known that AVP, on the short term, increases AQP2 transcription via an increase in cAMP, leading to phosphorylation of the cAMP responsive element binding protein (CREB), which stimulates transcription from the AQP2 promoter (71-73). We showed that Epac activation increases transcription as well (chapter 5), however, the pathway is not known. Besides AVP, we found many other factors to affect AQP2 transcription. Dopamine and ATP decreased cAMP levels (chapter 2), suggesting a reduction of AQP2 transcription would be by a decrease in PKA-mediated CREB phosphorylation or the pathway activated by Epac, counteracting directly the pathway activated by AVP. Demeclocycline decreases adenylate cyclase abundance and thereby cAMP generation (chapter 6), and the demeclocycline-mediated decrease of AQP2 transcription will therefore also likely be a direct effect on the pathway activated by AVP. Tonicity did not affect cAMP levels, and the tonicity-responsive elements were pin-pointed to nucleotides -283 to -252 and -157 to -126 bp of the AQP2 promoter (chapter 4). In these segments, binding sites for nuclear factor kappa B (NF-kappaB), SP1 and GATA were found. Like tonicity, lithium also decreases AQP2 independently of cAMP (75). We show in chapter 7 that the effect of lithium on AQP2 transcription involves the most proximal 200 bp of the AQP2 promoter. The exact transcription factor involved needs however to be identified.

Pharmacological regulation of AQP2

Regulation of AQP2 by demeclocycline

Demeclocycline is a bacteriostatic antibiotic of the tetracycline group, which has been shown to cause water diuresis and NDI (92; 93). Because of the effect on water diuresis,

demeclocycline is currently used to treat sustained hyponatremia in patients with SIADH (94). Demeclocycline has been shown to restore the sodium plasma concentration in SIADH patients to normal levels, permitting unrestricted water intake in these patients (95).

Wilson *et al.* showed that the demeclocycline-induced diuresis is exerted by selective inhibition of the water reabsorption in the distal part of the nephron (96). Others have shown that demeclocycine inhibits the AVP-induced osmotic water flow in the toad urinary bladder (92; 97; 98), a model system of the mammalian collecting duct. Just like demeclocycline, the tetracycline antibiotics minocycline, doxycycline and tetracycline have been shown to reduce the water flow in the toad bladder (97), and tetracycline has been reported to decrease urinary concentrating ability in men (96), suggesting that other tetracycline antibiotics affect collecting duct function and urinary concentrating ability as well.

In chapter 6, we showed that demeclocycline and minocycline down-regulated AQP2 abundance in the mpkCCD cell line in a time- and concentration-dependent manner. Demeclocycline down-regulates AQP2 at concentrations that are equivalent to those measured in urine from patients (99), illustrating that the effects observed are at pharmacologically-relevant doses and that the demeclocycline-mediated diuresis in patients is caused by a decrease in AQP2 abundance.

The protein synthesis inhibitor cycloheximide abolishes the effect of demeclocycline on AQP2, suggesting that a decrease in AQP2 production explains the decrease in AQP2 abundance. Using an AQP2 promoter-luciferase reporter construct, we showed that demeclocycline decreases AQP2 transcription. In regard to the intracellular cell signaling, we found a blunting of dDAVP-induced cAMP production, which is in line with findings of Dousa and Wilson showing a decreased basal and AVP-induced cAMP generation in renal medulla tissue (100). Our results also show a decreased abundance of both adenylate cyclase 3 and 6 after demeclocycline incubation, which explains the lower cAMP generation.

In conclusion, our data show that the diuretic effect of tetracycline antibiotics such as demeclocycline and minocycline is mediated via down-regulation of the water channel AQP2. Demeclocycline decreases AQP2 gene transcription by decreasing adenylate cyclase abundance and cAMP generation. Its effect on AQP2 transcription will therefore likely be by reducing AVP-mediated PKA or Epac activation. If demeclocycline has a similar effect *in vivo* needs further investigation.

Regulation of AQP2 in lithium-induced NDI

Lithium is regularly used to treat psychiatric diseases, such as bipolar disorders, schizoaffective disorders and depression. Unfortunately, approximately 20% of patients undergoing lithium treatment develop NDI (101-103). In fact, lithium-induced NDI is the most common form of NDI.

Studies in rats have shown that lithium-induced NDI occurs in conjunction with AQP2 down-regulation (104; 105) and in agreement with this, lithium treatment reduces urinary AQP2 excretion in humans, indicating a decreased renal AQP2 expression (103). Chronic lithium treatment also leads to a severe decrease in the fraction of principal cells in rats. This is 'compensated' by an increase in the fraction of intercalated cells, which are involved in acid/base balance regulation (106; 107).

Furthermore, lithium leads to inactivation of glycogen synthase kinase (Gsk) 3β and this is temporally related to an increased renal COX-2 abundance and increased urinary PGE_2 excretion (49; 108). As PGE_2 reduces AVP-stimulated water reabsorption, as discussed in chapter 3, this suggests an important role for PGE_2 in lithium-induced NDI development.

In chapter 7, we investigated the potential role of prostaglandins in lithium-induced down-regulation of AQP2 in mpkCCD cells. As *in vivo*, the reduced AQP2 abundance after lithium incubation coincided with Gsk3β inactivation and increased COX-2 abundance. The lithium-induced increase in COX-2 abundance did however not result in elevated prostaglandin levels in media of mpkCCD cells. This might be explained by the fact that the availability of free arachidonic acid, the substrate for production of prostaglandins, and not COX, is the rate-limiting step in in prostaglandin production in most tissues and cells (109).

Similarly to the increase in AQP2 abundance after treatment with the COX-blocker indomethacin (chapter 3), dexamethasone-induced down-regulation of COX-2 coincided with increased AQP2 abundance. However, dexamethasone did not affect the lithium-induced upregulation of COX-2 nor down-regulation of AQP2.

Indomethacin significantly reduced PGE_2 and $PGF_{2\alpha}$ levels in lithium-treated cells, suggesting that a reduction of PGE_2 and/or $PGF_{2\alpha}$ is involved in the attenuating effect of indomethacin on the lithium-induced AQP2 down-regulation. In line with this, addition of PGE_2 or $PGF_{2\alpha}$ to dDAVP-treated mpkCCD cells resulted in reduced AQP2 levels as discussed in chapter 3.

However, as no effect of lithium on prostaglandin production was found in mpkCCD cells, this suggests that the lithium-induced AQP2 down-regulation in these cells occurs independently of prostaglandins. This is also suggested by the observation that in the presence of indomethacin, lithium still decreased AQP2 abundance compared to cells incubated with indomethacin alone.

Addition of PGE_2 or $PGF_{2\alpha}$, reducing AQP2 protein abundance, did not decrease AQP2 transcription or mRNA levels. In line with this, indomethacin, blocking prostaglandin production, increased dDAVP-stimulated AQP2 protein abundance, but did not increase AQP2 transcription or mRNA levels. Prostaglandin-induced AQP2 down-regulation could be prevented by co-incubation with the lysosome inhibitor chloroquine, showing that the effect of

prostaglandins on AQP2 abundance in mkpCCD cells is mediated by increasing AQP2 degradation.

Lithium, however, did not affect AQP2 degradation, in agreement with previous results (75), but decreased AQP2 mRNA by decreasing AQP2 gene transcription. Our results show that the lithium-sensitive AQP2 promoter segment is within the most proximal 200 bp. As lithium decreases AQP2 transcription in contrast to prostaglandins, these data indicate that the effect of lithium on AQP2 transcription is independent of prostaglandin production.

In vivo data show that blocking prostaglandin production by indomethacin reduces urine volume and increases AQP2 abundance of lithium-treated rats (110) and reduces the urine volume of lithium-induced NDI patients as well (111; 112). Extrapolated to this *in vivo* data and our data showing that addition of PGE_2 to mpkCCD cells, simulating the increase in PGE_2 found *in vivo*, leads to AQP2 down-regulation in mpkCCD cells, this indicates that the increased prostaglandin levels in the kidney in lithium-NDI might contribute to the reduced AQP2 abundance observed. However, this does not contribute to the effect of lithium on AQP2 gene transcription.

A limitation of our cell model is the absence of the EP3 receptor, which is found *in vivo* in the collecting duct. EP3 activation inhibits cAMP generation via Gi (51), and might therefore inhibit AVP-stimulated AQP2 transcription by decreasing CRE-mediated transcription. Although this might contribute to the AQP2 down-regulation induced by PGE_2, earlier studies show no decrease in cAMP after lithium treatment in *vivo* (75), suggesting that an *in vivo* effect of lithium on AQP2 transcription is EP3-independent. Conclusive evidence for the roles of particular prostaglandin receptors in mediating a prostaglandin-induced down-regulation of AQP2 in lithium-induced NDI awaits studies using collecting duct specific knockout of these receptors.

In conclusion, our data reveal that in mpkCCD cells lithium decreases AQP2 protein abundance as well as AQP2 gene transcription. Lithium decreases AQP2 transcription independently of the endogenous prostaglandin production in mpkCCD cells. Our data furthermore show that in mpkCCD cells, released prostaglandins decrease AQP2 protein stability by increasing its lysosomal degradation, Based on these observations, *in vivo* paracrine produced prostaglandins might have an additional role in lithium-induced NDI by decreasing AQP2 protein stability, without affecting AQP2 transcription.

Protection of AQP2 down-regulation in lithium-induced NDI

To further investigate the mechanism of lithium-induced NDI and to investigate if lithium-induced NDI can be attenuated, we investigated in chapter 8 the effect of amiloride, blocking the epithelial sodium channel ENaC, which is located in the apical membrane of principal cells.

In the mouse collecting duct cell lines $mCCD_{c11}$ and mpkCCD, the AVP-induced AQP2 abundance was time- and dose-dependently reduced by clinically-relevant lithium concentrations, which could be prevented by co-incubation with the ENaC blockers amiloride or benzamil. Consistently, amiloride reduced transcellular lithium transport, the intracellular lithium concentration and lithium-induced inactivation of Gsk3β. Overall, our study shows that ENaC is the major cellular entry pathway for lithium and that blocking ENaC reduces lithium-induced Gsk3β inactivation and prevents AQP2 down-regulation.

In vivo, simultaneous treatment of rats with amiloride attenuated the AQP2 down-regulation, and completely prevented the reduction of principal/intercalated cell ratio. Amiloride treatment furthermore decreased urine volume and increased urine osmolality, although values were still different from those of control rats. Taken together, our data indicate that blocking ENaC with amiloride has a protective effect on lithium-induced NDI development by preventing the lithium-induced change in cellular composition and partial protection of AQP2 down-regulation, leading to a preserved concentrating ability of the collecting duct. As such, our data provide a rationale for the use of amiloride as a treatment for lithium-induced NDI.

The complete prevention of the change in cell ratio and the partial protection of AQP2 down-regulation by amiloride suggests that AQP2 expression is more sensitive to lithium than the integrity of the principal cells, which is in agreement with the fact that AQP2 down-regulation precedes the fractional decrease in principal cells in lithium-induced NDI development (104; 107). It furthermore suggests that the used amiloride concentration did not completely prevent the deleterious effect of lithium, as also seen in the mCCD cells. This could be due to an incomplete block of ENaC by amiloride, although the amiloride dose given to the rats and cell lines is expected to block ENaC completely, or this could be due to cellular lithium influx via other transporters or channels.

A recent paper showed that collecting duct specific αENaC knockout mice did not demonstrate the polyuria and reduction in urine osmolality induced by lithium treatment, and lithium treatment reduced AQP2 protein levels only in the inner medulla in these mice, not in the cortex or outer medulla (113). The protection from lithium-induced NDI by the absence of functional ENaC in the collecting duct supports our conclusion that ENaC is the main entry pathway into the principal cells. The remaining decrease of AQP2 in the inner medulla suggests an additional mechanism of AQP2 down-regulation, either another entry pathway into the principal cells, or increased prostaglandin release from interstitial cells. The absence of an NDI phenotype in the αENaC knockout mice suggests that the role of this additional mechanism is small. However, as the serum concentration of lithium in knockout mice given lithium was lower than the concentration in control mice administered lithium, and also below the clinically used concentrations, it cannot be excluded that a clinically relevant lithium dose

would induce a more severe AQP2 down-regulation and the development of an NDI phenotype in the αENaC knockout mice.

Based on our own studies and earlier data, we propose the following model for the mechanism of lithium-induced NDI (Fig. 4): lithium enters the principal cells predominantly through the epithelial sodium channel ENaC (chapter 8), leading to AQP2 down-regulation, and, on the long term, to an increase in apoptosis as well as proliferation, resulting in a decreased fraction of principal cells (106; 107; 114). Lithium entry in the principal cell leads to inactivation of Gsk3β (chapter 8, (49)). In line with an important role of Gsk3β in AQP2 expression, also other Gsk3β inhibitors lead to a down-regulation of AQP2 expression in mpkCCD cells, as shown in chaper 8. Inactivation of Gsk3β leads to an increased abundance of COX-2 (49; 115), resulting in increased production of PGE_2 (49; 108) by renal medullary interstitial cells and, possibly, renal principal cells, leading to an increase in AQP2 protein degradation.

Besides a change in COX-2 abundance, a change in the purinergic system could also be involved in the increased PGE_2 production seen with lithium treatment. Recently, lithium has been shown to cause a change in relative purinergic receptor expression, and a P2Y2 knockout mouse shows a reduced sensitivity to develop lithium-induced NDI (116; 117),

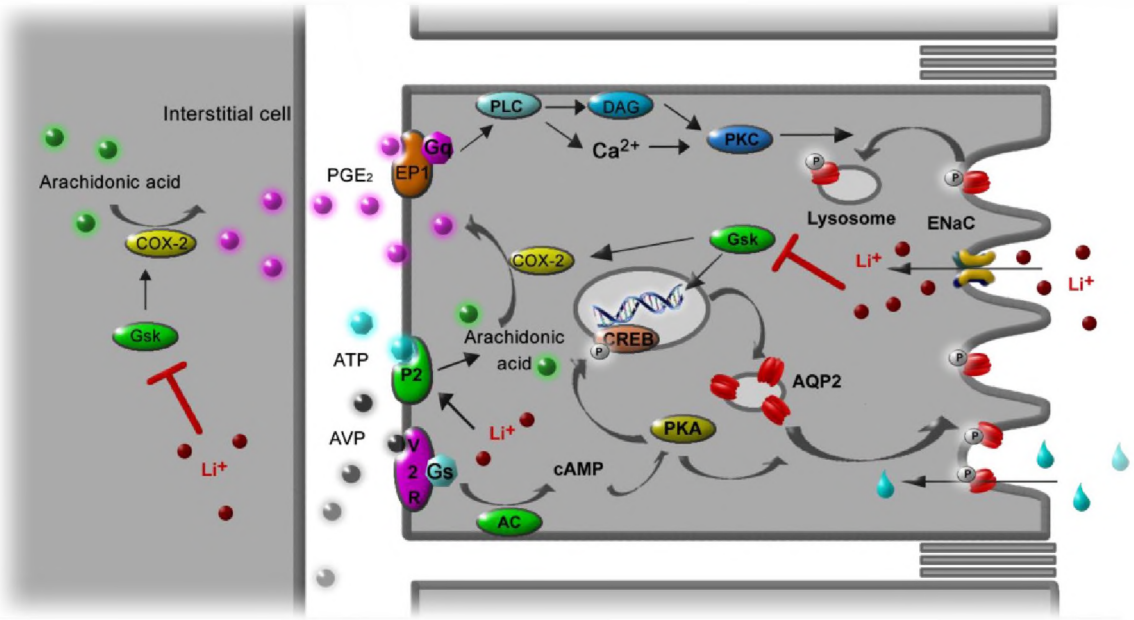

Figure 4. Model of the lithium-induced inhibition of AQP2-mediated water reabsorption. Lithium enters the principal cells through the epithelial sodium channel ENaC, leading to inactivation of Gsk3β, thereby reducing AQP2 transcription. Inactivation of Gsk3 in interstitial cells, and possibly principal cells, leads to an increased expression of COX-2, resulting in increased production of PGE_2. Binding of PGE_2 to the EP1 receptor leads to an increase in AQP2 protein degradation. Lithium might also effect purinergic receptor expression or activation, leading to increased arachidonic acid availability, further stimulating PGE_2 release. Indicated are adenylate cyclase (*AC*), aquaporin-2 (*AQP2*), vasopressin (*AVP*), cyclic adenosine monophosphate (*cAMP*), cyclooxygenase-2 (*COX-2*), Diacylglycerol (*DAG*), lithium (*Li*), purinergic receptor (*P2*), protein kinase A (*PKA*), protein kinase C (*PKC*), phospholipase C (*PLC*) and vasopressin V2 receptor (*V2R*).

suggesting a role of purinergic receptor activation in lithium-induced NDI.
Besides the effect on protein degradation, lithium also decreases AQP2 expression by reducing AQP2 gene transcription, which is prostaglandin independent (chapter 7), and is likely a consequence of inhibition of one of the other pleiotropic pathways steered by Gsk3β, like the pathways involving the transcription factors Nuclear factor of activated T-cells (NFAT), β-catenin, or hypoxia-inducible factor (HIF).
The two different pathways leading to a decrease in AQP2 abundance might also have consequences for the clinical treatment of lithium-induced NDI. Both COX inhibitors and the ENaC blocker amiloride have been shown to individually reduce the NDI phenotype (111; 112; 118-120). However, as the effect of lithium on prostaglandin production could be blocked by COX inhibitors, and the entry of lithium into the principal cells leading to a decrease in AQP2 transcription could be blocked by amiloride treatment, this suggest a benicifial effect of combining these two therapies for the treatment of lithium-induced NDI.

Concluding remarks

In this thesis, we unraveled a small part of the complex regulation of AQP2 trafficking and expression. Besides AVP, hormones as dopamine and ATP are tightly involved in AQP2 regulation, as they induce ubiquitination, internalization and degradation of AQP2, thereby inhibiting AVP-induced water permeability. One of the challenges of the future is to further identify the receptors and signaling pathways involved in these effects. Similarly, prostaglandins PGE_2 and $PGF_{2\alpha}$ decrease AVP-induced AQP2 abundance by increasing AQP2 degradation, leading to decreased water permeability. In the absence of AVP, PGE_2 stimulates AQP2 abundance, thereby increasing water permeability. This difference in PGE_2 effects might be explained by an AVP-induced change in relative receptor expression, increasing inhibitory pathways and decreasing stimulatory pathways. Tonicity also stimulates AQP2 expression, however, this is independent of AVP, and in contrast to the prostaglandin effects, this affects AQP2 transcription. Tonicity-responsive regions in the AQP2 promoter were identified. It remains to be established which transcription factors and their promoter elements are responsible for the tonicity effect on AQP2 transcription.
The regulation of AQP2 by AVP was further elucidated by showing that the PKA-CRE pathway is involved in the initial increase in AQP2 abundance after AVP stimulation, but not in the long-term effect of AVP. Instead, long-term regulation of AQP2 may involve the activation of Epac. Further studies are necessary to determine the role of Epac *in vivo*, and the signaling pathway activated by Epac.

The tetracycline antibiotic demeclocycline was shown to cause diuresis via down-regulation of AQP2. Demeclocycline decreases AQP2 protein abundance and gene transcription by decreasing adenylate cyclase abundance and cAMP generation. Further studies are necessary to confirm that demeclocycline has a similar effect *in vivo*.

Lithium was shown to mainly enter the principal cell by the epithelial sodium channel ENaC, leading to a decreased AQP2 gene transcription, which is prostaglandin independent. The *in vivo* observed increased PGE_2 levels in lithium-induced NDI might decrease AQP2 protein stability by increasing its lysosomal degradation. Blocking lithium entry with amiloride attenuates lithium-NDI by preventing AQP2 down-regulation and the change in principal/intercalated cell ratio. As such, our data provide a rationale for the use of amiloride as a treatment for lithium-NDI.

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CHAPTER 10

Samenvatting

Samenvatting

Behoud van de waterbalans door het reguleren van zowel de osmolaliteit als het bloedvolume is essentieel voor de meeste fysiologische processen in het menselijk lichaam. De waterbalans in het lichaam wordt nauw gereguleerd via de waterinname en de waterexcretie in de urine. Verhoging van de natriumconcentratie in het bloed zorgt voor een activatie van specifieke osmoreceptoren in de hypothalamus, wat resulteert in een dorstprikkel en de daaropvolgende inname van water. Tegelijkertijd wordt het hormoon arginine vasopressine (AVP) uitgescheiden door de hypofyse en komt in de bloedbaan terecht. Behalve door de natriumconcentratie, wordt AVP-uitscheiding ook beïnvloed door het bloedvolume en veranderingen van de bloeddruk, gedetecteerd door baroreceptoren. Het vrijgekomen AVP regelt de hoeveelheid water in het lichaam, doordat het zorgt dat de nieren water uit de voorurine reabsorberen en daardoor het urinevolume verminderen.

Zoals uiteengezet in dit proefschrift, speelt het aquaporine-2 (AQP2) waterkanaal een sleutelrol in de regulatie van de renale waterexcretie en is cruciaal voor de osmoregulatie en de waterbalans in het lichaam. De binding van AVP aan zijn type-2 receptor, aanwezig op het basolaterale membraan van de hoofdcellen in de verzamelbuis en de verbindingsbuis van de nier, induceert een signaalcascade, resulterend in de verplaatsing van AQP2 waterkanalen van intracellulaire blaasjes naar het apicale membraan. Water komt vervolgens via de AQP2 waterkanalen de cel binnen en stroomt vervolgens via de AQP3 en AQP4 waterkanalen in het basolaterale membraan richting het bloed, hetgeen leidt tot een concentrering van de urine. Naast het effect op AQP2-lokalisatie, verhoogt AVP ook AQP2-transcriptie. Zodra de waterbalans hersteld is, daalt de AVP-uitscheiding en wordt AQP2 geïnternaliseerd, waardoor het apicale membraan opnieuw water-ondoorlaatbaar wordt.

Door zijn belangrijke rol in de waterhomeostase, is het niet verwonderlijk dat AQP2 is betrokken bij verschillende ziekten die samenhangen met een verstoorde waterhomeostase, waaronder het syndroom van inadequate secretie van antidiuretisch hormoon (SIADH) en nefrogene diabetes insipidus (NDI). AQP2 wordt nauw gereguleerd, niet alleen door AVP, maar ook door andere hormonen, moleculen en medicijnen. Echter, de exacte moleculaire mechanismen die AQP2 in deze verschillende omstandigheden reguleren zijn nog niet bekend. Het doel van dit proefschrift was het verkrijgen van meer inzicht in de fysiologische en farmacologische regulatie van de renale waterreabsorptie en AQP2.

Naast AVP reguleren diverse andere hormonen de waterhuishouding door middel van inhibitie van AVP-geïnduceerd watertransport. Voorbeelden zijn extracellulaire purines, dopamine, endotheline, epidermale groeifactor en prostaglandine. Het werkingsmechanisme

van deze hormonen is onbekend. Het doel van hoofdstuk 2 was meer inzicht te krijgen in het mechanisme waarmee hormonen het effect van AVP op AQP2 tegengaan, door in detail de werking van ATP en dopamine op de AVP-geïnduceerde AQP2 expressie te analyseren, zowel in een celmodel van de verzamelbuis (mpkCCD-cellen) als in de nier.
Toevoeging van deze hormonen aan mpkCCD-cellen verlaagde zowel de totale hoeveelheid AVP-geïnduceerd AQP2 als de hoeveelheid op het plasmamembraan, onder meer door een verhoogde internalisatie in blaasjes. De vermindering van de AQP2-expressie werd geblokkeerd door co-incubatie met chloroquine, een remmer van lysosomale degradatie, wat aangeeft dat deze hormonen lysosomale afbraak van AQP2 veroorzaken. Eerder is aangetoond dat AQP2-internalisatie en degradatie veroorzaakt door AVP-verwijdering of door proteïne kinase C activatie wordt voorafgegaan door ubiquitinering van AQP2 op Lys270. Ook ATP en dopamine verhogen de ubiquitinering van AQP2. De AQP-internalisatie was ubiquitine-afhankelijk, aangezien de plasmamembraan-lokalisatie van AQP2-K270R, die niet kan worden geübiquitineerd, niet beïnvloed werd door deze hormonen. Eenzelfde proces vindt plaats *in vivo*, waar incubatie van dDAVP-behandelde nierplakjes met beide hormonen ook resulteerde in AQP2-internalisatie. Beide hormonen zorgden ook voor verlaagde cAMP en AQP2 mRNA niveaus, hetgeen wijst op een bijkomend effect op de AQP2-transcriptie.
Hieruit concludeerden we dat ATP en dopamine de AVP-geïnduceerde waterpermeabiliteit tegengaan door het verhogen van AQP2-degradatie in lysosomen, voorafgegaan door ubiquitine-afhankelijke internalisatie, en daarnaast door het verlagen van AQP2-transcriptie door het verminderen van de AVP-geïnduceerde cAMP hoeveelheid.
Onlangs is aangetoond dat, naast de fosforylering van Ser256, waarvan bekend is dat deze belangrijk is voor de AVP-geïnduceerde translocatie naar het apicale membraan, AQP2 ook kan worden gefosforyleerd op Ser261, Ser264 en Ser269. Interessant is dat zowel dopamine als ATP AQP2-fosforylering op Ser261 verhogen, zowel in mpkCCD cellen als in de nier. De exacte functie van deze fosforylering dient nog verder onderzocht te worden.

Zoals hierboven vermeld, zijn ook prostaglandines betrokken bij de regulatie van de waterbalans. Met name van prostaglandine E_2 (PGE_2) is aangetoond dat het de waterdoorlaatbaarheid van verzamelbuisjes vergroot in de afwezigheid van de AVP. In tegenstelling hiermee vermindert PGE_2 de waterreabsorptie in de aanwezigheid van AVP. Het mechanisme is echter nog onduidelijk. In hoofdstuk 3 gebruiken we daarom mpkCCD cellen als modelsysteem voor de renale hoofdcellen om te onderzoeken hoe prostaglandines deze uiteenlopende effecten op het waterreabsorptie in de aanwezigheid of afwezigheid van AVP uit kunnen oefenen.
In afwezigheid van AVP werd AQP2-expressie verhoogd door PGE_2, in overeenstemming met het hierboven vermelde effect van PGE_2 op de waterreabsorptie. In aanwezigheid van

de AVP-analoog dDAVP zorgde PGE_2 voor een verlaging van de AQP2-expressie. Naast PGE_2 zorgde ook $PGF_{2\alpha}$-toevoeging voor een verlaging van de AQP2-expressie, in overeenstemming met eerdere bevindingen dat $PGF_{2\alpha}$ AVP-gestimuleerde waterpermeabiliteiit in de verzamelbuis remt. Het blokkeren van de prostaglandine-productie door indomethacine in dDAVP-gestimuleerde mpkCCD cellen verhoogde de AQP2-expressie, waaruit blijkt dat onder fysiologische condities, dDAVP-gestimuleerde AQP2-expressie verminderd wordt door de werking van endogeen geproduceerde prostaglandines. MpkCCD cellen produceerden vooral PGE_2 en $PGF_{2\alpha}$. dDAVP-toevoeging zorgde voor een toename van de PGE_2 en PGD_2-productie, terwijl $PGF_{2\alpha}$-productie afnam. De prostaglandine-receptoren EP1, EP4, FP en TP werden gedetecteerd via RT-PCR. dDAVP-toevoeging verhoogde de expressie van de EP1 en FP-receptoren, terwijl EP4-expressie afnam.
Samenvattend blijkt uit ons onderzoek dat in dDAVP-gestimuleerde mpkCCD cellen zowel PGE_2 als $PGF_{2\alpha}$ de AQP2-expressie verlagen, terwijl in de afwezigheid van dDAVP, PGE_2 de AQP2-expressie verhoogt. dDAVP zorgt voor een toename in de productie van PGD_2 en PGE_2, en afname van $PGF_{2\alpha}$. Aangezien het remmende effect van PGE_2 op de AVP-geïnduceerde waterreabsorptie zeer waarschijnlijk ontstaat door activatie van EP1 en / of EP3 receptoren, terwijl het stimulerende effect van PGE_2 op het basale watertransport kan worden gemedieerd via de EP4 receptor, suggereert dit dat de verschillen in het PGE_2-gemedieerde effect op de waterpermeabiliteit hoogstwaarschijnlijk wordt veroorzaakt door de verschillende relatieve expressie van de prostaglandine-receptor subtypes.

Osmolaliteit beïnvloedt niet alleen de AVP-afgifte, maar lijkt ook een direct effect te hebben op AQP2-expressie en het urine-concentrerend vermogen. SIADH, bijvoorbeeld, gaat gepaard met te veel waterreabsorptie en verlaagde toniciteit in het bloed. De mate van hypotoniciteit is echter veel minder dan men zou verwachten naar aanleiding van de AVP-concentratie in het bloed. Dit wordt toegeschreven aan een afweermechanisme, de vasopressine-escape, wat gepaard gaat met een AQP2-afname. Het onderliggende mechanisme is echter onduidelijk. Om dit te onderzoeken, gebruikten we de mpkCCD cellijn.
In hoofdstuk 4 vonden we dat in mpkCCD cellen, getransfecteerd met een AQP2-promotor luciferase construct, de AQP2 eiwit-hoeveelheid en transcriptie werden verlaagd en verhoogd na een kweek in hypotoon en hypertoon medium, respectievelijk. Dit hing af van toniciteit in plaats van de osmolaliteit, en was onafhankelijk van de AVP-analoog dDAVP, intracellulaire cAMP-niveaus of proteïne kinase A (PKA)-activiteit. Hoewel prostaglandines en stikstofmonoxide de AQP2-eiwit hoeveelheid verlagen, had remming van hun synthese geen invloed op de toniciteit-geïnduceerde AQP2 transcriptie. Ook cellen waarin het cAMP of toniciteit responsieve element (CRE / TonE) in de AQP2-promotor werden gemuteerd reageerden op een vergelijkbare wijze op hypotoniciteit. In plaats daarvan werden toniciteit-

responsieve elementen gelokaliseerd in de nucleotiden -283 tot -252 en -157 tot -126 bp van de AQP2-promoter.
Concluderend blijkt uit onze gegevens dat hypotoniciteit de AQP2-eiwit expressie en transcriptie verlaagd, en dat dit effect onafhankelijk is van vasopressine, cAMP, en de bekende TonE en CRE in de AQP2-promoter. Verhoogde prostaglandine en stikstofmonoxide-concentraties, zoals gevonden *in vivo*, kunnen bijdragen aan een vermindering van AQP2 in vasopressine-escape, maar zijn niet betrokken bij het effect van hypotoniciteit op AQP2-transcriptie. Onze gegevens suggereren dat twee nieuwe segmenten (-283 tot -252 en -157 tot -126 bp) in de AQP2-promoter betrokken zijn bij de hypotoniciteit geïnduceerde AQP2-afname tijdens vasopressine-escape.

Zoals hierboven beschreven leidt AVP-stimulatie tot fosforylering van AQP2 op Ser256 en een daaropvolgende herverdeling van AQP2-waterkanalen van intracellulaire blaasjes naar het apicale membraan. Daarnaast verhoogt AVP de transcriptie van AQP2 en dus de AQP2 eiwit-hoeveelheid. Veranderingen in de AQP2-expressie zijn toegeschreven aan PKA-geïnduceerde fosforylering van het cAMP-responsieve element bindend eiwit (CREB), die de AQP2 transcriptie stimuleert via de CRE in de AQP2 promoter. Aangezien cAMP-niveaus afnemen kort na AVP-toevoeging, terwijl de AQP2-hoeveelheid nog steeds toeneemt en hoogt blijft, onderzochten we in hoofdstuk 5 het mechanisme verantwoordelijk voor de AQP2-verhoging na een lange termijn dDAVP-toevoeging met behulp van mpkCCD cellen.
Terwijl een korte termijn (30 minuten) dDAVP-stimulatie zorgde voor een sterke toename van de cAMP-hoeveelheid, was cAMP sterk verminderd met lange termijn (4 dagen) dDAVP, hoewel cAMP nog steeds significant meer was dan in ongestimuleerde controle cellen. Eén dag dDAVP-stimulatie zorgde voor een stijging van de AQP2-promoter afhankelijke transcriptie, wat kon worden geblokkeerd door H89, een specifieke remmer van PKA. Bovendien werd fosforylering van CREB en CRE-afhankelijke transcriptie waargenomen na korte termijn dDAVP-toevoeging. Met 4 dagen dDAVP-stimulatie bleef de AQP2-transcriptie verhoogd, maar deze stijging kon niet meer worden geblokkeerd door H89. Bovendien was de CRE-afhankelijke transcriptie en CREB-fosforylering niet toegenomen na 4 dagen dDAVP-stimulatie ten opzichte van ongestimuleerde controle cellen, wat aangeeft dat de lange termijn regulatie van AQP2 door dDAVP onafhankelijk is van PKA en CREB.
Onze resultaten in hoofdstuk 5 laten verder zien dat de lange termijn regulatie van AQP2 door dDAVP mogelijk deels wordt gemedieerd door het eiwit Epac. In overeenstemming met *in vivo* studies, waar zowel Epac1 en Epac2-eiwitten werden gevonden in de verzamelbuis, brengen mpkCCD cellen endogeen Epac1 en Epac2 tot expressie. Epac1-expressie werd gestimuleerd door dDAVP, terwijl Epac2 werd verminderd. Epac-activatie alleen, zonder dDAVP-voorincubatie, zorgde niet voor een toename van de AQP2-expressie. Echter,

stimulatie van de Epac-activiteit na een voorbehandeling met dDAVP zorgde voor een toename in zowel AQP2-transcriptie als AQP2-eiwit, suggererend dat Epac deel uitmaakt van de PKA-onafhankelijke signaalcascade zorgend voor een handhaving van een hoge AQP2-expressie tijdens een lange termijn dDAVP-incubatie.
Concluderend is de PKA-CRE signaleringscascade betrokken bij de initiële stijging van de AQP2-expressie na AVP-stimulatie, maar niet bij het lange termijn effect van AVP. In plaats daarvan wordt AQP2 op de lange termijn mogelijk gereguleerd door activatie van Epac.

Demeclocycline en minocycline zijn antibiotica van de tetracycline-groep, die een diuretisch effect hebben. Vanwege dit effect wordt demeclocycline momenteel gebruikt om langdurige hyponatriëmie te behandelen bij patiënten met SIADH. Hoewel van deze medicijnen wordt gedacht dat ze werken op het distale deel van het nefron is hun precieze werkingsmechanisme nog niet beschreven. In hoofdstuk 6 laten we zien dat demeclocycline en minocycline het eiwit-niveau van AQP2 in de mpkCCD-cellijn verlagen op een tijds- en concentratie-afhankelijke wijze. De eiwitsynthese remmer cycloheximide blokkeert het effect van demeclocycline op AQP2, wat impliceert dat een daling van de AQP2-productie de afname van de AQP2-expressie verklaart. Met behulp van een AQP2-promoter luciferase construct toonden we aan dat demeclocycline zorgt voor een afname van de AQP2-transcriptie. Ook vonden we een afname van de dDAVP-geïnduceerde cAMP-productie en een verlaagde expressie van zowel adenylaatcyclase 3 als 6, wat de afgenomen cAMP-productie verklaart.
Concluderend blijkt uit onze gegevens dat het diuretische effect van tetracycline antibiotica, zoals van demeclocycline en minocycline, wordt gemedieerd door het verlagen van adenylaatcyclase-expressie en cAMP-generatie, met als gevolg een verminderde AQP2-gentranscriptie en eiwit-expressie.

Lithium wordt regelmatig gebruikt voor de behandeling van psychiatrische ziektebeelden, zoals een bipolaire stoornis, schizoaffectieve stoornis en depressie. Helaas ontwikkelt ongeveer 20% van de lithium-behandelde patiënten NDI, een aandoening waarbij de nieren niet in staat zijn om de urine te concentreren. Lithium-geïnduceerde NDI gaat gepaard met een verminderde AQP2-expressie. Chronische lithium behandeling in ratten leidt ook tot een afname van de fractie van de hoofdcellen, wat wordt 'gecompenseerd' door een toename van de fractie van intercalaire cellen, die betrokken zijn bij de regulatie van het zuur / base-evenwicht. Bovendien leidt lithium *in vivo* tot een inactivatie van glycogeen synthase kinase (Gsk) 3β. Dit gaat gepaard met een verhoogde renale cyclooxygenase 2 (COX-2)-expressie, betrokken bij prostaglandine productie, en verhoogde PGE_2 uitscheiding in de urine. PGE_2

vermindert de AVP-gestimuleerde water reabsorptie en kan dus een belangrijke rol spelen in de ontwikkeling van lithium-geïnduceerde NDI.
In hoofdstuk 7 onderzochten we de mogelijke rol van prostaglandines in de lithium-geïnduceerde AQP2 afname, met behulp van mpkCCD cellen. In deze cellen ging de AQP2-afname na lithium-incubatie samen met Gsk3β-inactivatie en verhoogde COX-2-expressie. Remming van COX door indomethacine of dexamethason-geïnduceerde afname van COX-2-expressie verhoogden beide de AQP2-expressie. Echter, beide behandelingen voorkwamen niet de lithium-geïnduceerde AQP2-afname. Lithium heeft geen invloed op de prostaglandine-hoeveelheid geproduceerd door mpkCCD cellen. Lithium-toevoeging verlaagde de AQP2-eiwit expressie, mRNA niveaus en transcriptie. Prostaglandines verlaagden de AQP2-expressie door het verhogen van de eiwit-afbraak, maar hadden geen invloed op AQP2-gentranscriptie.
Concluderend blijkt uit onze gegevens dat in mpkCCD cellen, prostaglandines zorgen voor een afname van de AQP2-eiwitstabiliteit door verhoging van de lysosomale degradatie, hetgeen aangeeft dat *in vivo* paracrien geproduceerde prostaglandines mogelijk een rol spelen in lithium-geïnduceerde NDI. Echter, lithium heeft ook invloed op AQP2 gentranscriptie, en dit effect is prostaglandine-onafhankelijk. Onze resultaten tonen aan dat het lithium-gevoelige AQP2-promotor segment zich bevindt in de meest proximale 200 bp.

Om het mechanisme van lithium-geïnduceerde NDI verder te onderzoeken en om te onderzoeken of lithium-geïnduceerde NDI kan worden behandeld, onderzochten we in hoofdstuk 8 het effect van amiloride. Van amiloride is in een selecte groep patiënten aangetoond dat het lithium-geïnduceerde NDI vermindert, maar het mechanisme is nog onduidelijk. Aangezien amiloride het epitheliale natrium-kanaal (ENaC) in het apicale membraan van de hoofdcellen blokkeert, hadden we de hypothese dat ENaC de toegangspoort is voor lithium in de cel en dat amiloride daarom nuttig kan zijn door de toegang van lithium in de cel te blokkeren.
Inderdaad vonden we in de muis-verzamelbuis cellijnen mCCD en mpkCCD dat de AVP-geïnduceerde AQP2-expressie tijds- en dosisafhankelijk werd verminderd door klinisch relevante lithium-concentraties, wat kon worden voorkomen door co-incubatie met de ENaC-blokkers amiloride en benzamil. Amiloride verlaagde ook het transcellulair lithium-transport, de intracellulaire lithium-concentratie en de lithium-geïnduceerde inactivatie van Gsk3β. Samenvattend toont onze studie aan dat ENaC de belangrijkste cellulaire toegang voor lithium is en dat het blokkeren van ENaC AQP2-afname voorkomt.
In vivo zorgde gelijktijdige behandeling van ratten met amiloride voor een vermindering van de lithium-geïnduceerde AQP2-afname, en amiloride voorkwam volledig de vermindering van

de verhouding in hoofdcellen / intercalaire cellen. Amiloride-behandeling zorgde verder voor een afname van het urinevolume en een verhoogde urine-osmolaliteit. Concluderend blijkt uit onze gegevens dat het blokkeren van ENaC met amiloride een beschermend effect heeft op de ontwikkeling van lithium-geïnduceerde NDI door de lithium-geïnduceerde veranderingen in cellulaire samenstelling te voorkomen en de AQP2-expressie gedeeltelijk te beschermen, wat leidt tot een bewaard gebleven concentrerend vermogen van de verzamelbuis. Als zodanig verstrekken onze gegevens een onderbouwing voor het gebruik van amiloride als een behandeling voor lithium-geïnduceerde NDI.

In dit proefschrift hebben we een klein deel van de complexe regulatie van AQP2 ontrafeld. Naast AVP zijn dopamine en ATP betrokken bij de AQP2-regulatie, via ubiquitinering, internalisatie en degradatie van AQP2, zo de AVP-geïnduceerde waterpermeabiliteit verminderend. Ook prostaglandines PGE_2 en $PGF_{2\alpha}$ verlagen AVP-geïnduceerde AQP2-expressie door het verhogen van AQP2-degradatie, leidend tot een verminderde waterpermeabiliteit. In afwezigheid van AVP stimuleert PGE_2 de AQP2-expressie en waterpermeabiliteit. Dit verschil in PGE_2-effect kan worden verklaard door een AVP-geïnduceerde verandering in de relatieve prostaglandine-receptor expressie, leidend tot een verhoging van remmende signaleringscascades en een afname van stimulerende cascades. Toniciteit stimuleert ook de AQP2-expressie, maar dit is onafhankelijk van AVP, en in tegenstelling tot prostaglandines heeft toniciteit effect op AQP2-transcriptie. De regulatie van AQP2 door AVP werd verder opgehelderd door aan te tonen dat de PKA-CRE cascade betrokken is bij de initiële stijging van AQP2-expressie na AVP-stimulatie, maar dat het lange termijn effect van AVP PKA-CRE-onafhankelijk is, en mogelijk gaat via Epac-activatie. We toonden aan dat demeclocycline diurese veroorzaakt door middel van een afname in AQP2-eiwit, via een verminderde AQP2-transcriptie veroorzaakt door een verlaging van adenylaatcyclase-expressie en cAMP-productie. Verder toonden we aan dat lithium de hoofdcel voornamelijk binnengaat via het epitheliale natrium-kanaal ENaC, wat leidt tot een verminderde AQP2-transcriptie, wat onafhankelijk is van prostaglandines. Het blokkeren van lithium door middel van amiloride vermindert lithium-geïnduceerde NDI door AQP2-afname en de verandering in hoofdcel / intercalaire cel-verhouding te voorkomen. Als zodanig verstrekken onze gegevens een onderbouwing voor het gebruik van amiloride als een behandeling voor lithium-geïnduceerde NDI. Zoals beschreven voor lithium-geïnduceerde NDI, kan een beter begrip van de fysiologische regulatie van AQP2 bijdragen tot het vinden van een mogelijke therapie voor verschillende ziektebeelden, waarin AQP2 een rol speelt.

CHAPTER 11

Curriculum Vitae

List of abbreviations

List of publications

Dankwoord

Curriculum Vitae

Marleen Kortenoeven werd geboren op 13 januari 1981 in Tegelen. Zij behaalde in 1999 het VWO diploma aan het Bouwens van der Boijecollege te Panningen, en begon datzelfde jaar haar studie Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Binnen deze studie werden bijvakstages uitgevoerd bij de afdeling Experimentele Urologie van het Universitair Medisch Centrum St. Radboud, onder supervisie van Dr. Wim Jongmans, en de afdeling Pathologie, onder supervisie van Dr. Margit Schraders. Ook verrichte zij een hoofdvakstage bij de afdeling Lead Discovery Pharmacology van het bedrijf Organon, onder supervisie van Dr. Julia Oosterom. In 2005 begon zij met haar promotie-onderzoek bij de afdeling Fysiologie van het Universitair Medisch Centrum St. Radboud onder begeleiding van Prof. Dr. Peter Deen en Prof. Dr. Jack Wetzels, wat resulteerde in dit proefschrift. Tijdens haar promotieonderzoek begeleidde ze verschillende studenten van de opleidingen Biologie, Biomedische Wetenschappen, Hoger Laboratorium Onderwijs en Geneeskunde. Momenteel werkt zij bij de afdeling Biomedicine aan de Aarhus Universiteit te Denemarken, onder supervisie van Prof. Dr. Robert Fenton.

List of abbreviations

A

AQP	aquaporin
ANOVA	analysis of variance
ATP	Adenosine triphosphate
AVP	Arginine vasopressin

C

°C	degree Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
COX	cyclooxygenase
CRE	cAMP Responsive Element
CREB	cAMP responsive element binding protein

D

dDAVP	[deamino-Cys^{1}, D-arg^{8}]-vasopressin
DMEM	dulbecco modified eagle medium
DNA	deoxyribonucleic acid
DP	prostaglandin D receptor
DTT	Dithiothreitol

E

ECL	Enhanced Chemiluminescence
ENaC	epithelial sodium channel
EP	prostaglandin E receptor
Epac	Exchange factor directly activated by cAMP
ER	endoplasmic reticulum

F

FP	prostaglandin F receptor

G

Gs/Ms/Ms	Gas chromatography– tandem mass spectrometry
Gi	inhibitory G protein
Gq	phospholipase C activating G protein
Gs	stimulatory G protein

Gsk	glycogen synthase kinase

H

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	hour
HRP	horseradish peroxidase

I

Ig	immunoglobulin
IMBX	3-Isobutyl-1-methylxanthine
IP	prostaglandin I receptor

K

kb	kilobase
kDa	kilodalton(s)

L

L-NAME	L-NG-Nitroarginine methyl ester

M

mCCD	mouse cortical collecting duct cell line
MDCK	Madin Darby Canine Kidney
mpkCCD	mouse cortical collecting duct cell line
mRNA	messenger RNA

N

n	number in group
NDI	nephrogenic diabetes insipidus
NEM	*N*-ethylmaleimide
NFAT	Nuclear factor of activated T-cells
NF-kappaB	nuclear factor kappa- B
NO	nitric oxide

O

osm	osmole

P

p	probability of events
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PKA	protein kinase A
PKC	protein kinase C
PVDF	polyvinylidene fluoride

R

RNA	ribonucleic acid
RT	reverse transcriptase

S

SEM	standard error of the mean
SIADH	syndrome of inappropriate antidiuretic hormone secretion

T

TonE	tonicity responsive element
TonEBP	tonicity-responsive enhancer binding protein
TP	thromboxane receptor
TPA	12-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)-aminomethan
TxA_2	thromobaxane A_2

U

UTP	Uridine triphosphate

W

wt	wildtype

List of Publications

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Kortenoeven MLA, Van den Brand M, Li Y, Wetzels JFM, Deen PMT. In mpkCCD cells, long-term regulation of aquaporin-2 by vasopressin occurs independent of protein kinase A and CREB, but may involve Epac. *Submitted,* 2011.

Kortenoeven MLA*, Sinke AP*, Hadrup N, Fenton RA, Wetzels JFM, Deen PMT. Demeclocycline Attenuates Hyponatremia by Reducing Adenylate Cyclase and Aquaporin-2 Expression. *Manuscript in preparation.* *Authors contributed equally

Dankwoord

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