1. Introduction

1.1 Structure and function of the mammalian kidney

The mammalian kidney assures the maintenance of an optimal fluid environment, with concomitant excretion of soluble metabolic end products. Blood plasma osmolality, determined mainly by sodium and its anions, must be kept relatively constant for proper systemic body function despite alterations in water intake. Therefore, the kidney compensates plasma dilution due to increased water intake by excretion of a larger volume of a diluted urine, whereas a small volume of a highly concentrated urine is excreted when water intake is small. The mammalian kidneys are located outside the peritoneal cavity on either side of the spine. A longitudinal cross-section through the rat kidney reveals four concentrically arranged regions (see Fig. A), made up of the functionally and morphologically different sections of the radially arranged nephrons. The outmost region in the kidney is the renal cortex, followed by the outer medulla and the inner medulla which tapers into the renal papilla. The unilobular kidney is the basic kidney type, and is found in most small mammalian species. The multilobular kidney, as the human kidney, might be regarded as an adaptation to larger body size. It is composed of many lobes, each consisting of a cortex, and a medulla ending in a papilla.

The nephron. The functional unit of a kidney is the nephron. As illustrated, a nephron begins in the renal cortex with the renal corpuscle, consisting of the Bowman´s capsule and the glomerulum. The Bowman´s capsule is connected to the proximal convoluted tubule, which leads into the proximal straight tubule. Connected to the proximal straight tubule is the descending thin limb of the loop of henle. The apex of the loop of henle reaches far into the inner medulla for long-looped nephrons, whereas short looped nephrons may form their bends within the outer medulla or even in the cortex. At the apex, the loop of henle bends back and leads as the ascending limb of henle into the outer medulla, where it widens into the thick ascending limb. Just before leading into the distal convoluted tubule, the final portion of the thick ascending limb bearing the macula densa, a patch of renin-secreting cells, contacts the Bowman´s capsule. Multiple nephrons are connected via their individual connecting tubule to a collecting duct. The collecting ducts extend from the cortex into the papilla where the urine empties into the renal pelvis.

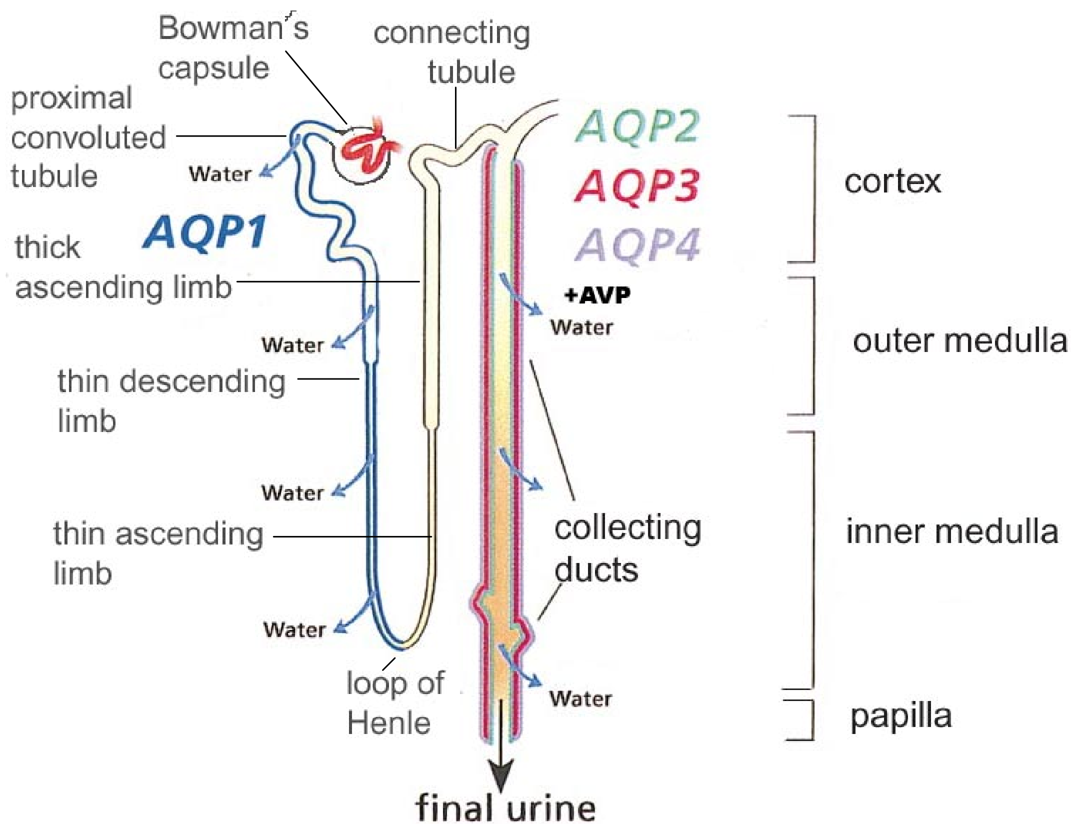

Fig. A. Schematic representation indicating the organization of the nephron and the sites of aquaporin expression (modified after Nielsen et al., 2002). AQP1 (blue) is constitutively present in the proximale tubule and the descending limb rendering the epithelium water permeable (blue arrows). AQP3 (red) and AQP4 (purple) are inserted into the basolateral membrane compartement of principal cells of the collecting duct. Water permeability is conferred to the collecting duct epithelium upon arginine-vasopressin (AVP) stimulation, which leads to the insertion of AQP2 into the apical membrane compartement of principal cells. For details, see text.

Kidney function. The formation of primary urine begins with ultrafiltration of the blood plasma in the Bowman´s capsule. Therein, the capillary endothelium is covered on the outside with podocytes, interdigitated epithelial cells of the tubular blind end. Between these epithelia, a double basal lamina forms a filter composed of a negatively charged glycoprotein network with a mesh-size of 6-9 nm. Therefore, this filter constitutes an effective barrier for larger, or negatively charged proteins. The driving-force for filtration is the difference between the hydrostatic pressure in the capillary network and the oncotic pressure of the plasma proteins remaining in the capillary lumen. Essential for the excretion of a concentrated urine is an osmotic gradient which increases from cortico-medullary boundary to papillary tip. This gradient is sustained even during diuresis, though its magnitude is increased in antidiuresis. The osmotic gradient is established for the most part by the different properties of individual segments along the renal tubular system in respect to their water permeability and sodium transporter abundance. The filtrate entering the descending limb of henle, contains a sodium concentration comparable to

the blood. Towards the base of the loop of henle, the luminal fluid becomes more concentrated because approximately 90 % of the free luminal water leaves the tubules via constitutively present AQP1 water channels, i.e. down the osmotic gradient created by the hypertonicity of the renal medulla. Medullary tonicity is upheld by active sodium transport from the lumen of the thick ascending limb of henle. The increase in tonicity of the surrounding interstitium provides the driving force for water reabsorption from the fluid entering the water permeable descending limb. The main transporters involved in delivering sodium, potassium and chloride from the luminal fluid across the highly water impermeable epithelium of the thick ascending loop of henle to the surrounding interstitium are $Na^+/K^+/2Cl^-$ cotransporters together with Na^+/H^+-cotransporters (sodium hydrogen exchangers, NHEs) on the luminal membrane and Na^+/K^+-ATPases and Na/HCO_3^- cotransporters on the basolateral membrane. The countercurrent flow in the ascending and descending limb together with sodium transport lead to the establishment of an osmotic gradient along the cortico-papillary axis. Due to the massive removal of ions, the fluid entering the cortical connecting tubule is hypotonic. Along the collecting duct lumen, urea is concentrated by water absorption via AQP2 inserted in the apical and AQP3 and AQP4 in the basolateral membrane compartment of collecting duct principal cells (see Fig. B). In the terminal part of the inner medullary collecting duct, urea is transported from the collecting duct lumen to the surrounding interstitium by the action of urea transporter UT-A1, which leads to a further increase in inner medullary osmolality. The water permeability of the collecting ducts is regulated by the action of the antidiuretic hormone arginine-vasopressin (AVP) on V_2R receptors, which activate a signalling cascade leading to the insertion of AQP2 water channels into the apical membrane of primary cells along the collecting duct.

The hydration state of an organism influences its plasma osmolality, which determines the level of synthesis and secretion of the antidiuretic hormone arginine-vasopressin (AVP). Minor increases in plasma osmolality are recognized by hypophyseal osmosensory neurons located in the supraoptic and paraventricular nuclei, the sites of AVP-precursor synthesis. These neurons reach into the posterior pituary, where AVP-containing neurosecretory vesicles are released into the blood stream (in Schnermann and Sayegh, 1998). In healthy humans, a threshold increase in plasma osmolality triggers a detectable increase in circulating AVP-levels. This threshold for AVP secretion varies between individuals (~0.5 mosmol/l to 5 mosmol/l). The plasma-AVP concentration affects the amount of AQP2 inserted into the apical membrane compartment of principal cells along the collecting duct and has thus a major influence on urine osmolality and -volume. *In vivo*, cells of the mammalian kidney are subjected to a generally elevated and fluctuating osmolality due to the renal concentrating mechanism. The high interstitial inner medullary osmolality is mainly derived from urea and NaCl concentrations. The concentrations

of NaCl and urea gradually increase from the kidney cortex to the medullary tip, creating an osmotic gradient required for water reabsorption. This osmotic gradient is maintained even during diuresis and its magnitude is increased during antidiuresis. The sodium concentration in the rat renal medulla varies between 140 mmol/l in hydrated animals and more than 400 mmol/l in dehydrated animals (Beck et al., 1984). The concentration of urea increases even more during dehydration than the sodium concentration (Garcia-Perez and Burg, 1991). The cells of the collecting duct are thus constantly challenged by fluctuations in sodium-derived tonicity (i.e. effective osmolality exerted by membrane impermeant compounds) and urea-derived osmolality. Nevertheless, the intracellular ion distribution of renal cells is kept relatively constant, with high potassium and low sodium concentrations, to maintain the transmembrane sodium concentration gradient as the driving force for many sodium coupled transport processes and to insure proper protein function. Steep increases in extracellular tonicity were shown to cause double-stranded DNA breaks and as a consequence, apoptosis in cultured renal cells (Kültz et al., 2001; Michea et al., 2000). High extracellular sodium is balanced by intracellular accumulation of organic osmolytes such as sorbitol, betaine, glycerophosphocholine, inositol and taurine (Bagnasco et al., 1986). Urea concentrations readily equilibrate between the interstitium and the interior of the cell, due to the solutes´ membrane permeating properties and the presence of urea transporters. The organic osmolytes, which in contrast to sodium, do not perturb protein function (non-perturbing osmolytes) have stabilizing effects on protein function and thus counteract the denaturing effect of urea (Yancey et al., 1982). The biosynthesis of many of the transporters responsible for the intracellular accumulation of organic osmolytes is induced by hypertonicity (reviewed in [1]Handler and Kwon, 2001). Moreover, the transcriptional regulation of UT-A urea transporters (UT-A1, UT-A3, and UT-A4), and thus urea accumulation in the inner medulla, is regulated by tonicity (Nakayama et al., 2000). Hypertonicity activates the the recently discovered tonicity-responsive element binding protein (TonEBP), which, bound to tonicity-responsive element (TonE) present in the regulatory region of these genes, increases transcription (Woo et al., 2002).

1.2 Aquaporins: structure and function

The first water channel was discovered in membranes of red blood cells as a membrane protein with 28,000 Da (Agre et al., 1987; Denker et al., 1988). After decoding of its cDNA (Preston and Agre, 1991), the protein was shown to dramatically increase the water permeability of hypotonically treated *Xenopus* oocytes injected with its mRNA (Preston et al., 1992). Pictures showing a number of burst oocytes revealed the protein´s function as a water channel, which was subsequently named CHIP28 (channel forming integral protein of 28 kDa). The

nomenclature was later standardized and CHIP28 was renamed AQP1. Since the discovery of the first aquaporin water channel, 10 members of the family have been found in mammals (AQP0-AQP9). They may be subdivided into the solely water permeable aquaporins (AQP0, AQP1, AQP2, AQP4) and aquaglyceroproteins (e.g. AQP3, AQP7, AQP9) which, in addition to their water permeability, are permeable to urea and glycerol (Agre et al., 1998). All aquaporins have 6 transmembrane domains connected by five loops (A to E). The two highly conserved asparagine-proline-alanine-motivs (NPA) have proved vital for the function of AQP1 as a water channel, inasmuch as mutation of the NPA motif abolished water permeability. This finding lead to a model in which the center of the pore is formed by two NPA motifs in loops B and E. The NPA motifs face each other midway between the two leaflets of the membrane with the protein folded into a structure somewhat reminiscent of an hour-glass (Jung et al., 1994). Aquaporins are inserted in the membrane as tetramers, with each monomer forming one water permeable pore. The tetrameric organization of AQP1 and the hour glass model were confirmed by electron chrystallographic analysis of AQP1 at a resolution of 6-Ǻ (Cheng et al., 1997; Li et al., 1997). A three-dimensional atomic model of AQP1 answered the question of how the selectivity for water, excluding protons, is achieved. Water enters the channel as a column of molecules connected by hydrogen bonds. At the very center of the pore each water molecule temporarily bonds with the two asparagine residues of the adjacent NPA motifs which excludes the passage of associated protons (Murata et al., 2000).

1.3 Molecular mechanisms regulating the expression and trafficking of AQP2 in renal principal cells: description of the classical AVP-triggered pathway

AQP2 is found only in the kidney and the male reproductive tract (Nelson et al., 1998; Stevens et al., 2000). The molecular mechanism underlying the tissue-specific expression of AQP2 are still unclear. In the kidney, AQP2 expression is confined to principal cells of the renal collecting duct, and abundance of the protein increases from cortex to papilla (Nielsen et al., 1993). It is widely accepted that AVP-mediated elevation of cellular cAMP is the main factor governing not only the trafficking of AQP2 in renal principal cells (Nielsen et al. 1995), but also the expression of this water channel (Matsamura et al. 1997). Binding of AVP to V_2R-receptors located within the basolateral membrane compartment triggers the liberation of the receptor-bound G-protein Gs from a complex consisting of Gαs, and Gβγ, a process involving receptor-mediated exchange of GDP to GTP. GTP-bound Gαs then activates adenylyl-cyclase, which catalyzes the formation of cAMP from ATP. After activation of adenylyl-cyclase, GTP is hydrolyzed to GDP, favoring the binding of Gαs to Gβγ. The G-protein complex binds again to a V_2R-receptor and is available for another cycle of activation. Elevation of intracellular cAMP leads to

the activation of protein-kinase A (PKA). The binding of two molecules cAMP to PKA enables the dissociation of the two catalytic subunits from the two regulatory subunits. PKA is anchored via PKA anchoring proteins (AKAPs) to AQP2 bearing vesicles and thus brought into close proximity to its substrate. With the IMCD cell model (Maric et al., 1998) used herein, it was shown that inhibition of PKA-binding to AKAPs impairs the targeting of AQP2 to the membrane of IMCD cells (Klussmann et al., 1999). The catalytic subunits then phosporylate AQP2 at Ser-256.

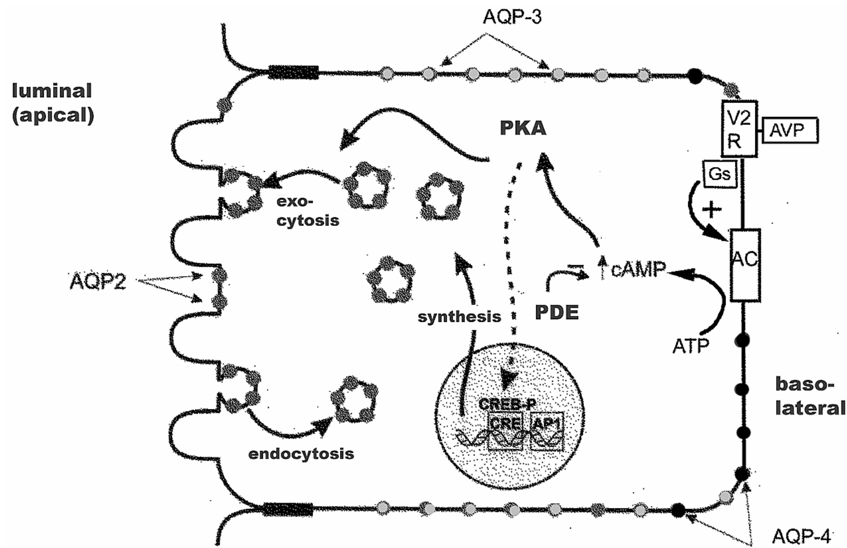

Fig. B. Simplified model for the regulation of AQP2 synthesis and trafficking in a renal principal cell in response to AVP (modified after Marples et al., 1999). Binding of AVP to V_2R triggers the liberation of G-protein G_s, which in turn activates the adenylyl-cyclase (AC). The rise in intracellular cAMP leads to the activation of protein kinase A (PKA). Phosphodiesterase (PDE) activity decreases intracellular cAMP. Activated PKA phosphorylates AQP2 located in vesicles, triggering their transport and insertion into the apical membrane. In addition to the acute increase in apical membrane water permeabiltiy (short-term regulation), elevated cAMP levels lead to increased synthesis of AQP2 (long-term regulation). cAMP-activated PKA phosphorylates the transcription factor cAMP response element binding protein (CREB) at Ser-133, which, bound to the cAMP response element (CRE) in the AQP2 promoter region, is a prerequisite for the initiation of AQP2 transcription.

The phosphorylation of AQP2 is required for its translocation to the apical membrane of principal cells, inasmuch as three of the four AQP2 molecules present in a tetramere need to be phosphorylated for the multimer´s proper transport to the apical plasma membrane (Kamsteeg et al., 2000). The molecular mechanisms involved in AQP2 translocation are not yet completely understood. Capacitance measurements on IMCD cells have shown, that the insertion of AQP2 bearing vesicles into the plasma membrane does not require transient rises in the intracellular

calcium concentration (Lorenz et al., 2001). Membrane targeting of AQP2 was also observed, when the cortical actin cytoskeleton was disassembled by Clostridium B toxin administration, whereas introduction of a constitutively active form of Rho, which favored the formation of actin stress fibers, inhibited the targeting of AQP2 to the membrane (Klussmann et al., 2001; Tamma et al., 2001).

The signalling cascade leading to an elevation of intracellular cAMP and thus PKA activation constitutes also a pathway regulating the expression of AQP2. Elevated cAMP levels and activated PKA are thought to be the main factors required for the induction of AQP2 transcription. A cAMP-responsive element (CRE) present in the 5´-flanking region of the AQP2 gene was reported to be involved in the activation of AQP2 by cAMP (Hozawa et al., 1996; Matsumura et al., 1997). PKA phosphorylates the transcription factor CRE-binding protein (CREB) within its kinase inducible domain (KID) at Ser-133. CREB- phosphorylation at Ser-133, which was shown to be crucial for the transcription of CRE-regulated genes (Gonzalez et al., 1989), promotes the binding of CREB to CRE and thus, subsequently activates AQP2 transcription. Moreover, the regulatory region of the human AQP2 gene harbors binding sites for the transcription factors AP-1 and a SP-1 site, which were shown to be responsive to cAMP in other cell types. Whereas the CRE-element is conserved in all species examined so far, the AP-1 and SP-1 binding sites were found only in the regulatory region of the human AQP2 gene, suggesting that these elements are not required for an action of elevated AVP on the transcription of AQP2 in general.

1.4 States with impaired water homeostasis due to altered AQP2 expression

Nephrogenic diabetes insipidus. Defects in the signalling cascade governing the expression and trafficking of AQP2 may result in severe urine concentrating disability, known as Diabetes insipidus (DI). The disease may be inherited or acquired. Among the inherited forms is the Diabetes insipidus centralis, a state, in which the synthesis of functional AVP is abrogated, in most cases due to disruptions in the AVP gene. Therefore, the thirst-response triggered by minor increases in plasma-osmolality does not result in pituary gland AVP synthesis and functional AVP cannot be secreted into the blood stream. Untreated patients excrete large amounts of dilute urine (up to 20 l/d). States of severely reduced urine concentrating ability, due to defects in the kidney-residing mediators of AQP2-dependent water-reabsorption, as mutations within the V_2R-receptor (Rosenthal et al., 1992) or the AQP2 gene (Deen et al., 1994) itself, are termed nephrogenic Diabetes insipidus (NDI). Inherited DI appears at a very low frequency. Acquired forms of DI, due to side-effects of medication, chronic metabolic disorders or chronic kidney disease have a far higher occurrence rate. Long-term lithium treatment

commonly used for the treatment of bipolar disorders leads to an often irreversible NDI in about one half of patients. According to data collected by Peet and Pratt, about 0.1 % of the US-American population has been treated with lithium (Peet and Pratt, 1993). Lithium is believed to impair the function of the Gαs/adenylyl-cyclase system and thereby prevent the formation of cAMP (Yamaki et al., 1991). Marples and co-workers have shown that a long-term lithium treatment in rats resulted in a 95 % reduction in AQP2 expression compared to controls (Marples et al., 1995).

Evidence for AVP-independent mechanisms participating in the expressional regulation of AQP2. Apart from the renal collecting duct, AQP2 expression was found in principal cells of the vas deferens in the male reproductive tract, where AQP2 expression was not governed by AVP (Stevens et al., 2000). There is also substantial, but mechanistically yet unexplained evidence for an AVP-independent regulation of the water permeability of renal principal cells in the intact animal (Nielsen et al., 2002). Elevated vitamin D production as a result of hyperparathyroidism leads to increased intestinal calcium reabsorption. Substantial elevation of plasma and urine calcium levels can cause NDI in humans. Rats treated with the vitamin D analogon dihydrotachysterol exhibited a 15 % increase in plasma calcium with concomitant down-regulation of AQP2 (Earm et al., 1998). Sands and co-workers suggested that the action of calcium on AQP2 expression is mediated via luminal calcium receptors which activate the G-protein Gi and thus impair the function of adenylyl-cyclase (Sands et al., 1997). Studies on primary cultured rat inner medullary collecting duct cells (IMCD cells), have shown that addition of 5 mM/l calcium to the culture medium decreased cAMP-dependent AQP2 expression by ~50 % compared to controls (Storm, 1999). By means of RT-PCR, the message for the calcium receptor was found only in RNA isolated from whole-kidney tissue, but not in RNA derived from IMCD cells, raising the question whether this receptor is involved in the calcium-elicited down-regulation of AQP2 observed in this model (Storm, unpublished observation). Puliyanda and co-workers have recently shown that AQP2 whithin purified endosomal vesicles is degraded in a calcium-sensitive fashion by μ-calpain (which is strongly expressed in the kidney inner medulla) and a metalloprotease, both of which were inhibited by leupeptin. It was argued that the down-regulation of μ-calpain and its endogenous inhibitor calpastatin in hyperparathyroid rats is suggestive for a chronic activation of this proteolytic pathway. The authors therefore proposed that increased activity of μ-calpain participates in the down-regulation of AQP2 protein observed in rats with elevated intracellular calcium levels (Puliyanda et al., 2003).

Multiple states with increased water-retention such as chronic heart failure, severe hepatic cirrhosis, and pregnancy have been linked to an over-expression of AQP2 (for rev. see Nielsen et al., 2002). The increase in AQP2 expression and improved targeting to the apical plasma

membrane in rats with experimentally induced chronic heart failure is thought to be due to increased circulating AVP levels (Xu et al., 1997). Elevated AVP levels may also be responsible for increased AQP2 expression observed in rats with experimentally induced severe hepatic cirrhosis by carbon tetrachloride administration (Fujita et al., 1995). Increased water retention has been observed in pregnant rats, which exhibit an enhanced AQP2 expression (150 % compared to controls; Ohara et al., 1998). The increase in AQP2 expression is thought to be due to increased AVP levels, but AVP-independent factors might be involved as well (Nielsen et al., 2001). It was shown, that AQP2 protein expression was nearly doubled in IMCD cells, cultured in the presence of the cAMP-analogon dibuturyl-cAMP (500 μM) in acidic medium (pH 6.4) compared to controls (pH 7.5), whereas AQP2 mRNA levels were unaffected. The NHE isoforms 1, 2, 3, and 4 were detected by RT-PCR in rat kidney tissue, whereas all but isoform 3 were found in cultured IMCD cells. The low pH-elicited increase in AQP2 protein expression was prevented by the presence of subtype-unspecific sodium-proton exchanger (NHE) inhibitors such as ethyl-isopropyl-amiloride or amiloride (Storm et al., 2000). Decreased AQP2 expression without changes in AVP or cAMP levels was observed in fasting and protein-deprived rats and humans (Sands et al. 1996, Amlal et al. 2001) and in senescent rats (Preisser et al. 2000). Water deprivation increased AQP2 expression in rats chronically given V_2R antagonists (Marples et al., 1998), whereas water loading decreased AQP2 expression, despite chronic administration of the V_2R-specific agonist dDAVP (Ecelbarger et al., 1997). These findings indicate that as yet unknown AVP-independent mechanisms participate in the regulation of renal AQP2 expression.

1.5 Introduction of the primary cultured inner medullary collecting duct (IMCD) cell model used in this work

Inner medullary collecting duct cells were obtained from inner medullae of rats. As described by Maric and co-workers, AQP2 expression rapidly declined on mRNA and protein levels when these cells were taken into culture. The sustained and robust AQP2 expression in this cell model was shown to rely on the addition of 500 μM dibuturyl-cAMP (DBcAMP) to the culture medium, which was routinely elevated to 600 mosmol/l by equimolar addition of sodium chloride and urea (Maric et al., 1998). RT-PCR analysis using gene specific primers revealed that these cells express the aquaporins AQP2, AQP3, AQP4 (Storm, unpublished observation) and the sodium proton exchanger isoforms NHE1, NHE2, and NHE4 (Storm et al., 2000). This cell model was used in a number of recent publications concerned with the mechanisms of AQP2 trafficking (Klussmann et al., 1999, 2000; Maric et al., 2001; Klussmann et al., [1]2001, [2]2001; Lorenz et al., 2003) and the expressional regulation of AQP2 (Storm et al., 2003).

1.6 The aim of this study

The above cited findings clearly point to the existence of pathways other than the classical AVP-elicited pathway with the potency to regulate AQP2 expression. It was shown that the expression of AQP2 increased in dehydrated rats and decreased in water-loaded animals (reviewed in Nielsen et al., 2002). The hydration status affects plasma osmolality and therefore the levels of circulating AVP. Nevertheless, AVP-independent mechanisms must also be involved, as water deprivation increased AQP2 expression in rats chronically given V_2R-antagonists (Marples et al., 1998). Water loading decreased AQP2 expression in spite of chronic administration of the V_2R-specific agonist desmopressin (DdAVP) (Ecelbarger et al. 1997). It can be speculated whether the postulated AVP-independent pathway(s) represent mechanisms compensating for inappropriate AVP levels or, whether they participate in AQP2 regulation under physiological conditions. Considering that renal principal cells are constantly exposed to elevated urea and sodium concentrations, osmolality and/or tonicity were promising candidates to influence the expression of AQP2 along the renal collecting duct. The study presented here was therefore undertaken to investigate whether extracellular osmolality potentially influences the expression of AQP2 in primary cultured IMCD cells (Maric et al., 1998).