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NEPHROGENIC DIABETES INSIPIDUS

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■ **Abstract** Nephrogenic diabetes insipidus, which can be inherited or acquired, is characterized by an inability to concentrate urine despite normal or elevated plasma concentrations of the antidiuretic hormone arginine vasopressin. Polyuria, with hyposthenuria, and polydipsia are the cardinal clinical manifestations of the disease. About 90% of patients with congenital nephrogenic diabetes insipidus are males with the X-linked recessive form of the disease (OMIM 304800) who have mutations in the arginine vasopressin receptor 2 gene (*AVPR2*), which codes for the vasopressin V_2 receptor. The gene is located in chromosomal region Xq28. In $<10\%$ of the families studied, congenital nephrogenic diabetes insipidus has an autosomal-recessive or autosomal-dominant (OMIM 222000 and 125800, respectively) mode of inheritance. Mutations have been identified in the aquaporin-2 gene (*AQP2*), which is located in chromosome region 12q13 and codes for the vasopressin-sensitive water channel. When studied in vitro, most *AVPR2* mutations result in receptors that are trapped intracellularly and are unable to reach the plasma membrane. A few mutant receptors reach the cell surface but are unable to bind arginine vasopressin or to properly trigger an intracellular cyclic AMP signal. Similarly, aquaporin-2 mutant proteins are misrouted and cannot be expressed at the luminal membrane. Chemical or pharmacological chaperones have been found to reverse the intracellular retention of aquaporin-2 and arginine vasopressin receptor 2 mutant proteins. Because many hereditary diseases stem from the intracellular retention of otherwise functional proteins, this mechanism may offer a new therapeutic approach to the treatment of those diseases that result from errors in protein kinesis.

INTRODUCTION

Resistance to the action of almost every hormone is now recognized to cause human disease (1), and nephrogenic diabetes insipidus (NDI) is an example: It is the nephrogenic failure to concentrate urine in response to the antidiuretic hormone. The antidiuretic hormone in humans and most mammals is 8-arginine vasopressin (AVP). NDI may be caused by a defect in the vasopressin-induced permeability of the distal tubules and collecting ducts to water, an insufficient buildup of the

TABLE 1 Causes of nephrogenic diabetes insipidus

Narrow definition of NDI: water permeability of the collecting duct not increased by AVP
Congenital (idiopathic)
Hypercalcemia
Hypokalemia
Drugs
Lithium
Demeclocycline
Amphotericin B
Methoxyflurane
Diphenylhydantoin
Nicotine
Alcohol
Broad definition of NDI: defective medullary countercurrent function
Renal failure, acute or chronic (especially interstitial nephritis or obstruction)
Medullary damage
Sickle-cell anemia and trait
Amyloidosis
Sjögren syndrome
Sarcoidosis
Hypercalcemia
Hypokalemia
Protein malnutrition
Cystinosis

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corticopapillary interstitial osmotic gradient, or to a combination of these two factors (2). Thus the broadest definition of the term NDI embraces any antidiuretic-hormone-resistant urinary concentration defect, including medullary disease with low interstitial osmolality, renal failure, and osmotic diuresis. In its narrower sense, NDI describes only those conditions in which AVP release fails to induce the expected increase in the permeability of the cortical and medullary collecting ducts to water (3; Table 1).

URINE CONCENTRATION AND THE COUNTERCURRENT SYSTEM

Urine is not concentrated by active transport of water from tubule fluid to blood; such a system would require a tremendous expenditure of metabolic energy. It has been estimated that >300 times the energy needed by an active salt transport and passive water equilibration system would be required because salt concentrations are ~0.15 mmol/liter whereas water concentrations are ~55 mmol/liter. Instead, urine is concentrated with relatively little expenditure of metabolic energy by a complex interaction between the loops of Henle, the medullary interstitium, the

medullary blood vessels or vasa recta, and the collecting tubules. This mechanism of urine concentration is called the countercurrent mechanism because of the anatomical arrangement of the tubules and vascular elements (Figure 1; Figure 2*A*). Tubular fluids move from the cortex toward the papillary tip of the medulla via the proximal straight tubule and the thin descending limbs. The tubules then loop back toward the cortex so that the direction of the fluid movement is reversed in the ascending limbs. Similarly, the vasa recta descend to the tip of the papilla and then loop back toward the cortex. This arrangement of tubule segments and vasa recta allows the two fundamental processes of the countercurrent mechanism—countercurrent multiplication and countercurrent exchange—to take place (2, 4).

For the following descriptions, osmolality within the medulla is considered to range from 300 mOsmol/kg at the corticomedullary tip junction to 1400 mOsmol/kg at the papillary tip (Figure 1). In keeping with tissue analysis (5), approximately half the medullary hypertonicity is assigned to NaCl and half is assigned to urea. It is also assumed that secretion of the antidiuretic hormone, AVP, occurs and that this hormone will interact with specific receptors on the collecting tubule (see below).

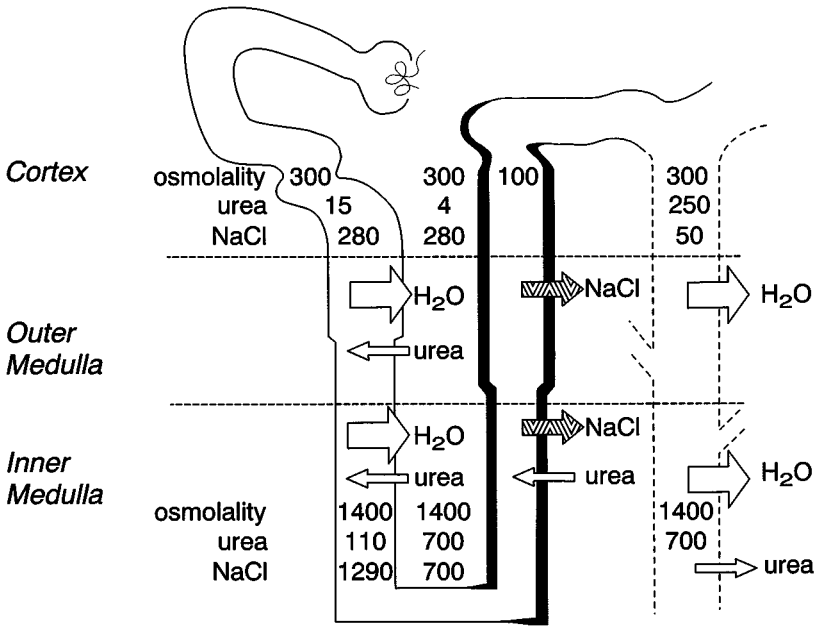

Figure 1 Schematic illustration of the model of Kokko & Rector for the renal concentrating mechanism. Heavy boundaries indicate very low permeability to water. Arrows indicate relative magnitudes of solute and water fluxes in the various segments. Note that active chloride transport in the thin ascending part of the loop of Henle is now demonstrated (14) (modified from 107 with permission).

The permeability and structural characteristics of the tubular and vascular segments responsible for the countercurrent mechanism are now described at a molecular level. The presence and abundance of the water channels of the aquaporin family seem to determine whether tubular or vascular structure is highly permeable or impermeable to water. Aquaporin-1 (AQP1), the first aquaporin to be characterized (6, 7), is present in both the apical and basolateral plasma membranes of proximal tubules, in thin descending limbs of Henle epithelia, and in descending vasa recta endothelia (8).

The facilitated urea transporter (UT2) is located in the last portion of descending thin limbs of short loops of Henle (9) (Figure 2). Isotonic fluid entering the highly water-permeable (but urea- and Na^+-impermeable) descending thin limb is concentrated almost entirely by water abstraction, so that fluid entering the ascending limb has a higher NaCl concentration and a lower urea concentration than the medullary interstitium. These passive driving forces between the lumen and interstitium poise the system for fluid dilution (10).

Thin and thick ascending loops of Henle are highly impermeable to water because they bear no water channels. The urea transporter present in the thin ascending segment is not precisely characterized, but as fluid moves up the water-impermeable thin limb, NaCl efflux from the lumen to the interstitium exceeds passive urea influx from the interstitium to the tubular fluid, resulting in tubular fluid dilution. The recently characterized chloride channel CLC-K1 (11, 12) is critically involved in active chloride transport in the thin ascending part of the loops of Henle (13, 14; see below). This kidney-specific member of the CLC chloride channel family is found exclusively in the thin ascending limbs of loops of Henle in both the apical and basolateral membranes (12).

In the thick ascending limb, tubular fluid is diluted further by the active transport of NaCl from the tubule to the interstitium (Figure 3). The NaCl reabsorption mechanisms for the thick ascending loop of Henle and the distal convoluted tubule depend on low intracellular Na^+ activity that is maintained by active extrusion of Na^+ from the cell by the basolateral Na^+/K^+-ATPase (the Na^+ pump). K^+ entering the thick ascending loop of Henle by the Na-K-2Cl cotransporter (NKCC2) recycles back to the tubular urine through potassium channels (15) (Figure 3). This has two major consequences: It replenishes the urinary K^+ that would otherwise be lost through absorption by NKCC2, and it results in a lumen-positive transepithelial voltage that provides the driving force for paracellular transport of one half of the Na^+ reabsorbed (15). It is now recognized that loss-of-function mutations in the genes coding for the NKCC2, the apical K^+ channel, or the basolateral chloride channel are responsible for Bartter's syndrome (16 –19) (Figure 3). Bartter's syndrome (OMIM 601678)[3] is a hereditary disease characterized by salt wasting, hypokalemic alkalosis, and deficits in diluting and concentrating capacity.

[3]Online Mendelian Inheritance in Man OMIM™. Center for medical genetics, Johns Hopkins University and National Center for Biotechnology Information, National Library of Medicine 1997. Http://www.ncbi.nlm.nih.gov/omim/.1998

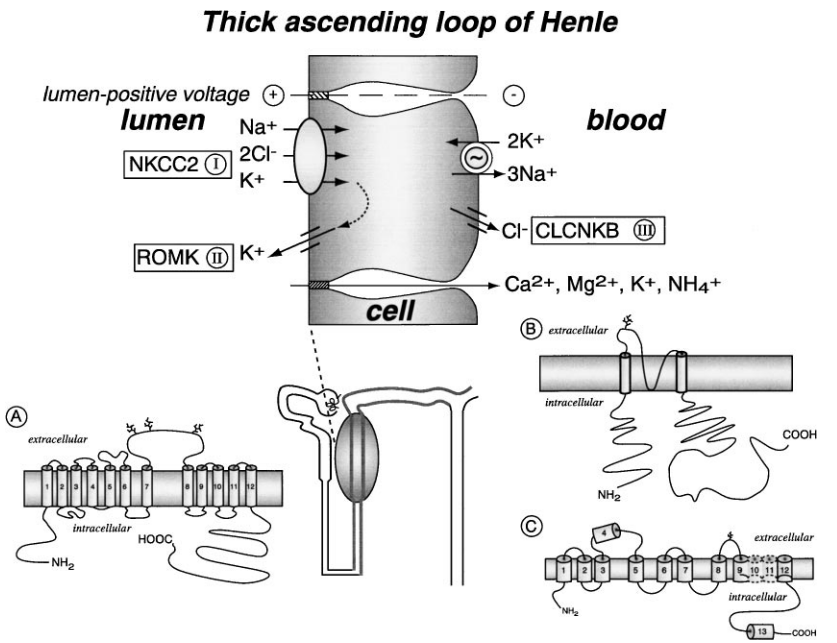

Figure 3 Polyuric-polydipsic symptoms are frequently observed in patients with Bartter's syndrome, which is secondary to loss-of-function mutations in three genes. Mutations in the bumetamide-sensitive Na-K-2Cl gene (*NKCC2*) cause type I Bartter's syndrome (16). *NKCC2* is expressed exclusively in apical membranes of thick ascending limb cells. (*A*) NKCC2 is a large protein with a core molecular mass of ~120 kDa, and its topology includes a large hydrophobic central region with at least 12 membrane-spanning helices. Sugar residues are linked to an extracellular loop between the seventh and eighth membrane-spanning segments, making this cotransporter a glycoprotein and increasing its apparent molecular mass on Western blots to 150–165 kDa. (*B*) Mutations in the gene that encodes the inwardly rectifying renal potassium channel, *ROMK*, cause type II Bartter's syndrome (17, 18). (*C*) Mutations in the gene that encodes the renal chloride channel, *CLCNKB*, cause type III Bartter's syndrome (19).

In the collecting duct, the first step in the antidiuretic action of AVP is its binding to the vasopressin V_2 receptor (Figure 4), located on the basolateral membrane of collecting duct cells. This step initiates a cascade of events—receptor-linked activation of the cholera toxin-sensitive G protein (G_s), activation of adenylyl cyclase, production of cyclic AMP (cAMP), and stimulation of protein kinase A (PKA)—that lead to the final step in the antidiuretic action of AVP. That is, the exocytic insertion of specific water channels, AQP2, into the luminal membrane results in increased permeability of the luminal membrane. AQP2 is the vasopressin-regulated water channel in renal collecting ducts. It is exclusively present in the principal cells of inner medullary collecting duct cells and is diffusely distributed in the cytoplasm in the euhydrated condition, whereas apical

staining of AQP2 is intensified in the dehydrated condition or after administration of 1-desamino[8-D-arginine]vasopressin (dDAVP), a synthetic structural analog of AVP. The short-term regulation of AQP2 by AVP involves the movement of AQP2 from intracellular vesicles to the plasma membrane, a confirmation of the shuttle hypothesis of AVP action that was proposed two decades ago (20). In long-term regulation, which requires a sustained elevation of circulating AVP levels for 24 h or more, AVP increases the abundance of water channels, which is thought to be a consequence of increased transcription of the *AQP2* gene (21). The activation of PKA leads to phosphorylation of AQP2 on serine residue 256 in the cytoplasmic carboxyl terminus. This phosphorylation step is essential for the regulated movement of AQP2-containing vesicles to the plasma membrane upon elevation of the intracellular cAMP concentration (22, 23). A second G protein (the first being the cholera-toxin-sensitive G protein; G_s) is also essential for the AVP-induced shuttling of AQP2. This G protein is sensitive to pertussis toxin and is involved in the pathway downstream of the cAMP/cAMP-dependent protein kinase signal (24). The molecular basis for the translocation of the AQP2-containing vesicles is known, but it is thought to be analogous to neuronal exocytosis (25). This is supported by the identification of various proteins known to be involved in regulated exocytosis—for example, Rab3a and synaptobrevin II (VAMP2) or synaptobrevin II-like protein—in the vesicles (26–28). In contrast to neuronal exocytosis, which is triggered by Ca^{2+}, cAMP and PKA appear to be crucial for the translocation process (29, 30). Vesicle trafficking probably involves the interaction of AQP2-containing vesicles with the cytoskeleton (31) (Figure 4). Drugs that disrupt microtubules or actin filaments have long been known to inhibit the hormonally induced permeability response in target epithelia (32). More recently, Sabolic and coworkers (33) have shown that microtubules are required for the apical polarization of AQP2 in principal cells. AQP3 and AQP4 are the constitutive water channels in the basolateral membranes of renal medullary collecting ducts.

AVP also increases the water reabsorption capacity of the kidney by regulating the urea transporter UT1 (Figure 2), which is present in the inner medullary collecting duct, predominantly in its terminal portion (34) (Figure 5). AVP also increases the permeability of principal collecting duct cells to sodium (35).

In summary, as stated by Ward et al (35), in the absence of AVP stimulation, collecting duct epithelia exhibit very low permeabilities to sodium, urea, and water. The low permeabilities of sodium, urea, and water permit the excretion of large volumes of hypotonic urine formed during intervals of water diuresis. In contrast, AVP stimulation of the principal cells of the collecting ducts leads to selective increases in the permeability of the apical membrane to water (P_f), urea (P_{urea}), and sodium (P_{Na}).

These actions of vasopressin in the distal nephron are possibly modulated by prostaglandin E_2 and by the luminal calcium concentration. High levels of E-prostanoid (EP_3) receptors are expressed in the kidney (36). However, mice lacking EP_3 receptors for prostaglandin E_2 were found to have quasinormal regulation of urine volume and osmolality in response to various physiological stimuli (36). An apical calcium/polycation receptor protein expressed in the terminal portion of

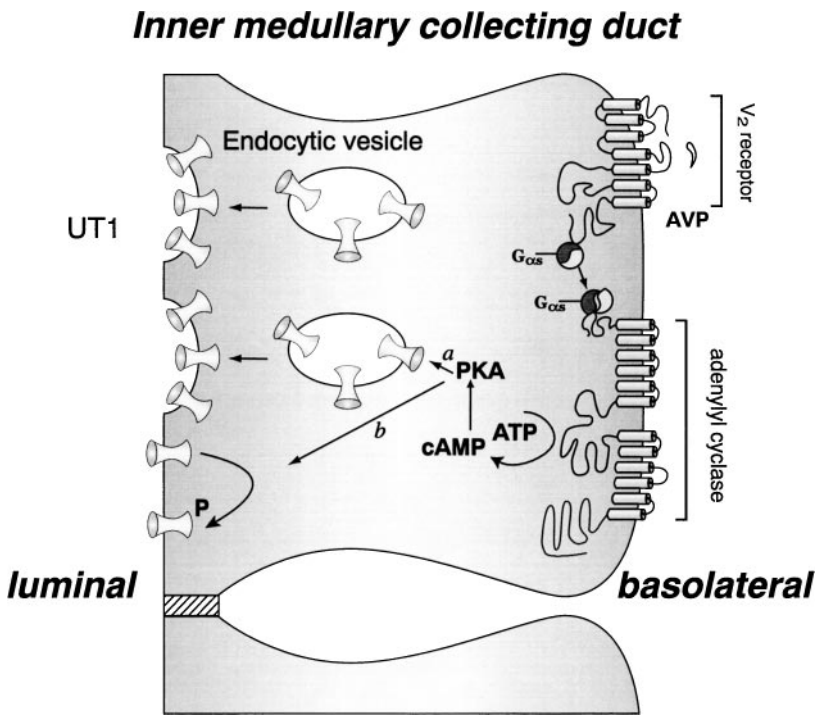

Figure 5 Potential mechanisms of urea transporter (UT1) activation by arginine vasopressin (AVP) in the inner medullary collecting duct. AVP binds to the V_2 receptor in the basolateral membrane. This results in the activation of the $G_{\alpha s}$ subunit, activation of adenylyl cyclase, production of cAMP, and stimulation of PKA. Two potential mechanisms for UT1 activation by PKA are indicated: (*a*) by increasing the insertion of vesicles containing the urea transporter and (*b*) by direct phosphorylation of urea transporter molecules. Current experimental evidence suggests that the latter mechanism is mainly involved in UT1 activation (modified from 9, with permission).

the inner medullary collecting duct of the rat has been shown to reduce AVP-elicited osmotic water permeability of the collecting duct when the luminal calcium concentration rises (37). This possible link between calcium and water metabolism may play a role in the pathogenesis of renal stone formation (37).

Knockout Mice with Urinary Concentration Defects

A useful strategy to establish the physiological function of a protein is to determine the phenotype produced by pharmacological inhibition of protein function or by gene disruption. Transgenic knockout mice individually deficient in AQP1, AQP3, and AQP4 (except AQP3 and AQP4 together) or in CLCNK1, NKCC2, or AVPR2 have been engineered (14, 38–43). To date, descriptions of knockout mice with inactivated *Aqp2* have not been published.

Aqp1 knockout mice were found to be normal in terms of survival, physical appearance, and organ morphology. However, they became severely dehydrated and lethargic after water deprivation for 36 h. Body weight decreased by 35% ± 2% (Figure 6), serum osmolality increased to ~500 mOsmol/kg (a value not compatible with life in humans), and urinary osmolality (657 ± 59 mOsmol/kg) did not change from that before water deprivation (39) (Figure 6). In the *Aqp1* knockout mice, a decrease in the superficial glomerular filtration rate was responsible for a normal distal flow despite decreased proximal reabsorption (44). This decrease in the single-nephron glomerular filtration rate was most probably caused by activation of a tubuloglomerular feedback mechanism. That the urinary flow rate was increased despite normal distal delivery suggests that the diuresis seen in *Aqp1* knockout mice results primarily from reduced fluid absorption in the collecting duct. This defect is secondary to a low interstitium medullary tonicity as suggested more recently by Chou et al (40). Freeze-fracture electron microscopy of rat thin descending limb of Henle demonstrated an exceptionally high density of intramembranous particles that may represent tetramers of AQP1 (45). A striking decrease in the density of these intramembranous particles was observed in the thin descending limb of Henle of *Aqp1*-deficient mice (40). In isolated perfused segments of thin descending limb of Henle of wild-type mice, transepithelial osmotic permeability to water (P_f) was very high; it was reduced 8.5-fold in the *Aqp1* knockout mice. These results demonstrate that osmotic equilibration along the thin descending limb of Henle by water transport plays a key role in the renal concentrating mechanism. By contrast, inactivation of *Aqp4* (38) has little or no effect on development, survival, and growth and causes only a small defect in urinary concentration ability (46), consistent with *AQP4* expression in the medullary collecting duct. The relatively mild defect in urine concentration ability in the *Aqp4* knockout mice suggests that another water channel (e.g. AQP3) may be more critical than AQP4 for the

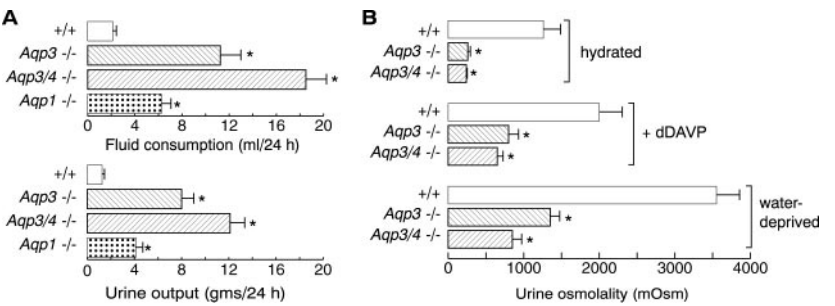

Figure 6 Urine-concentrating function in *Aqp3* single-knockout and *Aqp3 Aqp4* double-knockout mice. (*A*) Fluid consumption (*upper panel*) and urine output (*lower panel*) over 24 h in mice of indicated genotype (error bars indicate SEM; $n = 16$ mice per genotype]. (*B*) Urine osmolalities measured while mice were given free access to food and water before and after administration of 1-desamino[8-D-arginine]vasopressin (dDAVP) and after a 36-h water deprivation (error bars indicate SEM; $n = 12$ mice per genotype). $P < 0.005$ compared with wild-type mouse (modified from 41, with permission).

formation of concentrated urine. The *Aqp3*-null mice exhibited normal perinatal survival and postnatal growth but were remarkably polyuric and polydipsic (Figure 6). After dDAVP administration or water deprivation, the *Aqp3*-null mice were able to concentrate their urine partially, to ∼30% of that of wild-type mice. *Aqp3 Aqp4* double-knockout mice had greater impairment of urine concentration ability than did the *Aqp3* single-knockout mice (41) (Figure 6). These findings establish the existence of a form of NDI caused by the impaired permeability of collecting duct basolateral membranes to water.

The *Aqp1* knockout mouse has no human counterpart, as demonstrated by the fact that *AQP1*-null individuals have no obvious symptoms (47). Three women bearing loss-of-function mutations of *AQP1* were identified by the presence of high titers of circulating antibodies to the Colton blood group that apparently developed during pregnancy. Linkage between the Colton blood group and *AQP1* was demonstrated (48), and subsequent sequencing of DNA samples from individuals with defined Colton phenotypes demonstrated that the Colton antigen results from a missense mutation at residue 45 of the first extracellular loop of *AQP1* (49). Members of five kindreds were found to totally lack the Colton antigen. Blood and urine specimens were obtained from three probands of three different kindreds, and DNA analysis confirmed that each was homozygous for a different *AQP1* mutation (47). Two Colton-null individuals had no detectable AQP1 in erythrocytes or renal sediment: The first was homozygous for deletion of the entire exon 1, whereas the second was homozygous for a frameshift mutation after glycine residue 104. A third Colton-null individual was homozygous for the missense mutation *P38L* at the top of the first bilayer-spanning domain. This mutation resulted in unstable AQP1 protein when expressed in oocytes and corresponded to a 99% reduction in AQP1 in erythrocytes (47). The reasons for the difference between *Aqp1* knockout mice and AQP1-null humans are unknown.

When dehydrated, *Clcnk-1* knockout ($Clcnk^{-/-}$) mice were also lethargic; they exhibited a 27% decrease in body weight, compared with the 13% decrease seen in wild-type mice (14). Serum osmolality increased 360 to 381 osmol/kg in $Clcnk^{-/-}$ mice, compared with 311–323 osmol/kg in heterozygous ($Clcnk^{+/-}$) and wild-type ($Clcnk^{+/+}$) mice, and urinary osmolality was minimally increased after dDAVP administration (636 $\pm$ 31 mOsmol/kg preadministration versus 828 $\pm$ 25 mOsmol/kg postadministration).

The absence of the gene coding for NKCC2 in the luminal membrane of the thick ascending loop of Henle in the mouse also caused polyuria that was not compensated elsewhere in the nephron and recapitulated many features of the human classical Bartter's syndrome (42). The absence of transcellular NaCl transport via NKCC2 probably abolished the positive transepithelial voltage in the lumen that enables paracellular reabsorption of Na and K across the wall of the thick ascending tubule. The combined absence of transcellular and paracellular transport of NaCl across the thick ascending limb cells prevents the establishment of the normal osmotic gradient necessary for urine concentration.

Yun et al (43) introduced the *E242X* nonsense mutation into the mouse vasopressin V_2 receptor coding sequence and generated vasopressin receptor-deficient

male pups that exhibited decreased urinary osmolality, failure to thrive, and death within the first week after birth as a result of hypernatremic dehydration.

CLINICAL ASPECTS OF X-LINKED NEPHROGENIC DIABETES INSIPIDUS

X-linked NDI (OMIM 304800) is secondary to *AVPR2* mutations that result in the loss of function or a dysregulation of the V_2 receptor. The *AVPR2* gene has three exons and two small introns (50, 51). The cDNA sequence predicts a polypeptide with seven transmembrane, four extracellular, and four cytoplasmic domains that belongs to the family of G protein–coupled receptors (Figure 7).

Rareness and Diversity of *AVPR2* Mutations

We estimated the incidence of X-linked NDI in the general population from persons born in the Canadian province of Quebec during 1988–1997 to be ~8.8 per million (standard deviation = 4.4 per million) male live births (52). Thus X-linked NDI is generally a rare disorder. On the other hand, NDI is known to be a common disorder in Nova Scotia (53). Thirty affected males, who at the time of the study resided mainly in two small villages with a combined population of 2500 (54), are descendants of members of the Hopewell pedigree studied by Bode & Crawford (53) and carry the nonsense mutation W71X (55, 56). This is the largest known pedigree with X-linked NDI and has been referred to as the Hopewell kindred, named after the Irish ship Hopewell, which arrived in Halifax in 1761 (53). Descendants of Scottish Presbyterians who migrated to Ireland's Ulster Province in the 17th century emigrated from Ireland in 1718 and settled in northern Massachusetts. A later group of immigrants were passengers on the ship Hopewell and settled in Colchester County, Nova Scotia. Members of the two groups were subsequently united in Colchester County (53). Thus it is likely that Ulster Scottish immigrants, perhaps on more than one occasion, brought the W71X mutation to North America. To date, we have identified the W71X mutation in 38 affected males, who predominantly reside in the Maritime Provinces of Nova Scotia and New Brunswick. We estimated the incidence in these two Maritime Provinces to be 6 in 104,063, or ~58 per million (standard deviation = 24 per million) male live births for the 10-year period 1988–1997.

To date, 155 putative disease-causing AVPR2 mutations have been identified in 239 NDI families (57) (additional information is available in the NDI Mutation Database at http://www.medcor.mcgill.ca/~nephros/). Of these, we identified 82 different mutations in 117 NDI families referred to our laboratory. Half of the mutations are missense mutations. Frameshift mutations caused by nucleotide deletions or insertions (27%), nonsense mutations (11%), large deletions (5%), in-frame deletions or insertions (4%), splice site mutations (2%), and one complex mutation account for the remainder of the mutations. Mutations have been identified in

every domain, but on a per nucleotide basis about twice as many mutations occur in transmembrane domains as occur in the extracellular or intracellular domains. We previously identified single mutations (identified only once), recurrent mutations, and mechanisms of mutagenesis (58, 59). The 10 recurrent mutations (D85N, V88M, R113W, Y128S, R137H, S167L, R181C, R202C, A294P, and S315R) were found in 35 ancestrally independent families (52). The occurrence of the same mutation on different haplotypes was considered evidence of recurrent mutation. In addition, the most frequent mutations—D85N, V88N, R113W, R137H, S167L, R181C, and R202C—occurred at potential mutational hot spots [a C-to-T or G-to-A nucleotide substitution occurred at a CpG (2-bp sequence) dinucleotide].

Benefits of Genetic Testing

The natural history of untreated X-linked NDI includes hypernatremia, hyperthermia, mental retardation, and repeated episodes of dehydration in early infancy (60–63). Mental retardation, a consequence of repeated episodes of dehydration, was prevalent in the Crawford & Bode study (63), in which only 9 of 82 patients (11%) had normal intelligence. Early recognition and treatment of X-linked NDI, with an abundant intake of water, allows a normal life span with normal physical and mental development (64). Familial occurrence in males and mental retardation in untreated patients are two characteristics suggestive of X-linked NDI. Skewed X inactivation is the most likely explanation for clinical symptoms of NDI in female carriers (52, 65, 66).

The identification of the molecular defect underlying X-linked NDI is of immediate clinical significance because early diagnosis and treatment of affected infants can prevent the physical and mental retardation resulting from repeated episodes of dehydration. Diagnosis of X-linked NDI was accomplished by mutation testing of chorionic villus samples ($n = 4$), cultured amniotic cells ($n = 5$), or cord blood ($n = 17$). Three infants whose mutation testing was done on amniotic cells ($n = 1$) or chorionic villus samples ($n = 2$) also had their diagnoses confirmed by cord blood testing. Of the 23 offspring tested, 12 were found to be affected males, 7 were unaffected males, and 4 were noncarrier females (DG Bichet, unpublished data). The affected males were immediately treated with abundant water intake, a low-sodium diet, and hydrochlorothiazide. They have not experienced severe episodes of dehydration, and their physical and mental development remain normal; however, their urinary output is decreased by only 30%, and a normal growth curve is still difficult to attain during the first 2–3 years of life despite the above-described treatments and intensive attention. Water should be offered every 2 h day and night, and temperature, appetite, and growth should be monitored. Hospitalization for continuous gastric feeding may be necessary. The voluminous amounts of water kept in patients' stomachs will exacerbate physiological gastrointestinal reflux in infants and toddlers, and many affected boys frequently vomit and have a strong positive Tuttle test (esophageal pH test). These young patients often improve with the absorption of a H-2 (histamine-2 receptor)

blocker and with metoclopramide (which may induce extrapyramidal symptoms) or with domperidone, which is better tolerated and efficacious.

DEFECTIVE INTRACELLULAR TRANSPORT AND RESCUE OF MUTANT V_2 RECEPTORS

Most Mutant V_2 Receptors Are Not Transported to the Cell Membrane and Are Retained in the Intracellular Compartments

The classification of the defects of mutant V_2 receptors is based on that of the low-density lipoprotein receptor, for which mutations have been grouped according to the function and subcellular localization of the mutant protein whose cDNA has been transiently transfected in a heterologous expression system (67, 68). In this classification system, type 1 mutant receptors reach the cell surface but display impaired ligand binding and are consequently unable to induce normal cAMP production. The presence of mutant V_2 receptors on the surface of transfected cells can be determined pharmacologically. By carrying out saturation-binding experiments using a tritiated AVP, the number and apparent binding affinity of cell surface mutant receptors can be compared with those of the wild-type receptor. In addition, the presence of cell surface receptors can be assessed directly by using immunodetection strategies to visualize epitope-tagged receptors in whole-cell immunofluorescence assays.

Type 2 mutant receptors have defective intracellular transport. This phenotype is confirmed by carrying out, in parallel, immunofluorescence experiments on cells that are intact (to demonstrate the absence of cell surface receptors) or permeabilized (to confirm the presence of intracellular receptor pools). In addition, protein expression is confirmed by Western blot analysis of membrane preparations from transfected cells. It is likely that these mutant type 2 receptors accumulate in a pre-Golgi compartment because they are initially glycosylated but fail to undergo glycosyl-trimming maturation. This is readily detected by performing pulse-chase metabolic-labeling studies and comparing the receptor maturation profiles of wild-type and mutant receptors. All complex N-linked glycosylated proteins undergo addition of a large high-mannose sugar moiety in the endoplasmic reticulum (ER) that is progressively processed by the glycosyl enzymes of the Golgi complex. This large addition and processing can be readily detected as a mobility shift of the protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Asglycosylated proteins are processed past the medial Golgi complex, they are no longer sensitive to endoglycosidase H and are considered mature proteins. Metabolically labeled wild-type V_2 receptors acquire endoglycosidase H resistance, whereas mutant type 2 receptors do not, which confirms that these receptor species are being retained in a pre-Golgi compartment. The vast majority of naturally occurring mutations in the V_2 receptor that cause NDI are type 2 mutations (see below).

Type 3 mutant receptors are ineffectively transcribed. This subgroup seems to be rare because Northern blot analysis of transfected cells reveals that most V_2 receptor mutations do not affect the quantity or molecular size of the receptor mRNA.

Of the 12 mutants that we tested (N55H, L59P, L83Q, V88M, 497CC→GG, ΔR202, I209F, 700delC, 908insT, A294P, P322H, and P322S) only 3 (ΔR202, P322S, and P322H) were detected on the cell surface. Similarly, the 10 mutant receptors (Y128S, E242X, 803insG, 834delA, ΔV278, Y280C, W284X, L292P, W293X, and L312Y) tested by Schöneberg et al (69, 70) did not reach the cell membrane and were trapped in the interior of the cell. Similar results were obtained for the following mutants: L44F, L44P, W164S, S167L, and S167T (71); R143P and ΔV278 (72); and Y280C, L292P, and R333X (73).

Other genetic disorders are also characterized by protein misfolding. AQP-2 mutations responsible for autosomal-recessive NDI are characterized by misrouting of the misfolded mutant proteins and entrapment in the ER (74). The ΔF508 mutation in persons with cystic fibrosis is also characterized by misfolding and retention in the ER of the mutated cystic fibrosis transmembrane conductance regulator, which is associated with calnexin and Hsp70 (for a review see 75). The C282Y mutant HFE protein, which is responsible for 83% of hemochromatosis in the Caucasian population, is retained in the ER and middle Golgi compartment, fails to undergo late Golgi processing, and is subject to accelerated degradation (76). Other mutant renal membrane proteins, responsible for Gitelman's syndrome (77) and cystinuria (78), are also retained in the ER.

Missense mutations responsible for these various diseases are often situated in regions of the protein that are not part of the active site, the binding site, or the site of interaction with other proteins. These mutations decrease the half-life of the affected protein (79). Missense mutations and short in-frame deletions or insertions that impair the propensity of the affected polypeptide to fold into its functional conformation have led to the coining of the term conformational diseases (80). The NDI missense mutations are likely to impair folding and lead to rapid degradation of the affected polypeptide rather than to accumulation of toxic aggregates because the other important functions of the principal cells of the collecting duct (where V_2 receptors are expressed) are entirely normal. These cells express the epithelial Na channel. A decrease in function of this channel will result in a state of Na loss (81). This has not been observed in patients with *AVPR2* mutations.

In Vitro Adenovirus-Mediated Gene Transfer Experiments Have Been Successful for a Limited Number of Mutations

Schöneberg et al (69, 70) genetically rescued truncated or missense V_2 receptors by coexpression of a polypeptide consisting of the last 130 amino acids of the V_2 receptor in COS-7 cells. Four of the six truncated receptors (E242X, 804delG, 834delA, and W284X) and the missense mutant Y280C regained considerable functional activity, as demonstrated by an increase in the number of binding sites

and stimulation of adenylyl cyclase activity, but the absolute number of expressed receptors at the cell surface remained low, and the precise mechanism of the rescue phenomenon (dimerization?) (82) was unclear. Most of the loss-of-function mutations secondary to *AVPR2* missense mutations are unlikely to be improved by this coexpression strategy, and delivery of the gene transfer vehicle is a major unresolved problem.

Nonpeptide Vasopressin Antagonists Act as Pharmacological Chaperones to Functionally Rescue Misfolded Mutant V_2 Receptors Responsible for X-Linked NDI

Several orally active nonpeptide AVP receptor antagonists have been reported (83, 84), and one, SR121463, is a potent and selective V_2 receptor antagonist (85). This extremely stable molecule is highly selective for V_2 receptors from several species, including humans, and exhibits powerful intravenous and oral aquaretic effects (85). SR121463 inhibits AVP-evoked cAMP formation in human kidney membranes and reverses extrarenal V_2 receptor antagonism of dDAVP-induced release of hemostatic factors in dogs (86). VPA-985 is a similar aquaretic compound (84). From a therapeutic point of view, V_2 receptor-specific antagonists able to block the action of AVP at the level of the renal collecting duct specifically promote water excretion.

As described above, a number of genetic diseases with the common property of producing intracellular, misfolded proteins that are recognized and retained by the cell's quality control system have been identified. Although the activities of the quality control system of the cell are generally advantageous to the cell, this stringent monitoring system can lead to intracellular retention and, ultimately, destruction of otherwise-salvageable proteins. Recently, members of a class of compounds called chemical chaperones were shown to reverse the intracellular retention of a number of misfolded proteins (74, 87–93). Among these, glycerol and other polyols have been reported to stabilize protein conformations (94), increase the rate of in vitro protein refolding (95), and increase the kinetics of oligomeric assembly (96). Such compounds likely facilitate the folding of mutant proteins into conformations that resemble the wild-type protein, allowing them to escape the quality control system.

Taking the concept of chemical chaperones a step farther, Loo & Clarke (91) characterized functional mutations of the multidrug resistance 1 gene that codes for the P glycoprotein transporter, which interacts with a variety of cytotoxic agents. Artificial mutations in this gene led to intracellular, misfolded proteins. Surprisingly, P glycoprotein synthesis and folding in the presence of specific substrates or modulators that bind this transporter resulted in a considerable increase in correctly folded, functionally active protein.

We recently assessed whether these selective V_2 vasopressin receptor antagonists could facilitate the folding of mutant proteins that are responsible for NDI and are retained in the ER. We monitored the biosynthesis of mutant V_2

receptors in the presence of SR121463 and VPA-985. These cell-permeant antagonists were able to convert precursor forms of mutant V_2 receptor into fully glycosylated mature receptor proteins that were now targeted to the cell surface, as determined by pulse-chase analysis and cell surface immunofluorescence microscopy. Once at their correct cellular location, these receptors were able to bind AVP and produce an intracellular cAMP response that was 15 times higher than that produced in cells not exposed to these antagonists (92) (Figure 8). This effect could not be mediated by nor competed with V_2 receptor antagonists that are membrane impermeant, indicating that SR121463A was mediating its effects intracellularly.

On the basis of these data, we propose a model in which small nonpeptide V_2 receptor antagonists permeate into the cell and bind to incompletely folded mutant receptors. This would then stabilize a conformation of the receptor that allows its release from the ER quality control apparatus. The stabilized receptor would then be targeted to the cell surface, where upon dissociation from the antagonist it could bind vasopressin and promote signal transduction. Given that these antagonists are specific to the V_2 receptor and that they perform a chaperone-like function, we termed these compounds pharmacological chaperones (92, 97).

AQP2 GENE AND MUTANTS

The human *AQP2* gene is located in chromosome region 12q13 and has four exons and three introns (98–100). It is predicted to code for a polypeptide of 271 amino acids that is organized into two repeats, oriented at 180° to each other, and has six transmembrane, three extracellular, and four cytoplasmic domains. AQP2 is a member of the major intrinsic protein family of transmembrane channel proteins and has the characteristic NPA motifs at residues 68–70 and 184–186 (10) (Figure 9). AQP1 and, by analogy, AQP2 are homotetramers containing four independent water channels. To date, 26 putative disease-causing *AQP2* mutations have been identified in 25 NDI families (Figure 9). By type of mutation, there are 65% missense mutations, 23% frameshift mutations caused by small nucleotide deletions or insertions, 8% nonsense mutations, and 4% splice site mutations. Additional information is available in the NDI Mutation Database at http://www.medcor.mcgill.ca/∼nephros/.

Reminiscent of expression studies done with AVPR2 proteins, misrouting of AQP2 mutant proteins is the major cause underlying autosomal-recessive NDI (74, 101, 102). To determine if the severe AQP2 trafficking defect observed with the naturally occurring mutations T126M, R187C, and A147T is correctable, Chinese hamster ovary and Madin-Darby canine kidney cells were incubated with the chemical chaperone glycerol for 48 h. Using immunofluorescence microscopy, redistribution of AQP2 from the ER to the plasma membrane-endosome fractions was observed. This redistribution was correlated with improved water permeativity measurements (74, 103). It will be important to correct this defective AQP2 trafficking in vivo.

In contrast to the *AQP2* mutations seen in persons with autosomal-recessive NDI, which are located throughout the gene, the dominant mutations are predicted to affect the carboxyl terminus of AQP2 (104). One dominant mutation, *E258K*, has been analyzed in detail in vitro; AQP2-E258K had reduced water permeativity compared with wild-type AQP2 (105). In addition, AQP2-E258K was retained in the Golgi apparatus; this differs from the mutant *AQP2* seen in cases of recessive NDI, which is retained in the ER. The dominant action of *AQP2* mutations can be explained by the formation of heterotetramers of mutant and wild-type *AQP2* that are impaired in their routing after oligomerization (105, 106).

CONCLUSIONS

The deconstruction of congenital NDI allowed further characterization of key proteins involved in the countercurrent system. Clinically, greater awareness of the disease is leading to earlier diagnosis, and mutational analysis has revealed the diversity of *AVPR2* mutations. Functional studies underscore the frequent occurrence of misfolded mutant receptor proteins trapped in the ER and unable to reach the plasma membrane. The large number of different mutations, with varying functional defects, hinders the development of a specific therapy. However, pharmacological chaperones such as vasopressin receptor antagonists might constitute a general strategy to rescue a large number of different misfolded mutant V_2 receptors.

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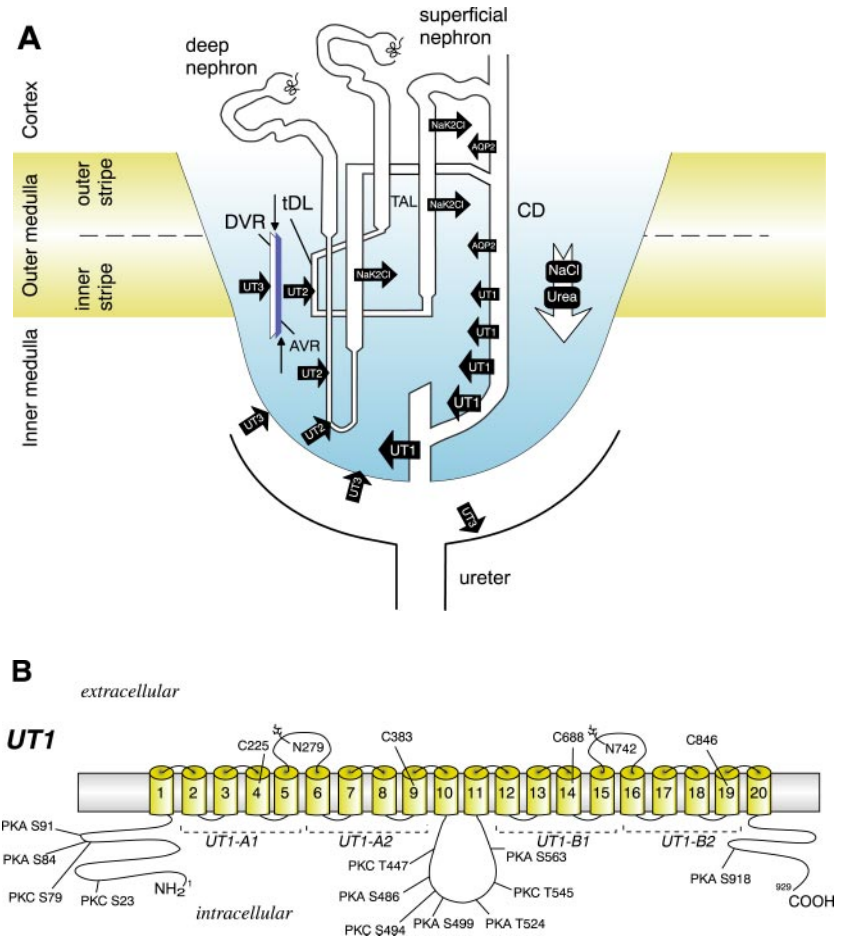

Figure 2 See legend next page

Figure 2 (*A*) Schematic representation of urea transporters in the rat kidney. A superficial nephron with a short loop and a deep nephron with a long loop of Henle are represented. Urea transporter *UT1* is present in the apical membrane of the terminal inner medullary collecting duct and is involved in vasopressin-regulated urea reabsorption. *UT2* is located in the late part of descending thin limbs of short loops and participates in urea recycling. In the inner stripe of the outer medulla, vascular structures of ascending vasa recta (*AVR*), descending vasa recta (*DVR*), and tubule components of thin descending limb *tDL*) are arranged together to form vascular bundles. *UT3* is present in descending vasa recta and allows efficient countercurrent exchange between ascending vasa recta and descending vasa recta as well as between descending vasa recta and thin descending limbs. The vertical arrow on the right-hand side of the figure indicates the corticopapillary osmolality gradient, which is primarily formed by NaCl and urea. TAL, thick ascending limb; CD, collecting duct. [Modified from Tsukaguchi et al (9) with permission.] (*B*) Hypothetical structural model and hydropathy analysis of *UT1*, the vasopressin-regulated urea channel. *UT1* cDNA encodes a 929-amino-acid protein consisting of two similar halves, each composed of two extended hydrophobic membrane-spanning stretches (UT1A and UT1B). In addition, each half of UT1 can be further subdivided into two homologous hydrophobic domains (UT1-A1/UT1-A2 and UT1-B1/UT1-B2). UT1 has 12 potential phosphorylation sites, 7 for protein kinase A (PKA) and 5 for protein kinase C (PKC). Although there is no significant homology between water channels and urea transporters, the urea transporter repeats UT1-A1, UT1-A2, UT1-B1, and UT1-B2 contain a similar motif, Asn-Pro-Leu/Trp. This motif could conceivably form part of the urea translocation pathway. [Modified from Shayakul et al (34) with permission.]

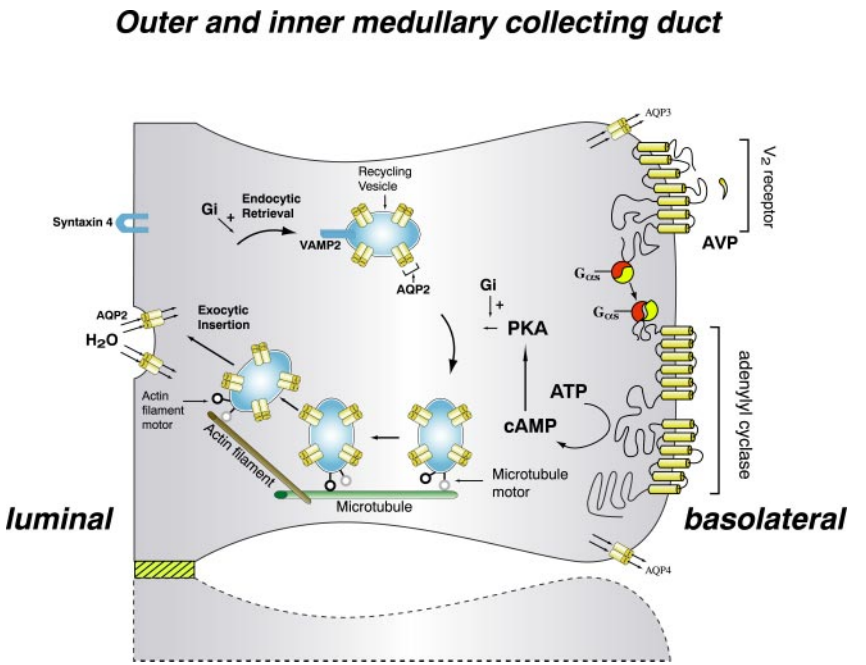

Figure 4 Schematic representation of the effect of *AVP* that increases the permeability of the principal cells of the collecting duct to water. Arginine vasopressin (*AVP*) is bound to the V_2 receptor (a G protein—linked receptor) on the basolateral membrane. The basic process of G protein—coupled receptor signaling consists of three steps: A heptahelical receptor detects a ligand (in this case, AVP) in the extracellular milieu, a G protein dissociates into α subunits bound to GTP and $\beta\gamma$ subunits after interaction with the ligand-bound receptor, and an effector (in this case, adenylyl cyclase) interacts with dissociated G protein subunits to generate small-molecule second messengers. AVP activates adenylyl cyclase, thereby increasing the intracellular concentration of cyclic AMP (cAMP). Adenylyl cyclase is characterized by two tandem repeats of six hydrophobic transmembrane domains separated by a large cytoplasmic loop, and it terminates in a large intracellular tail. Generation of cAMP follows receptor-linked activation of the heteromeric G protein (G_s) and interaction of the free $G_{\alpha s}$ chain with the adenylyl cyclase catalyst. Protein kinase A (PKA) is the target of the generated cAMP. Cytoplasmic vesicles carrying the water channel proteins (represented as homotetrameric complexes) are fused to the luminal membrane in response to AVP, thereby increasing the permeability of this membrane to water. Microtubules and actin filaments are necessary for vesicle movement toward the membrane. The mechanisms underlying docking and fusion of aquaporin-2 (*AQP2*)-bearing vesicles are not known. The detection of the small GTP-binding protein Rab3a, synaptobrevin 2, and syntaxin 4 in principal cells suggests that these proteins are involved in *AQP2* trafficking (24). When AVP is not available, water channels are retrieved by an endocytic process and water permeativity returns to its original low rate. Aquaporin-3 (*AQP3*) and aquaporin-4 (*AQP4*) water channels are expressed on the basolateral membrane.

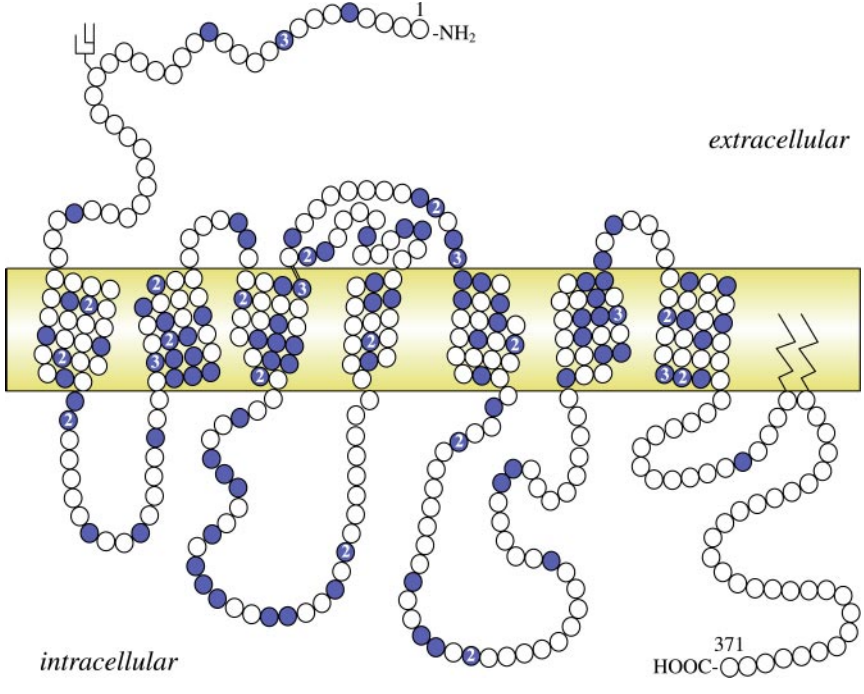

Figure 7 Schematic representation of the V_2 receptor and identification of 155 putative disease-causing *AVPR2* mutations. For a figure including the numbering of individual amino acids, please contact author at d-binette@crhsc.umontreal.ca. A solid circle indicates the location of (or the closest codon to) a mutation; a number indicates more than one mutation in the same codon. The names of the mutations were assigned according to recommended nomenclature (108). The extracellular (E), transmembrane (TM), and cytoplasmic (C) domains are defined according to Mouillac et al (109). The common names of the mutations are listed by type. The 78 missense mutations are as follows: L43P, L44F, L44P, L53R, N55D, N55H, L59P, L62P, H80R, L81F, L83P, L83Q, A84D, D85N, V88L, V88M, Q92R, L94Q, P95L, W99R, R106C, G107E, C112R, R113W, G122R, M123K, S126F, S127F, Y128S, A132D, L135P, R137H, [C142W; R143G], R143P, A147V, W164S, S167L, S167T, Q174L, R181C, G185C, D191G, G201D, R202C, T204N, Y205C, V206D, T207N, I209F, F214S, P217T, L219P, L219R, M272K, V277A, Y280C, A285P, P286L, P286R, P286S, L289P, L292P, A294P, L309P, S315R (AGC > AGA), S315R (AGC > AGG), N317K, C319R, N321D, N321K, N321Y, P322H, P322S, W323R, and W323S. The 17 nonsense mutations are as follows: W71X, Q119X, Y124X, W164X, S167X, Q180X, W193X (TGG > TAG), W193X (TGG > TGA), Q225X, E231X, E242X, W284X, W293X, W296X, L312X, W323X, and R337X. The 42 frameshift mutations are as follows: in E_I, 15delC, 27–54del, 46–47delCT, 54–55ins28, and 102delG; in TMI, 137–138delTA; in C_I, 185–219del, 206–207insG, and [225delC; 223C > A]; in TM_{II}, 247–248ins7, 268–269delCT, and 295delT; in TM_{III}, 331–332delCT, 335–336delGT, 340delG, and 407–446del; in C_{II}, 418delG, 430–442del, 442–443insG, 452delG, 457–463del, and 460delG; in E_{III}, 567–568insC, 572–575del, 612–613insC, and 614–615delAT; in TM_V, 631delC; in C_{III}, 682–683insC, 692delA, 717delG, 727–728delAG, 738delG, 738–739insG, 763delA, 784delG, and 785–786insT; in TM_{VI}, 838–839insT, 847–851del, and 851–852ins5; and in TM_{VII}, 907delG, 930delC, and 969delG. The six_in-frame deletions or insertions are as follows: in C_I, 185–193del; in TM_{II}, 252–253ins9; in TM_{III}, Y128del; in TM_{IV}, F176del; in E_{III}, R202del; and in TM_{VI}, V279del. The three splice site mutations are as follows: IVS2-1delG, IVS2-1G > A, and IVS2-2A > G (52, 55, 56, 58, 65–67, 110–136). Eight large deletions and one complex mutation are not shown (52, 58, 65, 126, 130).

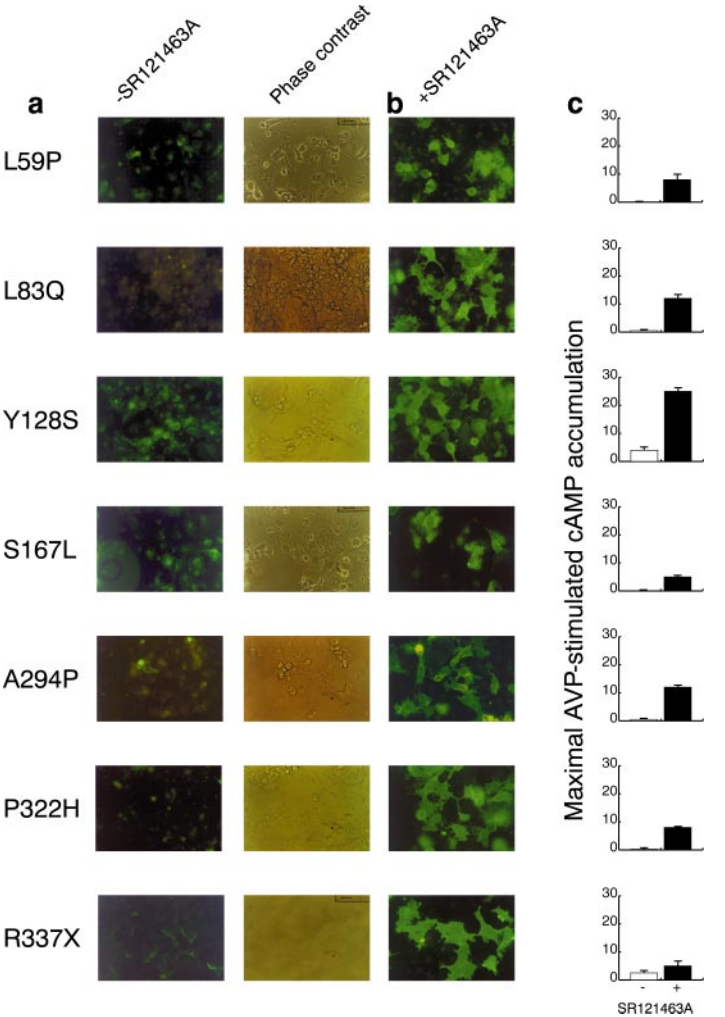

Figure 8 SR121463A increases the cell surface expression and signaling activity of several distinct nephrogenic diabetes insipidus (NDI) V_2 receptor mutants. (*a*) COS-1 cells transiently transfected with plasmids encoding the indicated Myc-tagged mutant V_2 receptors were visualized by whole-cell immunofluorescence and phase-contrast microscopy. All mutant receptors were found to be poorly expressed at the cell surface. (*b*) Treatment with 10^{-5} M SR121463A for 16 h led to appearance of these receptor mutants on the cell surface as assessed by fluorescence microscopy. Bar = 50 mm. (*c*) Effects of a 16-h pretreatment with 10^{-5} M SR121463A on vasopressin-stimulated cyclic AMP (cAMP) accumulation in COS-1 cells expressing the various mutants. The position and nature of the amino acid replacement in each NDI-causing mutation is indicated by the single-letter amino acid code. *X* indicates a stop codon. Data represent results from three separate experiments. [Reprinted from Morello et al (92) with permission].

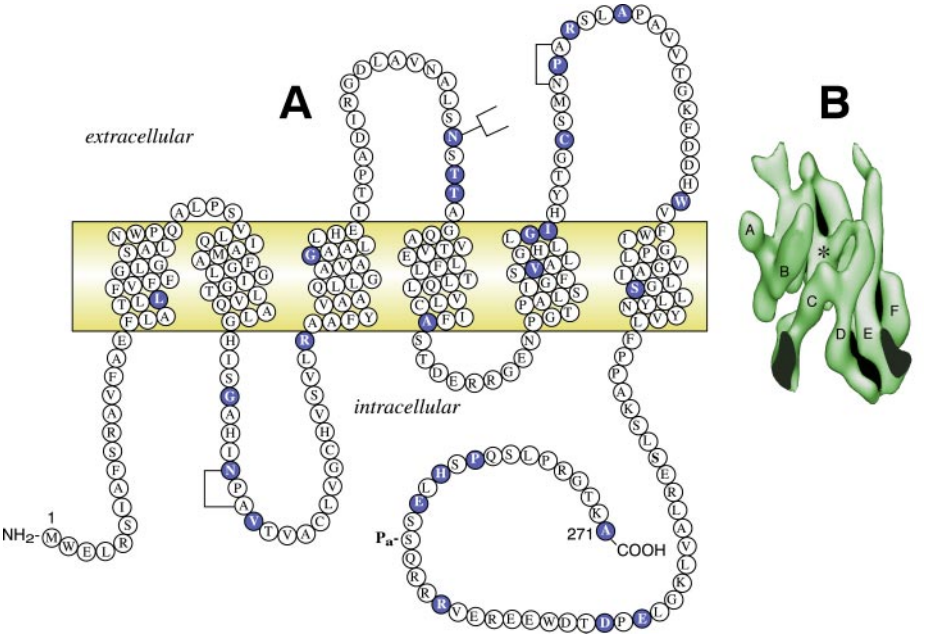

Figure 9 (*A*) Schematic representation of the aquaporin-2 (AQP2) protein and identification of 26 putative disease-causing *AQP2* mutations. A monomer with six transmembrane helices is represented. The location of the protein kinase A phosphorylation site (P_a) is indicated. This site is possibly involved in the arginine vasopressin-induced trafficking of AQP2 from intracellular vesicles to the plasma membrane and in the subsequent stimulation of endocytosis (22, 23). The extracellular (E), transmembrane (TM), and cytoplasmic (C) domains are defined according to Deen et al (98). As in Figure 7, solid circles indicate the locations of the mutations. See author at d-binette@crhsc.umontreal.ca for figure including numbering of individual amino acids. The common names of the mutations are listed by domain as follows: in TM_I, L22V; in C_{II}, G64R, N68S, V71M, and R85X; in TM_{III}, G100X; in E_{II}, 369delC, T125M, and T126M; in TM_{IV}, A147T; in TMV, V168M, G175R, and IVS2-1G > A; in E_{III}, C181W, P185A, R187C, A190T, and W202C; in TM_{VI}, S216P;and in C_{IV}, 721delG, 727delG, 756–765del, E258K, 779–780insA, P262L, and 812–816del (98, 102, 105, 127, 132, 137–141). (*B*) Representation of the six-helix barrel of the AQP1 protein viewed parallel to the bilayer. [Modified from Cheng et al (142) with permission.]

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