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Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane

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ABSTRACT **Water excretion by the kidney is regulated by the peptide hormone vasopressin. Vasopressin increases the water permeability of the renal collecting duct cells, allowing more water to be reabsorbed from collecting duct urine to blood. Despite long-standing interest in this process, the mechanism of the water permeability increase has remained undetermined. Recently, a molecular water channel (AQP-CD) has been cloned whose expression appears to be limited to the collecting duct. Previously, we immunolocalized this water channel to the apical plasma membrane (APM) and to intracellular vesicles (IVs) of collecting duct cells. Here, we test the hypothesis that vasopressin increases cellular water permeability by inducing exocytosis of AQP-CD-laden vesicles, transferring water channels from IVs to APM. Rat collecting ducts were perfused *in vitro* to determine water permeability and subcellular distribution of AQP-CD in the same tubules. The collecting ducts were fixed for immunoelectron microscopy before, during, and after exposure to vasopressin. Vasopressin exposure induced increases in water permeability and the absolute labeling density of AQP-CD in the APM. In parallel, the APM:IV labeling ratio increased. Furthermore, in response to vasopressin withdrawal, AQP-CD labeling density in the APM and the APM:IV labeling ratio decreased in parallel to a measured decrease in osmotic water permeability. We conclude that vasopressin increases the water permeability of collecting duct cells by inducing a reversible translocation of AQP-CD water channels from IVs to the APM.**

Vasopressin (the antidiuretic hormone) is a 9-amino acid peptide hormone, secreted by the neurohypophysis, which acts on the kidney via the adenylyl cyclase-coupled vasopressin receptor (V_2 receptor) to regulate water excretion. Vasopressin reduces urinary water excretion in part by increasing the water permeability of the renal collecting duct, thereby accelerating the osmotically driven absorption of water from the collecting duct lumen to the blood. Although the mechanism by which the water permeability increases is controversial, it presumably involves an increase in the number or unit conductance of water channels in the apical plasma membrane (APM), the rate-limiting barrier for net transepithelial water transport (1, 2). Several molecular water channels of the aquaporin (AQP) family are expressed in the kidney (3–5). One of these, AQP-CD (also called WCH-CD or AQP-2), appears to be expressed exclusively in the renal collecting duct (6, 7) and has been documented to be the major pathway responsible for vasopressin-regulated water transport across collecting duct cells (8, 9). Several years ago, it was hypothesized (10) that vasopressin induces translocation of water channels from intracellular vesicles (IVs) to the APM by exocytosis (the "shuttle hypothesis"). However, direct experimental evidence for translocation of water channels is lacking. We recently demonstrated that the AQP-CD water channel is present in the APM and IVs of collecting duct cells, consistent with requirements of the shuttle hypothesis (7). The purpose of this study is to test the hypothesis that the vasopressin-induced increase in the collecting duct water permeability is due to translocation of AQP-CD water channels from IVs into the APM. For this, we employed high-resolution immunoelectron microscopy in collecting ducts that had been perfused *in vitro* in the presence and absence of vasopressin. The use of isolated perfused tubules (rather than intact animals) for these experiments allowed us to measure water permeability in the same collecting ducts used to make observations of subcellular AQP-CD distribution and also allowed precise control of dose and duration of vasopressin exposure.

METHODS

Preparation of Anti-AQP-CD Antibody. Polyclonal antisera were raised in rabbits against a 23-amino acid synthetic peptide corresponding to the C terminus of AQP-CD (7). One of the antisera (LL127) was affinity purified using a column on which we immobilized the synthetic peptide via a sulfhydryl linkage to an N-terminal cysteine incorporated into the peptide sequence (ImmunoPure immobilization kit no. 2, Pierce).

Electrophoresis and Immunoblotting of Membrane Proteins. A crude membrane fraction was prepared from kidney inner medulla as described (7, 9). The membranes were solubilized in SDS (Laemmli sample buffer) and SDS/PAGE was run with 1 μg of membrane protein per lane according to the technique of Laemmli (11). After electroelution onto nitrocellulose membranes, the blots were probed with affinity-purified anti-AQP-CD (40 ng of IgG per ml), preimmune serum (1:1000), or anti-AQP-CD preadsorbed with excess synthetic peptide (100 μg of peptide per 40 ng of IgG). Labeling was visualized using a horseradish peroxidase-conjugated secondary antibody (1:3000, Dako) and luminol-based enhanced chemiluminescence (Amersham).

Perfusion of Isolated Inner Medullary Collecting Ducts. Collecting ducts were dissected from the inner half of the renal inner medullae of 100- to 125-g Sprague–Dawley rats that had been deprived of water for 24 hr. The collecting ducts were mounted on glass pipets and perfused *in vitro* at 37°C by the method of Burg (12). Fluorescein sulfonate (1 mM), an impermeant luminal marker, was added to the luminal perfusate to measure fluid flux in response to an imposed 200

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Abbreviations: AQP, aquaporin; AQP-CD, vasopressin-regulated water channel of collecting duct; AVP, [Arg^8]vasopressin; IV, intracellular vesicle; APM, apical plasma membrane.

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mosm/kg H_2O bath > lumen osmotic gradient as described (13). Fluorescein sulfonate concentrations in perfusate and collected fluid were measured by continuous-flow fluorometry and the osmotic water permeability (P_f) was calculated by standard methods (13).

P_f and the subcellular distribution of AQP-CD were determined in each perfused tubule. These observations were made in three groups of collecting ducts that were fixed for immunoelectron microscopy before (group A), during (group B), and after (group C) vasopressin exposure as described in the following:

Group A. The tubules (n = 11) were perfused for 40 min without [Arg^8]vasopressin (AVP) and the tubules were fixed for electron microscopy as described below.

Group B. The tubules (n = 10) were initially perfused for 40 min without AVP exposure. Then AVP (100 pM) was added to the peritubular bath and the tubules were perfused for an additional 40 min prior to addition of fixative.

Group C. The tubules (n = 9) were initially perfused in the absence of AVP for 40 min and then in the presence of AVP (100 pM) for 40 min; this was followed by washout of the AVP. After washout, the tubules were perfused for an additional 40 min before addition of fixative.

In all protocols, P_f was measured over the entire time course. The collecting ducts were fixed while still on the perfusion pipets by addition of 4% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.2) to the peritubular bath. The final water permeability measurement was made 1–2 min before the fixative was added to allow virtually simultaneous observation of water permeability and subcellular distribution of AQP-CD.

Preparation of Isolated Perfused Tubules for Immunocytochemistry. After paraformaldehyde fixation, the tubules were retrieved from the perfusion apparatus and embedded in 1% agarose in sodium cacodylate for temporary storage at 4°C in 2% paraformaldehyde in 0.1 M cacodylate buffer. Subsequently, they were cryoprotected in 2.3 M sucrose containing 2% paraformaldehyde in 0.1 M cacodylate, mounted on holders, and rapidly frozen in liquid nitrogen. The tubules were embedded in Lowicryl HM20 following a freeze-substitution protocol designed to preserve antigenicity (14). Initially the samples were equilibrated in 0.5% uranyl acetate in methanol at −80°C with a gradual increase to −70°C over 3 days. Then, they were exchanged to 100% methanol at −70°C with a gradual increase in temperature to −45°C over 24 hr. At −45°C, the samples were equilibrated with 1:1 Lowicryl HM20 in methanol, followed by 2:1 HM20:methanol, and finally pure HM20 before UV polymerization for 2 days at −45°C and 2 days at 0°C.

Immunoelectron Microscopy. Immunoelectron microscopy was performed on ultrathin sections (40 nm) of the Lowicryl-embedded tubules after cryosubstitution. The sections were incubated with affinity-purified anti-AQP-CD (100 ng of IgG per ml) and visualized with goat anti-rabbit-IgG gold (1:50 dilution, 10-nm gold particles, British BioCell, Cardiff, U.K.). The sections were stained with saturated uranyl acetate for 10 min. Controls were performed with nonimmune rabbit IgG (100 ng of IgG per ml) or anti-AQP-CD preadsorbed with the immunizing peptide (200 μg of peptide per 100 ng of IgG).

Immunohistochemistry. For light microscopic observations of medullary tissue, procedures for tissue processing, cryosectioning, and immunohistochemistry were as described (7) using affinity-purified anti-AQP-CD at 100 ng of IgG per ml.

Quantification of AQP-CD Immunolabeling in Inner Medullary Collecting Duct Cells. All quantification of immunolabeling was carried out with the observer blinded with regard to the identity of tissue specimens. The ratio of gold particles in APM to IVs was determined on electron micrographs (15,000×) taken of complete cross sections of tubules. Gold particles associated with the APM and IV were counted in at least 10 cells from each tubule (300–500 gold particles). IV counts included all IVs including multivesicular bodies. Labeling background was determined by use of nonimmune IgG at 0.1 μg/ml, and average background labeling was <5% of the total. We also determined the linear labeling density of APM (D_{APM}), the number of gold particles per unit length of APM. A computerized digitizing tablet was used to measure distance along the apical membrane (resolution < 4 nm). Statistical comparisons used ANOVA with Bonferroni contrasts.

RESULTS

The specificity of the affinity-purified anti-AQP-CD antibody was assessed by immunoblotting (Fig. 1*A*) and immunohistochemistry (Fig. 1*B*). The antibody recognized a band at the predicted molecular mass of AQP-CD (29 kDa) and a larger band (35–47 kDa) corresponding to glycosylated AQP-CD (15) (Fig. 1*A*, left panel). Both bands were fully ablated by preadsorption of the antibody with the immunizing peptide (Fig. 1*A*, middle panel) and were absent when preimmune serum was used (Fig. 1*A*, right panel). Immunohistochemistry in thin sections from rat cortex (not shown) and inner medulla (Fig. 1*B*) revealed exclusive labeling of collecting duct prin-

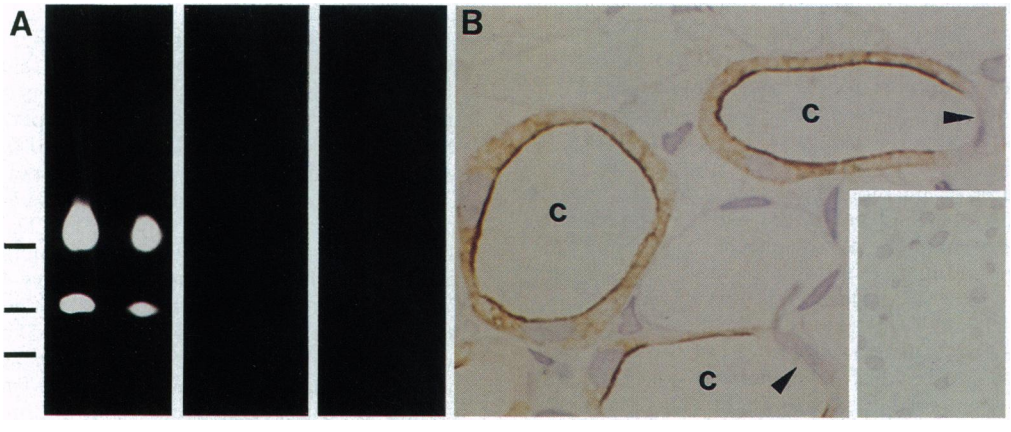

FIG. 1. Characterization of affinity-purified anti-AQP-CD antibody by immunoblotting (*A*) and by immunohistochemistry (*B*). (*A*) Immunoblots. Each of the three panels shows two lanes loaded with renal inner medullary membrane fractions prepared from different rats (1 μg of protein per lane). (Left panel) Blot probed with affinity-purified anti-AQP-CD (40 ng of IgG per ml). (Middle panel) Blot probed with anti-AQP-CD preadsorbed with an excess of the synthetic peptide used for immunization (100 μg of peptide per 40 ng of IgG). (Right panel) Blot probed with preimmune serum (1:1500 dilution). Bars indicate 21.5, 31, and 45 kDa. (*B*) Immunohistochemistry. Collecting ducts (c) alone were labeled with anti-AQP-CD antibody (100 ng of IgG per ml). Intercalated cells were unlabeled (arrowheads). No labeling was seen when antibody was preadsorbed with synthetic peptide as described above (*Inset*). (*B*, ×420; *Inset*, ×270.)

Table 1. P_f of tubules fixed for high-resolution immunoelectron microscopy

Group	n	P_f, μm/s		
		Basal	AVP (40 min)	Washout
A	11	65 ± 16	—	—
B	10	84 ± 19	454 ± 45*	—
C	9	163 ± 58	646 ± 77*	204 ± 68*

Tubules were fixed after basal P_f measurement (group A), after exposure to 100 pM AVP in peritubular bath for 40 min (group B), or 40 min after washout of AVP from peritubular bath (group C). For all tubules (n = 30): length, 0.83 ± 0.05 mm; inner diameter, 26.5 ± 0.5 μm. Values are mean ± SE.

*Statistical significance ($P < 0.001$) vs. preceding value by paired t test (group B) or ANOVA with Bonferroni contrasts (group C).

cipal cells. Labeling was absent when peptide-preadsorbed anti-AQP-CD was utilized (Fig. 1*B Inset*).

Inner medullary collecting ducts were microdissected from the rat kidney and perfused *in vitro* for measurement of P_f prior to fixation for immunocytochemistry. Table 1 shows the measured values of P_f for three groups of tubules: group A, in which tubules were fixed after basal P_f measurements (40-min perfusion in the absence of AVP); group B, in which tubules were fixed after perfusion for 40 min with AVP; and group C, in which tubules were fixed 40 min after washout of AVP from the peritubular bath. As shown, the collecting ducts responded to AVP with a large increase in P_f, which reversed upon withdrawal of AVP (Table 1).

The fixed tubules were embedded in Lowicryl for high-resolution quantitative immunoelectron microscopy using a freeze-substitution procedure designed to preserve antigenicity (14). With this approach, substantial labeling of the APM and IVs could be observed (Fig. 2), permitting valid quantitative analysis of labeling distribution. Distribution of AQP-CD labeling in representative collecting duct cells from groups A–C is presented in Fig. 2. Prior to vasopressin exposure, AQP-CD labeling was predominantly localized to IVs (in apical and basal parts of the cells) but was also seen in the APM (Fig. 2*A*). After exposure of the tubules to 100 pM AVP (Fig. 2*B*), the AQP-CD labeling of the APM increased markedly relative to labeling of IVs. The remaining labeled

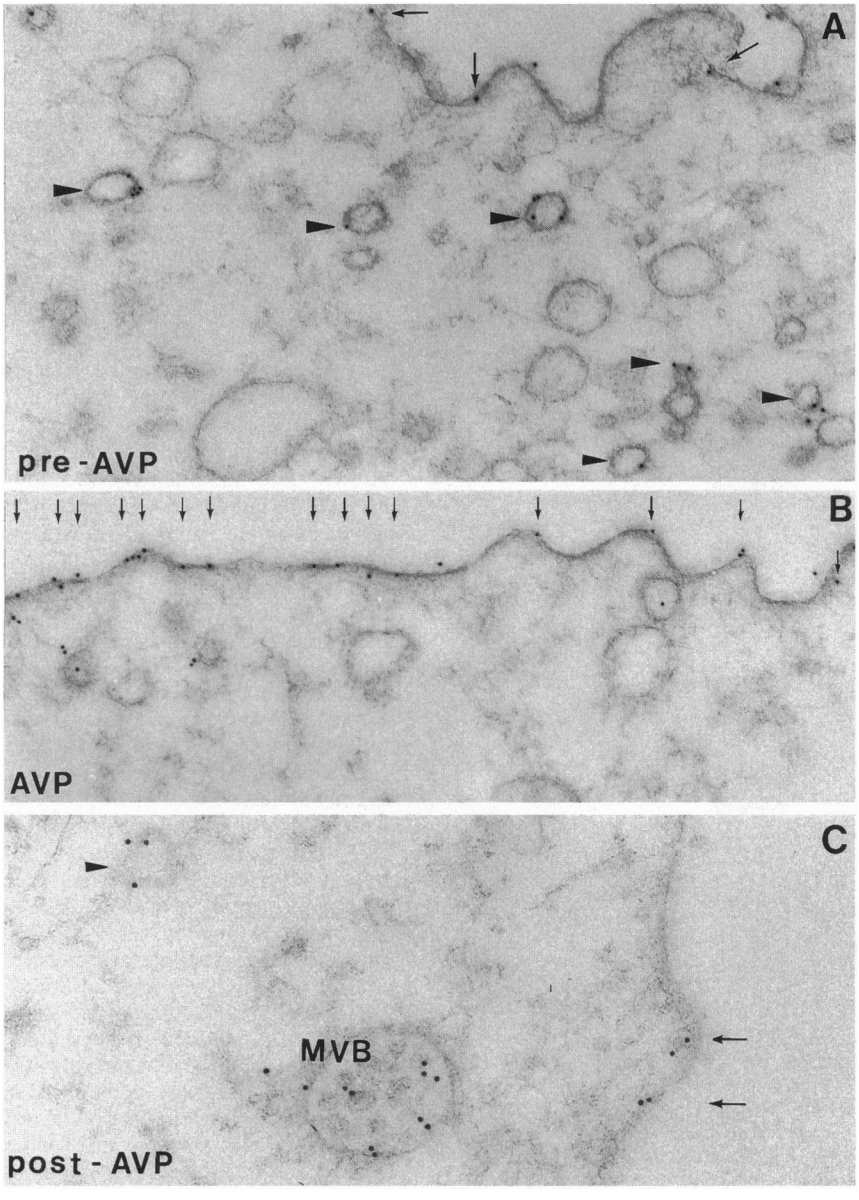

FIG. 2. Immunogold labeling of AQP-CD water channels in ultrathin sections (40 nm) from isolated perfused inner medullary collecting ducts fixed in absence of vasopressin (*A*), after 40-min exposure to AVP (*B*), and 40 min after washout of AVP from peritubular bath (*C*). Arrows point to labeled APM and arrowheads indicate labeled vesicles. MVB, multivesicular body. (*A*, ×56,700; *B*, ×29,700; *C*, ×79,200.)

vesicles were predominantly in the subapical region. Following washout of AVP from the peritubular bath, there was a decrease in AQP-CD labeling of the APM relative to the IVs (Fig. 2C). The labeled vesicles were distributed throughout the cells. After AVP washout, some of the vesicular labeling was associated with multivesicular bodies, although this represented a small fraction of the total labeling.

Sections prepared from all 30 fixed collecting ducts were analyzed quantitatively to document whether AQP-CD labeling shifted reversibly from the IVs to the APM after vasopressin. As shown in Fig. 3A, AVP induced a large increase in the APM:IV labeling ratio (from 0.32 ± 0.03 to 1.38 ± 0.14, $P < 0.001$) in parallel with a large increase in P_f in the same tubules (from 65 ± 16 to 454 ± 45 μm/s, Table 1). Thus, there is a shift in the distribution of AQP-CD water channels from IVs into the APM in association with the vasopressin-induced increase in water permeability, consistent with exocytic insertion of water channels into the APM. Conversely, when AVP was removed from the peritubular bath the APM:IV labeling ratio fell to 0.35 ± 0.04 ($P < 0.001$ vs. AVP), consistent with internalization of the AQP-CD water channel. Therefore, the fall in the water permeability of the collecting duct cells with AVP withdrawal is due at least in part to endocytosis of APM containing AQP-CD water channels.

The AQP-CD labeling density of the apical plasma membrane (D_{APM}) was also quantified (Fig. 3B). D_{APM} increased to four times the basal value with AVP stimulation ($P < 0.001$). Conversely, after removal of AVP, D_{APM} fell ($P < 0.001$) to a level close to the pre-AVP value. Thus, there is a direct correlation between AQP-CD labeling density of the APM and the transepithelial water permeability, consistent with the identification of AQP-CD as the major vasopressin-regulated water channel of the APM in collecting duct cells.

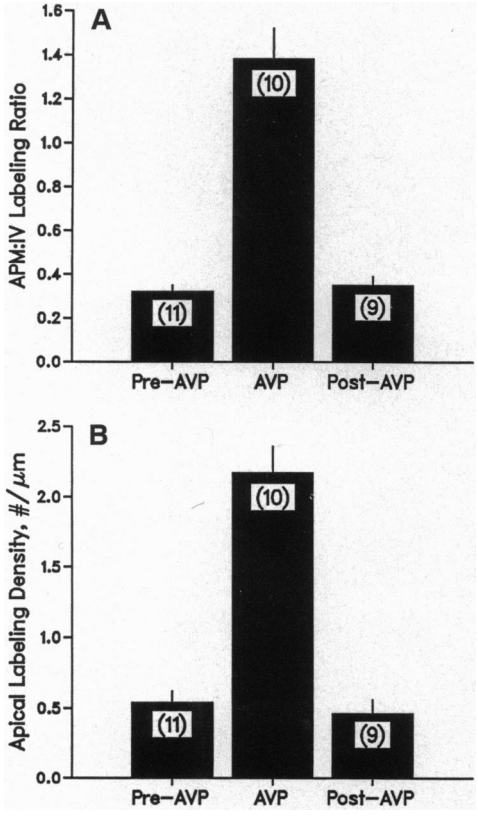

FIG. 3. Quantification of AQP-CD water channel labeling in inner medullary collecting duct cells. (A) Ratio of gold particles in APM to IVs. Electron micrographs (15,000×) were taken of complete cross sections of tubules. Gold particles associated with the APM and IV were counted in at least 10 cells from each tubule (300–500 gold particles). The APM:IV ratio with AVP was significantly greater than pre-AVP and post-AVP values ($P < 0.001$, ANOVA with Bonferroni contrasts). (B) D_{APM} of APM. Apical labeling density, D_{APM}, with AVP was significantly greater than pre-AVP and post-AVP values ($P < 0.001$, ANOVA with Bonferroni contrasts).

DISCUSSION

The AQPs are a family of integral membrane proteins that function as water-selective channels in the plasma membranes of highly water-permeable cells (3–5). Although several AQPs have been identified, two are believed to be responsible for most of the renal tubular water transport in the kidney: (i) aquaporin-1 (or AQP-CHIP) is heavily expressed in the proximal tubule and thin descending limb of Henle's loop (16–18); (ii) aquaporin-2 (or AQP-CD) is the predominant water channel of the collecting duct (6, 7) and has been demonstrated to be the major pathway responsible for vasopressin-regulated water transport across the collecting duct cells (8, 9).

Using a monospecific polyclonal antibody to AQP-CD, we have investigated the mechanism by which the water permeability of the APM of collecting duct cells increases in response to vasopressin. Following vasopressin exposure, a marked change in the intracellular distribution of AQP-CD labeling was seen, with an increased labeling of the APM at the expense of labeling of IVs (Fig. 3). Thus, AQP-CD was translocated from IVs into the APM of collecting duct cells, presumably by exocytosis. This redistribution paralleled the vasopressin-induced increase in epithelial water permeability measured in the same tubules (Table 1). The observed apical redistribution was reversed when vasopressin was washed out of the peritubular bath (Fig. 3), paralleling a decrease in P_f measured in the same tubules. These data, therefore, directly show that vasopressin regulates collecting duct water permeability largely by externalization and internalization of AQP-CD water channels.

The notion that water permeability may be regulated by membrane trafficking was originally proposed (10) chiefly on the basis of observations that aggregates (or "clusters") of intramembrane particles appear in the APM of toad urinary bladders and mammalian collecting ducts coincident with a vasopressin-induced rise in water permeability (19–22). These aggregates were proposed to represent sites of water movement (10). Brown and Orci (23) colocalized the particle clusters to clathrin-coated pits in the APM of collecting duct cells, suggesting the clusters are associated with the endocytic apparatus. It remains to be determined whether the particle clusters contain AQP-CD water channels—e.g., by use of freeze-fracture labeling techniques.

Previous studies have demonstrated that AVP increases apical endocytosis in collecting duct principal cells (24–27). The increase in endocytosis is believed to be a dynamic secondary response to a directly stimulated increase in exocytosis (28). In addition, consistent with the present findings, AVP withdrawal is associated with a transient increase in apical endocytosis (24, 27), which presumably is responsible for the fall in apical membrane AQP-CD labeling seen in the present study. Indeed, AQP-CD has been recently demonstrated to be present in water-permeable vesicles of endocytic origin isolated from the renal inner medulla (29).

The vasopressin-stimulated rise in water permeability in renal collecting ducts is mediated by cyclic AMP. Thus, vasopressin-stimulated externalization of water channels in collecting duct cells is apparently triggered by cyclic AMP, presumably through activation of protein kinase A. Although the fundamental mechanism by which cyclic AMP alters the rate of exo- and endocytosis is unknown, the mechanism may be shared by processes that regulate transporters via trafficking in other tissues—e.g., the histamine-mediated regulation of H^+,K^+-ATPase in gastric parietal cells (30) and insulin-

mediated regulation of the glucose transporter GLUT4 in adipocytes (31).

There exist a large number of disorders of water balance that are due to inherited or acquired defects in regulation of collecting duct water permeability (32). Many of these are associated with defects in the vasopressin receptor (33, 34), in intracellular signaling (32), or in the structure of the water channels themselves (8). The findings of this study add an additional possibility—namely, defective trafficking of water channels. We propose that dysregulation of collecting duct water permeability may arise from disruption of vasopressin-regulated exo- and endocytosis. This appears particularly likely with drug-induced nephrogenic diabetes insipidus associated with drugs such as vinblastine and colchicine (32), which may disrupt cytoskeletal elements involved in water channel trafficking.

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