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Regulation of collecting duct water channel expression by vasopressin in Brattleboro rat

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ABSTRACT **AQP-CD is a vasopressin-regulated water channel expressed exclusively in the renal collecting duct. We have previously shown that AQP-CD is present in the apical plasma membrane and subapical vesicles of collecting duct cells, consistent with membrane-shuttling mechanisms that have been proposed to explain the short-term action of [Arg^8]vasopressin (AVP) to regulate apical water permeability. We propose here that AVP may also have long-term actions on the collecting duct to regulate the expression of the AQP-CD water channel. We used immunoblotting, immunohistochemistry, and *in vitro* perfusion of renal tubules to investigate water channel regulation in collecting ducts of diabetes insipidus (Brattleboro) rats treated with a 5-day infusion of AVP or vehicle. Immunoblotting and immunohistochemistry demonstrated that collecting ducts of vehicle-infused Brattleboro rats had markedly reduced expression of AQP-CD relative to normal rats. In response to AVP infusion there was a nearly 3-fold increase in AQP-CD expression as detected by immunoblotting. Immunocytochemistry demonstrated that the increased expression was predominantly in the apical plasma membrane and subapical vesicles of collecting duct cells. Inner medullary collecting ducts of AVP-infused Brattleboro rats displayed a 3-fold increase in osmotic water permeability relative to vehicle-infused controls, in parallel with the change in AQP-CD expression. Based on these findings, we conclude that (*i*) long-term infusion of AVP, acting either directly or indirectly, regulates expression of the AQP-CD water channel and (*ii*) AQP-CD is the predominant AVP-regulated water channel.**

Total body water is maintained by a delicate balance between intake and excretion. This balance depends critically on the regulation of renal water excretion by the neuroendocrine peptide hormone vasopressin {[Arg^8]vasopressin (AVP); antidiuretic hormone}. AVP regulates water transport in the terminal portion of the renal tubule (the collecting duct), controlling the rate of water transport from the tubule lumen to the peritubular capillaries. AVP acutely alters the water permeability by increasing the number of functional water channels in the apical plasma membrane [the rate-limiting barrier for water transport in the collecting duct (1)] probably by regulated trafficking of vesicles containing the water channels to and from the apical membrane (the "shuttle" hypothesis) (2).

Until recently, definitive evidence for the existence of specific water channel proteins was lacking. In 1988, Denker *et al.* (3) reported the purification of an integral membrane protein from erythrocytes called CHIP28, which in 1992 was proved to be a water-selective channel (4). CHIP28 (renamed aquaporin-CHIP or AQP-CHIP) water channels are abundant in the proximal tubule and thin descending limb of Henle's loop in the kidney (5). AQP-CHIP, however, was not found in the collecting duct (5) and therefore could not be involved in vasopressin-regulated water transport. In 1993, using a PCR cloning technique, Fushimi *et al.* (6) determined the nucleotide sequence of a cDNA encoding another member of the aquaporin family found in the renal collecting duct, AQP-CD. AQP-CD (formerly called WCH-CD) was proposed to be the vasopressin-regulated water channel based primarily on its exclusive location in the collecting duct and on the functional expression of water transport activity when AQP-CD cRNA was injected into *Xenopus* oocytes (6).

In a recent study, we described the cellular and subcellular distribution of AQP-CD in the kidney by immunohisto- and immunocytochemistry using polyclonal antibodies to a synthetic peptide based on the deduced amino acid sequence of the carboxyl terminus of AQP-CD (7). The AQP-CD protein was found in the principal cells and inner medullary cells of the collecting duct (IMCD cells), but not in intercalated cells, which are not believed to possess vasopressin-sensitive water transport. We demonstrated that AQP-CD is localized in the apical plasma membrane, in subapical vesicles, and in multivesicular bodies, a localization consistent with the predicted distribution of water channels according to the shuttle hypothesis.

Previous studies by Lankford *et al.* (8) demonstrated long-term regulation of water permeability in the rat IMCD in response to changes in water intake of the animals. This long-term regulatory response, characterized by a marked increase in the basal water permeability in response to *in vivo* thirsting, was not dependent on changes in intracellular cAMP levels. Thus, the mechanism of this long-term regulation was initially unclear. Subsequently, however, Nielsen *et al.* (7) demonstrated by both immunoblotting and immunohistochemistry that rats thirsted for 24 hr had markedly increased expression of AQP-CD protein in their collecting ducts (7). In addition, Sasaki *et al.* (9) and Ma *et al.* (10) have demonstrated that levels of AQP-CD mRNA in the medulla increased with water restriction of the animals. We hypothesized that long-term increases in circulating vasopressin concentration associated with thirsting may contribute to increased expression of the water channel protein and water permeability. In the current study, we use Brattleboro rats, which manifest an absolute lack of circulating vasopressin (central diabetes insipidus) to investigate long-term regulation of AQP-CD expression. Using this model, we were able to make physiological observations in the presence or absence of AVP by giving a continuous infusion of AVP or vehicle. This allowed us to determine (*i*) whether AQP-CD water channel expression is decreased in control Brattleboro rats, (*ii*) whether long-term infusion of AVP alters the expression of AQP-CD protein, and (*iii*) whether induced changes in AQP-CD water channel expression are associated

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Abbreviations: AQP-CD, vasopressin-regulated water channel; IMCD, inner medullary collecting duct; P_f, osmotic water permeability; AVP, [Arg^8]vasopressin; AU, absorbance unit(s).

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with changes in AVP-stimulated osmotic water permeability (P_f) in the collecting ducts. The results provide evidence for long-term regulation of AQP-CD water channel expression by AVP and physiological evidence that AQP-CD is an AVP-sensitive water channel.

METHODS

Experimental Animals. The study utilized male Brattleboro homozygous (*di/di*) rats (age, 6–10 weeks, Harlan–Sprague–Dawley). The weights of control and experimental rats were carefully matched in each protocol. The rats were equilibrated in individual cages on standard rat chow and ad libitum water intake for at least 1 week after delivery. We used a vasopressin-infusion protocol originally described by Gellai *et al.* (11). Osmotic minipumps (Alza) were inserted subcutaneously in the flank under light general anesthesia with methoxyflurane. The minipumps administered AVP solution or vehicle at a rate of 0.5 μl/hr. AVP was diluted with 5% dextrose and 0.05% acetic acid in water to deliver 21 ng/hr. With this protocol, urinary osmolality typically reaches a nearly steady level within 5 days (mean, 1000–1200 mosM/kg of H_2O) (11).

Four Long Evans rats, the parent strain of the Brattleboro, were maintained for 1 week on the standard rat chow and ad libitum water. The kidneys were removed and pooled for the preparation of membranes to serve as a control lane on immunoblots and to assess the linearity of the densitometry procedure (see below).

Antibodies. We used two antibodies raised against synthetic peptides corresponding to the carboxyl-terminal end of AQP-CD (7). These antisera give identical labeling on immunoblots and in immunohistochemistry. All labeling was prevented by preabsorption of the antibody with an excess of the synthetic peptide.

Preparation of Membrane Fractions. The rats were killed by decapitation and both kidneys were removed. The kidneys were rinsed with isolation solution (250 mM sucrose/10 mM triethanolamine) and the inner medullas were dissected out. The tissue was then homogenized in isolation solution with added protease inhibitors (1 μg of leupeptin per ml and 1 mg of phenylmethylsulfonyl fluoride per ml). A crude membrane fraction was prepared as described by Turner and Silverman (12).

Electrophoresis and Immunoblotting of Membrane Proteins. The quantity of protein in the membrane fraction was determined spectrophotometrically on all samples in a single assay (Pierce BCA protein assay). One microgram of total protein was loaded in each lane. The samples were electrophoresed on SDS/PAGE minigels as described by Laemmli (13). The proteins were transferred to nitrocellulose membrane by electroelution (14). Enhanced chemiluminescence autoradiography (LumiGlo chemiluminescent kit, Kirkegaard & Perry Laboratories) was used to visualize the sites of antigen–antibody reaction. Triplicate gels were run. Two gels were used for immunoblotting and a third gel was used for Coomassie blue staining to confirm equal loading of protein in each lane. Band densities were measured on the exposed x-ray film (Kodak X-Omat AR) using an LKB Ultroscan XL laser densitometer. A standard curve of band density vs. quantity of inner medullary membrane protein from Long Evans rats was constructed to ensure that all experimental measurements were made within the linear range of the chemiluminescent reaction and x-ray film.

Perfusion of Microdissected IMCD Segments. Collecting ducts were dissected from the mid-portion of the inner medulla and were perfused at 37°C by the method of Burg *et al.* (15). The P_f was measured using 1 mM fluorescein sulfonate as a luminal volume marker as described by Wall *et al.* (16). The peritubular bath was identical to the perfusate except that it did not contain fluorescein sulfonate and had sufficient additional NaCl to increase its osmolality to 490 mOsm—i.e., 200 mOsm greater than the perfusate. The fluorescein sulfonate concentration was measured in perfusate and collected fluid using a continuous flow fluorometer (17) (coefficient of variation, 2%). Leak of fluorescein sulfonate from perfused IMCDs is generally negligible (16) and the presence of the dye in the lumen provided a means of ensuring an absence of bulk leak in all tubules. P_f was calculated using the equation of Al Zahid *et al.* (18). In each experiment, the perfused tubule was equilibrated for 30 min after warming to allow the P_f to fall to its basal level (16, 19) and then three collections were made over the next 10 min to determine the basal value of P_f. Then the peritubular bath was exchanged to one containing 200 pM AVP (Sigma). After 30 more min, three additional collections were made to determine the AVP-dependent P_f.

Immunohistochemistry and Immunocytochemistry. Immunolocalization studies were carried out as described (7). AVP-infused and vehicle-infused Brattleboro rats were anesthetized with pentobarbital and the kidneys were fixed by retrograde perfusion through the abdominal aorta with 8% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Tissue blocks from cortex, outer medulla, and four levels of the inner medulla were postfixed for 2 hr, cryoprotected in 2.3 M sucrose/2% paraformaldehyde for 30 min, mounted on holders, and rapidly frozen in liquid nitrogen. Thin (0.85 μm) and ultrathin (80 nm) cryosections were incubated with anti-AQP-CD antisera in 1:2000 dilution. The labeling was visualized as described (7) with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) or goat anti-rabbit IgG conjugated to 10-nm gold particles or protein A-gold (5 or 10 nm). Controls were prepared with (*i*) preimmune sera, (*ii*) antiserum absorbed with excess synthetic peptide, or (*iii*) omission of primary and/or secondary antibody. All controls revealed an absence of labeling.

Statistics. Data are presented as mean ± SEM. The band densities for AVP-infused and vehicle-infused Brattleboro rats were compared with each other using the Mann–Whitney nonparametric rank sum test. All other statistical comparisons utilized *t* tests.

RESULTS

The expression of the AQP-CD water channel in kidney inner medulla was assessed by immunoblotting. Fig. 1 shows representative lanes from an immunoblot demonstrating relative levels of AQP-CD expression in Long Evans rats, vehicle-infused Brattleboro rats, and AVP-infused Brattleboro rats. As demonstrated in our previous study, there are two bands, one at 29 kDa and another broader band between 35 and 45 kDa, which is believed to be the glycosylated form of the protein (7). Relative quantification of AQP-CD protein expression was determined by densitometry of autoradiograms. For this, equal amounts of membrane protein from the inner medullas of all control and all AVP-infused Brattleboro rats were loaded into individual lanes of a single gel to allow a direct comparison of band densities. To establish whether this approach is linear in the range of experimental observations, an immunoblot was done using serial dilutions of inner medullary membranes from Long Evans rats. Band densities of the 29- and 35- to 45-kDa bands were linear to a level of 3 absorbance units (AU) × mm, a value in excess of the largest value seen on experimental blots. Fig. 2 shows mean densitometric areas for both bands from the pooled Long Evans rat sample and the vehicle-infused ($n = 6$) and AVP-infused Brattleboro rats ($n = 6$). The mean area for the 29-kDa band in the vehicle group was 0.27 ± 0.06 AU × mm. After 5 days of AVP infusion, the mean area rose almost 3-fold to 0.78 ± 0.17 AU × mm ($P < 0.01$). There also was an almost 3-fold

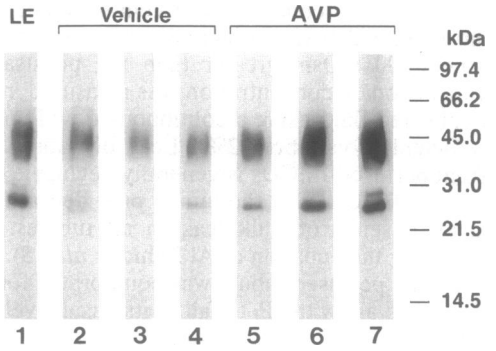

FIG. 1. Western blot of membrane proteins probed with anti-AQP-CD. Representative lanes shown here are from the same full blot, which compared membrane samples from all vehicle-infused and AVP-infused rats. One microgram of membrane protein was loaded in each lane. Equality of loading was checked by comparison of Coomassie blue staining. Lane 1, Long Evans (LE) control; lanes 2–4, vehicle-infused Brattleboro rats; lanes 5–7, AVP-infused Brattleboro rats. This figure was prepared using a scanned image with one grey scale subtraction.

difference in the band density of the 35- to 45-kDa band (0.7 ± 0.06 vs. 1.92 ± 0.4 AU × mm, $P < 0.01$). The densitometric value from the pooled Long Evans rat sample for the 29-kDa band was 0.86 AU × mm and 1.34 AU × mm for the broader band, values that were significantly greater than equivalent bands from vehicle-infused Brattleboro rats ($P < 0.01$).

Immunoblots were prepared using membranes isolated from renal cortices of the same vehicle-infused and AVP-infused Brattleboro rats. Densitometry of the 29-kDa band revealed a highly significant increase in AQP-CD expression in AVP-infused rats (vehicle-infused, 0.50 ± 0.10 AU × mm; AVP-infused, 1.26 ± 0.10 AU × mm, $P < 0.001$). Parallel changes were also seen in the 35- to 45-kDa band.

IMCDs from AVP-infused and vehicle-infused Brattleboro rats were dissected out and perfused *in vitro* to measure P_f (Fig. 3). In both groups, AVP significantly increased P_f. However, the measured permeabilities were substantially greater in IMCD segments from AVP-infused rats than in IMCDs from vehicle-infused rats. The mean AVP-dependent

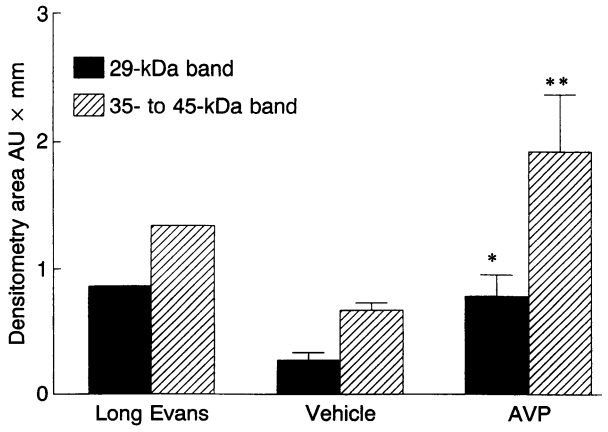

FIG. 2. Integrated band densities for immunoblot prepared with inner medullary membrane protein from four Long Evans rats, six vehicle-infused Brattleboro rats, and six AVP-infused Brattleboro rats on the same gel. The membranes from the Long Evans rats were pooled and loaded in a single lane to accommodate all samples in 13 lanes of the gel. *, $P < 0.01$ compared to the vehicle-treated 29-kDa band; **, $P < 0.01$ compared to the vehicle-treated 35- to 45-kDa band. Serial dilutions of membranes prepared from Long Evans rats demonstrated that the densitometric values are within the linear range of the instrument (data not shown). Urinary osmolalities: Long Evans rats, 624 ± 130 mOsm; vehicle-infused Brattleboro rats, 258 ± 25 mOsm; AVP-infused rats, 1280 ± 146 mOsm.

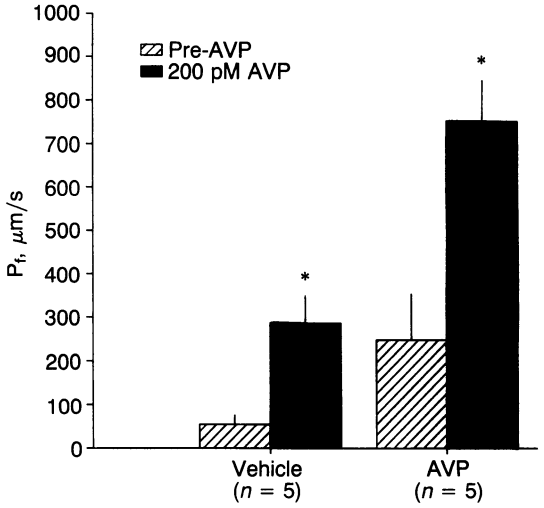

FIG. 3. Results of *in vitro* experiments in isolated perfused IMCD segments from Brattleboro rats. An asterisk (*) indicates that *in vitro* water permeability in presence of AVP (200 pM) was significantly greater than in the absence of AVP in both groups ($P < 0.01$). AVP-dependent water permeability in tubules from AVP-infused rats was significantly greater than AVP-stimulated water permeability from vehicle-infused rats (compare solid bars, $P < 0.01$). An apparent difference in basal values for vehicle- and AVP-infused rats was not statistically significant (compare hatched bars, $P = 0.055$). Urinary osmolalities: vehicle-infused, 202 ± 10 mOsm; AVP-infused, 1495 ± 185 mOsm.

P_f value was increased nearly 3-fold by the prior 5-day *in vivo* AVP infusion (from 286 ± 62 to 753 ± 93 μm/s, $P < 0.01$). Thus, these studies demonstrate that AVP has both a short-term and a long-term effect to increase water permeability in the IMCD.

In contrast to the heavy labeling seen in previous studies using the same antibodies in inner medullas from Sprague–Dawley and Wistar rats (7), the anti-AQP-CD gave sparse labeling of IMCD cells from vehicle-infused Brattleboro rats (Fig. 4*a*). The low expression was confirmed by parallel studies in Wistar rats with identical dilutions of antisera (not shown). A 5-day AVP infusion in Brattleboro rats resulted in a marked increase in AQP-CD labeling, chiefly in the apical region (Fig. 4 *b* and *c*). Chronic AVP infusion also produced an increase in AQP-CD labeling in principal cells of the cortical collecting duct (not shown) in parallel with demonstrated increases in AQP-CD in cortical membranes by immunoblotting (see above). Intercalated cells (arrowheads) and noncollecting duct elements (thin limbs of Henle's loops, vasa recta, and interstitial cells) did not label (Fig. 4 *b* and *c*).

To localize the subcellular sites of labeling in IMCD cells of AVP-infused Brattleboro rats, we carried out immunoelectron microscopy (Fig. 5). Extensive AQP-CD labeling was seen of both the apical plasma membrane and subapical vesicles. Occasionally, labeled vesicles were also seen in other regions of the cytoplasm (not shown).

DISCUSSION

We carried out the present studies in the Brattleboro rat, which manifests an absolute lack of circulating vasopressin. Use of this animal model enabled us to make physiological observations in the presence or absence of AVP. Gellai *et al.* (11) have established that a vasopressin infusion via minipump at the rate used in this study produced plasma vasopressin levels of 10.2 pg/ml at steady state. This is consistent with plasma levels measured in normal rats during antidiuresis. In that study, the authors also demonstrated that by day 5, urinary osmolality, urine flow rate, and plasma osmolality

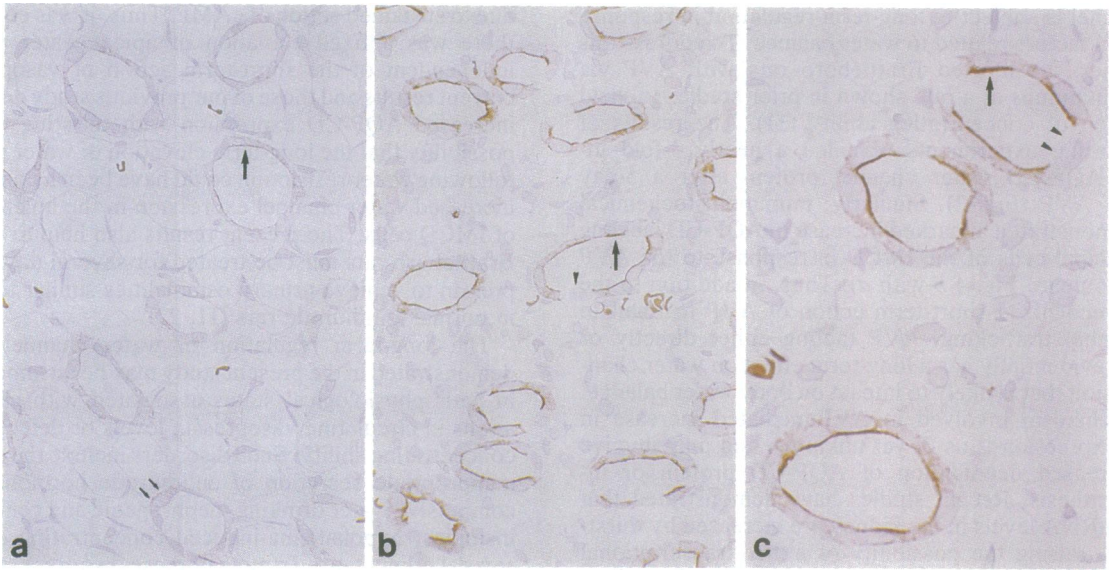

FIG. 4. Immunohistochemical localization of AQP-CD water channel protein in inner medulla from vehicle-treated (*a*) or AVP-treated Brattleboro rats (*b* and *c*). Labeling was done under identical conditions in *a–c*. (*a*) Low magnification of inner medulla of vehicle-infused Brattleboro rat. Weak apical and basolateral labeling is observed in some IMCD cells (arrows), whereas the labeling is at or below the level of detection in other IMCD cells. (×345.) (*b*) Low magnification of inner medulla of AVP-treated rat. Note extensive labeling of principal cells (arrow), whereas intercalated cells (arrowheads) are unlabeled. (×345.) (*c*) High magnification of inner medulla of AVP-treated rat. The extensive labeling is confined to the apical region (arrow), with little or no labeling of basolateral region. Intercalated cells are totally without labeling (arrowheads). There is no labeling of vasculature or thin limbs. (×670.)

had reached a plateau. Hence, we chose to perform our experiments on rats treated for 5–6 days. Because the AVP was infused in an otherwise intact animal, however, the effects of AVP on the collecting duct could be either a direct result of AVP-induced signaling in the collecting duct or a secondary effect associated with systemic actions of AVP. Nonetheless, the data support two general conclusions: (*i*) long-term infusion of AVP, directly or indirectly, regulates the expression of AQP-CD water channel in the medullary collecting duct and (*ii*) AQP-CD is the predominant AVP-regulated water channel. In the remaining text, we will discuss the general physiological and potential pathophysiological significance of the observations.

Preliminary immunohistochemical observations revealed that labeling by anti-AQP-CD was markedly diminished in the collecting ducts of Brattleboro rats relative to Wistar and Sprague–Dawley rats. The present controlled studies demonstrated that labeling was very low in collecting ducts of vehicle-infused Brattleboro rats (Fig. 4*a*). Quantification of AQP-CD expression by immunoblotting confirmed this observation (Figs. 1 and 2), showing decreased AQP-CD expression in the inner medullas of vehicle-infused Brattleboro rats relative to that seen in Long Evans rats, the parent strain of the Brattleboro rat. This finding in combination with our prior observation that thirsting increases AQP-CD expression in collecting ducts of normal rats (7) suggested that this

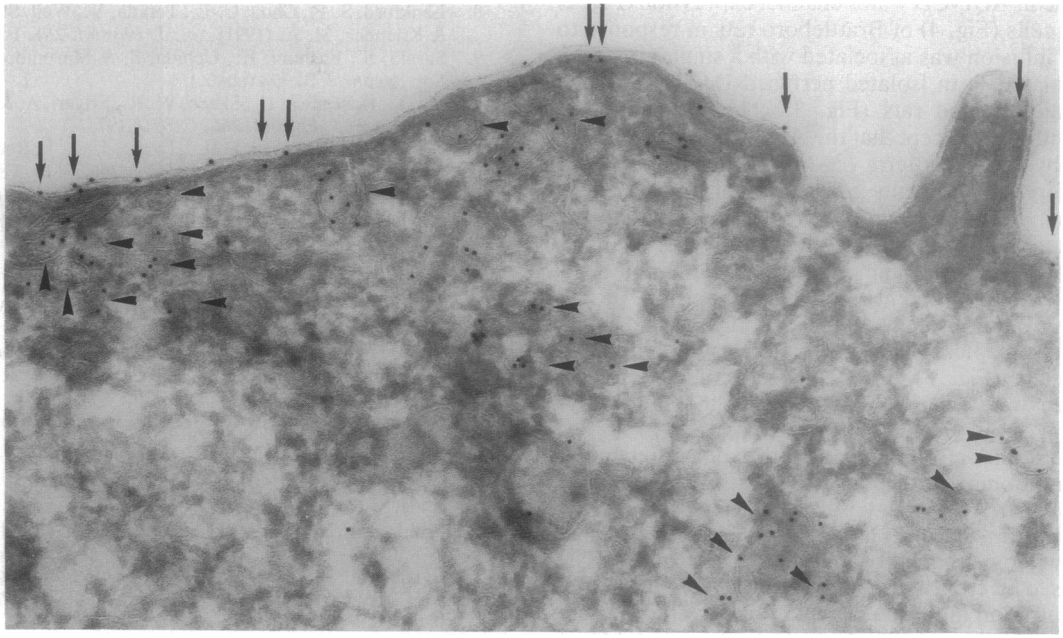

FIG. 5. Immunocytochemical localization of AQP-CD water channel protein of IMCD cells. IMCD cells exhibit intense labeling of apical plasma membranes (arrows) and of subapical vesicles (arrowheads). (×62,400.)

water channel is subject to long-term regulation in response to unknown factors related to water balance. To address this issue further, we infused Brattleboro rats with AVP via osmotic minipumps at a rate shown in prior studies to yield a restoration of concentrating ability (11). The results of immunoblotting experiments revealed a nearly 3-fold increase in AQP-CD water channel protein after a 5-day infusion of AVP (Fig. 2). Similarly, immunohistochemical studies demonstrated a marked increase in AQP-CD labeling in the principal cells of the IMCD in response to the AVP infusion (compare Fig. 4 *b* with *a*). Thus, in addition to the generally recognized short-term action of AVP to regulate water channel trafficking, AVP (acting either directly or indirectly) evidentially has a long-term effect on water channel expression that is likely to impact on body water balance.

The mechanism involved in AVP-mediated increase in AQP-CD expression is as of yet unknown and may involve either decreased degradation of AQP-CD protein or increased synthesis. Recent studies have demonstrated that AQP-CD mRNA levels in the kidney are increased by thirsting (9, 10), raising the possibility of either transcriptional regulation or regulation of AQP-CD mRNA stability. Furthermore, we do not yet know whether the increased AQP-CD protein expression is a direct response to AVP-induced intracellular signaling or to indirect effects of AVP. However, based on the observed AVP-induced increase in AQP-CD expression in the collecting duct of the cortex (which is always surrounded by an isotonic interstitium), it appears unlikely that changes in local osmolality are necessary to trigger the response.

In the initial report of the cloning of the AQP-CD water channel, the conclusion that AQP-CD was a vasopressin-sensitive water channel was based on its localization exclusively to the kidney collecting tubule and the expression of water channel activity by the injection of the AQP-CD cRNA in *Xenopus* oocytes (6). Recently, a report by Deen *et al.* (20) has described a patient who manifests nephrogenic diabetes insipidus and different mutations in the two AQP-CD alleles (located on chromosome 12). These results establish a clear link between the AQP-CD water channel and the regulation of renal water excretion, greatly strengthening the view that AQP-CD is the predominant AVP-regulated water channel. Our studies provide further evidence for this view. A nearly 3-fold increase in AQP-CD water channel expression (Fig. 2) in the IMCD cells (Fig. 4) of Brattleboro rats in response to chronic AVP infusion was associated with a similar increase in AVP-stimulated P_f in isolated perfused IMCD segments from identically treated rats (Fig. 3). These findings are compatible with the concept that the AQP-CD water channel is regulated by the short-term (cAMP-mediated) action of AVP. Therefore, these results add important functional evidence to the already-strong body of evidence that the AQP-CD water channel is an AVP-regulated water channel. The data provided by the immunohisto- and immunocytochemistry are also consistent with this conclusion, as the amount of AQP-CD protein increased only in the apical membrane and subapical vesicles of collecting duct principal and IMCD cells (Fig. 5), as would be expected for an AVP-regulated water channel according to the shuttle hypothesis (2).

The current studies also offer a possible explanation for the long-term conditioning in response to thirsting observed by Lankford *et al.* (8). These studies demonstrated that *in vivo* water restriction in Sprague–Dawley rats induced a stable increase in water permeability in isolated perfused IMCD segments dissected from these animals (8, 21). Because the cAMP concentration in these cells was not elevated and the tubules did not display an elevation of urea permeability, it was concluded that the increased water permeability was not due to sustained action of cAMP. Thus, it was concluded that there was a fixed elevation of apical water permeability independent of the short-term action of vasopressin. The current results and those of our previous study demonstrating increased AQP-CD expression with thirsting (7) raise the possibility that the long-term elevation of water permeability following water restriction could have been a consequence of increased water channel expression in the apical membrane of IMCD cells. The present results also help to explain why Brattleboro rats must be treated for several days with vasopressin to achieve urinary osmolalities similar to those seen in normal antidiuretic rats (11, 22).

The long-term regulation of water channel expression demonstrated in the present study may be an important factor in pathophysiological states associated with chronic alterations in circulating vasopressin levels or defects in urinary concentrating ability. Such disorders include the syndrome of inappropriate secretion of antidiuretic hormone (SIADH), compulsive water drinking, nephrogenic and central diabetes insipidus, hypokalemia-induced concentrating defects, and several other states. In most of these disorders, correction of the abnormality in urinary concentration lags significantly behind the acute correction of the proximate abnormality. This has been amply illustrated in studies involving forced water drinking or water restriction in normal subjects which demonstrate that a return to normal water balance often requires several days following cessation of the perturbation in water intake (23, 24). The results from our study suggest that this lag could be due in part to an up- or down-regulation of AVP-sensitive water channels.

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