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The AQP2 Water Channel: Effect of Vasopressin Treatment, Microtubule Disruption, and Distribution in Neonatal Rats

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Abstract. Aquaporin 2 is a collecting duct water channel that is located in apical vesicles and in the apical plasma membrane of collecting duct principal cells. It shares 42% identity with the proximal tubule/thin descending limb water channel, CHIP28. The present study was aimed at addressing three questions concerning the location and behavior of the AQP2 protein under different conditions. First, does the AQP2 channel relocate to the apical membrane after vasopressin treatment? Our results show that AQP2 is diffusely distributed in cytoplasmic vesicles in collecting duct principal cells of homozygous Brattleboro rats that lack vasopressin. In rats injected with exogenous vasopressin, however, AQP2 became concentrated in the apical plasma membrane of principal cells, as determined by immunofluorescence and immunogold electron microscopy. This behavior is consistent with the idea that AQP2 is the vasopressin-sensitive water channel. Second, is the cellular location of AQP2 modified by microtubule disruption? In normal rats, AQP2 has a mainly apical and subapical location in principal cells, but in colchicine-treated rats, it is distributed on vesicles that are scattered throughout the entire cytoplasm. This is consistent with the dependence on microtubules of apical protein targeting in many cell types, and explains the inhibitory effect of microtubule disruption on the hydroosmotic response to vasopressin in sensitive epithelia, including the collecting duct. Third, is AQP2 present in neonatal rat kidneys? We show that AQP2 is abundant in principal cells from neonatal rats at all days after birth. The detection of AQP2 in early neonatal kidneys indicates that a lack of this protein is not responsible for the relatively weak urinary concentrating response to vasopressin seen in neonatal rats.

Key words: Water channels — Vasopressin — Rat kidney — Immunocytochemistry — Microtubules — Cell polarity

Introduction

A collecting duct water channel, initially referred to as WCH-CD and now renamed aquaporin 2 (AQP2), has been recently sequenced, and its distribution in adult rats has been reported (Fushimi et al., 1993). This protein, which is 42% identical to CHIP28 (Preston & Agre, 1991), is located in the apical membrane and apical vesicles in collecting duct principal cells (Fushimi et al., 1993; Nielsen et al., 1993a). This water channel is believed to be the vasopressin-sensitive water channel that is located on specialized intracellular vesicles that are inserted into the apical plasma membrane of principal cells as a result of antidiuretic hormone stimulation (Wade, Stetson & Lewis, 1981; Brown 1991; Harris, Strange & Zeidel, 1991; Verkman, 1992). With the availability of specific anti-AQP2 antibodies, it becomes feasible to test this hypothesis directly by examining the distribution of AQP2 in vasopressin-deficient Brattleboro rats, before and after vasopressin administration. Examination of this question was the first aim of the studies described in this report.

It has been described, both in vivo and in isolated perfused tubules that microtubule disrupting agents such as colchicine and nocodazole inhibit the vasopressin-induced permeability increase in renal collecting ducts,

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presumably by interfering with the targeted insertion of vesicles that contain water channels into the apical plasma membrane (Dousa & Barnes, 1974; Dousa & Valtin, 1976; Phillips & Taylor, 1989). Our previous work has shown that colchicine treatment of rats disrupts the normal membrane recycling process that modulates the cell surface expression of certain plasma membrane proteins, including the proximal tubule protein gp330 (Gutmann et al., 1989), and the proton pumping ATPase in collecting duct intercalated cells (Brown, Sabolić & Gluck, 1991). Many other studies have demonstrated a permissive role for microtubules in the apical targeting of both membrane and secreted proteins (Orci et al., 1973; Patzelt, Brown & Jeanrenaud, 1977; Malaisse-Lagae et al., 1979; Busson-Mabillot et al., 1982; Pavelka, Ellinger & Gangl, 1983; Hasegawa et al., 1987; Ojakian & Schwimmer, 1988; Achler et al., 1989; van Zeijl & Matlin, 1990; De Almeida & Stow, 1991). The second aim of this report was to determine whether the normal apical distribution of the AQP2 water channel in principal cells was also disrupted by colchicine, thus providing a cell biological explanation for the inhibitory effect of microtubule disruption on epithelial water permeability.

Finally, neonatal mammals including man, rat, pig and rabbit have a reduced ability to concentrate their urine that progressively increases with age (Spitzer & Schwartz, 1992). It has been shown that several factors combine to produce this deficient concentrating process, including a reduced interstitial osmolality in the papilla as well as a reduced sensitivity of collecting ducts to the antidiuretic hormone, vasopressin. While neonatal rabbit collecting ducts have a higher basal water permeability than those of adult animals, this permeability is only moderately increased by vasopressin, in contrast to the case in adults (Horster & Zink, 1982). The vasopressin-induced stimulation of adenylyl cyclase is markedly reduced in neonates compared to adults, which could in part explain the relative lack of vasopressin sensitivity (Schlondorff et al., 1978). However, a further possibility is that neonatal collecting ducts either do not express, or express lower levels of the vasopressin-sensitive water channel, which would limit their capacity to respond to hormonal stimulation. It has recently been shown that there is a large induction of another renal water channel, CHIP28, at birth in rat kidneys, which explains the ability of rat kidneys to begin partially concentrating soon after birth (Smith et al., 1993). The final aim of this study was, therefore, to determine whether AQP2 is present in neonatal rat kidneys.

Materials and Methods

Generation of Polyclonal Antipeptide Antibodies

A peptide containing the sequence homologous to the last 16 amino acids of the AQP2 (CVELHSPQSLPRGSKA) (Fushimi et al., 1993) was synthesized and coupled to KLH. Rabbits were immunized intradermally with the conjugated peptide in complete Freunds adjuvant, and subsequently in incomplete adjuvant for booster immunizations. Sera were collected every three weeks and tested by indirect immunocytochemistry for positive staining of collecting duct principal cells, by Western blotting for staining of the specific protein bands in membranes from renal papilla (*see below*), and by ELISA assay against the immunizing peptide.

Affinity Purification of Anti-AQP2 Antibody

Total membranes from normal rat papilla were isolated, proteins dissolved in Laemmli sample buffer at 65°C, separated through a 12% gradient gel, and transferred to Immobilon as described below. Horizontal strips of Immobilon related to the nonglycosylated and glycosylated forms of AQP2 protein bands, whose localization on the Immobilon sheet had been previously verified by Western blotting of a vertically cut strip, were excised, washed 2 × 30 min with milk-containing blotting buffer, followed by 5× PBS and an incubation in concentrated immune serum for 5 hours at room temperature to adsorb anti-AQP2 antibodies. The strips were then extensively washed with PBS, and the immunoaffinity-adsorbed antibodies were released by incubation in 0.1 M citric acid buffer, pH 2, for 1 min under constant vortexing, followed by neutralization to pH 7–7.4 with 1 M Tris/HCl buffer, pH 10.5. The sample with released antibodies was then centrifuged at 30,000 *g* for 15 min to remove clumps and small Immobilon particles (this maneuver also removes significant background in immunocytochemical studies), and was used diluted with PBS 1:2 for immunocytochemical studies, or diluted 1:200 in blotting buffer for Western blotting.

Vasopressin Treatment of Rats

All studies and vesicle isolations were performed on tissues from either untreated Sprague-Dawley rats or Brattleboro rats (vasopressin deficient) that had been intravenously injected with 0.25 U arginine vasopressin (in 0.5 ml PBS) via the jugular vein 15 and 30 min prior to killing (vasopressin-treated rats) or PBS alone (control rats). Rats were anesthetized with sodium pentobarbital as described below prior to injection and perfusion. Three rats were used per time point for each treatment, and both kidneys from each rat were examined by immunocytochemistry. A further group of three rats was deprived of water for 17–25 hours (dehydrated rats) prior to sacrifice. For vesicle isolation, the papilla from six kidneys were used for each preparation.

Neonatal Rats

Rat pups of ages ranging from 24 h to 12 days were anesthetized with sodium pentobarbital and the kidneys were removed and fixed by immersion (*see below*). Older pups were fixed by perfusion fixation via a cardiac catheter (*see below*). After overnight fixation, they were rinsed in PBS and immersed in 30% sucrose for at least 1 h prior to cryosectioning.

Colchicine Treatment

Adult Sprague-Dawley rats were injected intraperitoneally with 0.5 mg/100 g colchicine in PBS (Gutmann et al., 1989). After 6, 8 and 12 h, the rats were anesthetized with sodium pentobarbital and the kidneys were fixed by perfusion as described below. Control rats were injected

with the inactive isomer of colchicine, lumicolchicine, prepared as previously described (Ercolani & Schultz, 1983).

Preparation of Membrane Fractions

To obtain total membranes from renal papilla, the inner medullas from 2–5 rats were pooled, dispersed in 15 ml cold PBS, and homogenized in a Dounce tissue grinder (about 50 strokes by hand). The homogenate was centrifuged at 2,500 *g* for 15 min. The pellet containing nuclei and cell debris was discarded; the supernatant was centrifuged at 48,000 *g* for 60 min. The resulting pellet, that was enriched in cell plasma membranes was dispersed in a small volume of PBS and prepared for SDS-PAGE.

Cortical brush border membrane vesicles (BBMV) were isolated by the Mg/EGTA-aggregation method of Biber et al. (1981). Cortical basolateral membrane vesicles (BLMV) and endocytic vesicles (EV) were isolated by the differential and Percoll density gradient centrifugation method of Scalera et al. (1981) and Sabolić and Burckhardt (1990), respectively. Endocytic vesicles from renal papilla were isolated by the method of Sabolić et al. (1992b). Isolated vesicles were either used immediately or were kept in liquid nitrogen until required. All homogenization steps were performed in the presence of 1 mM phenylmethyl-sulfonyl fluoride.

Plasma membranes from rat red blood cells (RBC-PM) were prepared by first releasing hemoglobin by lysis in distilled water, followed by three washings and centrifugation of membranes in distilled water, followed by a final centrifugation at 30,000 *g* for 30 min.

CHIP28 from human red blood cells and MIP26 from rat lens were isolated by the methods described by Van Hoek and Verkman (1992) and Verbavatz et al. (1994), respectively.

SDS-PAGE and Western Blotting

Because the detection of AQP2 was found to be sensitive to boiling (*see later*) all membrane preparations or purified CHIP28 and MIP26 were solubilized by heating at 65°C for 15 min in sample buffer (1% sodium dodecyl sulfate (SDS), 30 mM Tris/HCl, pH 6.8, 5% 2-β-mercaptoethanol, 12% (v/v) glycerol). Proteins (5 μg/lane of purified CHIP28 and MIP26, and 150 μg/lane of all other samples) were separated by electrophoresis through 12% Laemmli SDS-polyacrylamide gradient slab gels (SDS-PAGE), and transferred to Immobilon membrane (Millipore, Bedford, MA). The membranes were briefly stained with Coomassie blue to check the efficiency of the transfer. Destained membranes were blocked in blotting buffer comprising 5% nonfat dry milk, 0.15 M NaCl, 1% Triton-X-100, 20 mM Tris-HCl, pH 7.4, followed by incubation with either preimmune or immune serum (diluted 1:500 in blotting buffer), or affinity-purified antibodies (diluted with PBS 1:200) at room temperature for four hours. The membranes were then washed by several changes of blotting buffer, incubated for 60 min in blotting buffer which contained 0.1 mg/ml of goat anti-rabbit IgG conjugated to alkaline phosphatase (Vector, Burlingame, CA), washed again, and stained for alkaline phosphatase activity by using BCIP/NBT Phosphate Substrate System (Kirkegaard & Perry, Gaithersburg, MD).

Tissue Fixation

Brattleboro rats or Sprague-Dawley rats were anesthetized with Nembutal (65 mg/kg). The kidneys were perfused via the abdominal aorta (or the left ventricle in the case of immature rats) first with Hank's balanced salt solution for 2–3 min and then with a fixative containing 3% paraformaldehyde, 0.1% glutaraldehyde, 5% sucrose in PBS for 10 min. Some kidneys were fixed in paraformaldehyde-lysine-periodate (McLean & Nakane, 1974)—no difference in the staining pattern was observed with the two fixatives. Organs were removed, sliced, and kept overnight in the same fixative at 4°C, followed by washing (three times) with phosphate-buffered saline (PBS). The tissue slices were then kept in PBS containing 0.02% sodium azide at 4°C until further use.

Immunofluorescence

To cut 4–5 μm frozen sections for indirect immunofluorescence, tissue blocks were infiltrated with 30% sucrose overnight, frozen in liquid nitrogen, and sectioned on a Reichert Frigocut cryomicrotome. Sections were placed on Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), kept in PBS for 10 min, preincubated for 15 min with 1% BSA in PBS, and then incubated at room temperature for 90 min with either preimmune or immune serum (diluted 1:400 with PBS) or affinity-purified antibody (diluted with PBS 2:1). This was followed by washing two times for 5 min in PBS containing 2.7% NaCl (high-salt PBS), plus two times in regular PBS. Washing with a high-salt buffer decreases nonspecific binding of antibodies. The sections were then incubated for 60 min with CY3-labeled goat anti-rabbit immunoglobulin antibody (Jackson Imm.), followed by washing two times for 5 min in high-salt PBS, and two times in regular PBS. For double labeling studies, sections were first incubated as above to detect AQP2, and were then sequentially exposed to a monoclonal antibody against the 31 kD subunit of the vacuolar proton pumping ATPase (provided by Dr. Steven Gluck, Washington University), and goat anti-mouse FITC. Sections were mounted in Gelvatol and were examined and photographed with a Nikon FXA photomicroscope equipped for epifluorescence. Double stained sections were also examined with a Bio-Rad 600 confocal microscope.

Immunogold Electron Microscopy

Sections for ultrastructural labeling studies were prepared from a vasopressin-injected (30 min) and a control Brattleboro rat fixed in 3% paraformaldehyde, 0.1% glutaraldehyde, 5% sucrose in PBS as detailed above. After immersing pieces of papilla in 2.3 M sucrose for at least 1 hr tissue was frozen in liquid nitrogen and ultrathin, frozen sections were cut at approximately 80 nm thickness on a Reichert FC4D ultracryomicrotome. Sections were incubated on a drop of PBS/1% BSA for 10 min, then on a drop of affinity-purified anti-AQP2 antibody (undiluted) for 2 hr at room temperature. Grids were washed 3 × 5 min in PBS, and incubated for 1 hr on a drop of protein A-gold solution prepared from 10 nm gold particles as previously described (Slot & Geuze, 1985). After 3 more washes in PBS, the sections were post fixed in 1% glutaraldehyde for 10 min, washed in distilled water, and embedded and stained by incubation on 3% methylcellulose containing 0.2% uranyl acetate for 10 min at room temperature. The sections were examined and photographed using a Philips CM10 electron microscope. The final magnification of prints used for gold particle counting was 45,000×.

The number of gold particles per μm of apical plasma membrane was counted on 20 principal cells from a vasopressin-treated rat, and 17 principal cells from a control Brattleboro rat. The plasma membrane was highlighted on each photograph with a line that had a width of 5–6 gold particle diameters, and all particles falling within this band were considered to represent membrane staining. Background staining levels were estimated by counting the number of gold particles on apical plasma membranes of thin limbs of Henle, which should not contain the antigen of interest. Measurements were collected using a Numonics

graphic digitizer model 2210 (Numonics, Montgomeryville, PA) and Jandel Sigma Scan software (Jandel Scientific, Corte Madera, CA). Data were collated using the Statworks program (Cricket Software, Philadelphia, PA) and analyzed with Student *t*-test for unpaired samples.

Results

Characterization of the AQP2 Antibody

The AQP2 antibody used in these studies was raised against a synthetic peptide (VELHSPQSLPRGSKA) deduced from the C-terminal cDNA sequence of the cDNA clone. When applied in Western blots to papillary membrane fractions, the crude antiserum labeled a sharp band at 28 kD, a diffuse band around 40 kD, and some higher molecular weight bands (Fig. 1, lane 2). Following affinity purification against Immobilon strips that contained either the 28 kD region (lane 3) or the 40 kD region (lane 4) of the gel, an identical staining pattern was seen, except that the higher molecular weight bands were no longer detected. This indicates that the protein sequence recognized by the antiserum is present in both the 28 kD and the 40 kD bands—they probably represent deglycosylated and glycosylated forms of the protein, respectively. A test of specificity, preimmune serum was used (lane 1), and gave no staining of papillary membranes. Furthermore, antibody that was preincubated with the immunizing peptide gave a much reduced signal (lane 6), compared to the untreated antiserum (lane 5).

Although the C-terminal peptide used to raise the antibody does not share homology with other members of the CHIP28/MIP26 protein family, we tested the antibody against these purified proteins, as well as against membrane fractions from other kidney regions, and other cells, that are known to contain CHIP28. Fig. 1*B* shows that while MIP26 purified from lens is recognized by anti-MIP26 antiserum (lane 1), it is not stained with anti-AQP2 (lane 3). Similarly, purified red cell CHIP28 is stained with anti-CHIP28 antibodies (lane 2), but not by anti-AQP2 (lane 4). Other membranes known to contain CHIP28 were not stained with the anti-AQP2 antibodies (lanes 5–8; *see* figure legend for details). The effect of sample preparation is well illustrated in lanes 9 and 10 of Fig. 1*B*. Whereas both the 28 kD and 40 kD bands in papillary membranes are stained after denaturation at 65°C (lane 9), these bands are completely undetectable if the sample is heated to 100°C (lane 10). Finally, lane 11 shows that AQP2 is abundant in papillary endosomes. Compare this to its absence from cortical endosomes (lane 8). A similar result has been recently reported by Harris et al. (1994). This confirms that AQP2 is present in endosomes from papillary principal cells, as predicted by the membrane shuttle hypothesis of vasopressin action.

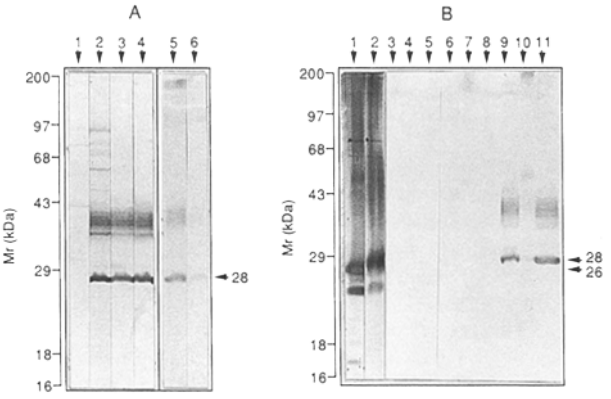

Fig. 1. Characterization of anti-AQP2 antibody by Western blotting. (*A*) Immunoblot showing reactivity of the preimmune serum, anti-AQP2 immune serum, and affinity-purified antibodies with proteins in total membranes from rat renal papilla. (1) Preimmune serum weakly stained three protein bands unrelated to the AQP2 protein. (2) Immune serum strongly stained a sharp band of 28 kDa and a more diffuse one at 40 kDa which presumably represent the nonglycosylated and glycosylated forms of AQP2, respectively. In addition, several other bands between 45 and 97 kDa were somewhat stained. (3) Antibodies that had been immunoadsorbed from the immune serum on Immobilon strips containing the 28-kDa band stained the 28 kDa and the 40 kDa protein bands only. The higher molecular weight bands were not stained. (4) Antibodies that had been immunoadsorbed from the immune serum on Immobilon strips containing 40 kDa band also stained both the 28 kDa and 40 kDa protein bands, thus indicating that both protein bands belong to the two different forms of the same protein. When the affinity-purified antibodies were preincubated with the peptide (10 mg/ml) for 60 min, the labeling of the 28 kDa and 40 kDa bands was considerably reduced (lane 6) compared to the labeling with the same antibody preparation not exposed to the peptide (lane 5). (*B*) Absence of crossreactivity of the affinity-purified anti-AQP2 antibodies with other members of the MIP family; heat inactivation of the AQP2 antigenicity. (Lane 1) Purified MIP26 is labeled with anti-MIP26 antibodies. (Lane 2) Purified CHIP28 is labeled with anti-CHIP28 antibodies. In contrast, affinity purified anti-AQP2 antibodies failed to label purified MIP26 (lane 3), purified CHIP28 (lane 4), red blood cell plasma membranes (lane 5), renal cortical brush-border (lane 6), basolateral (lane 7), and cortical endosomal membranes (lane 8). The 28 kDa and the diffuse 40 kDa protein bands of the AQP2 were stained in total papillary membranes denatured in sample buffer at 65°C for 15 min (lane 9), but the staining was absent in membranes that had been denatured at 100°C for 5 min (lane 10). Staining of both bands was present in an endosomal preparation from renal papilla. All protein and membrane samples, except for that in lane 10, were denatured at 65°C.

Effect of Vasopressin on Distribution of AQP2

Immunofluorescence

In untreated Brattleboro homozygous rats, staining for AQP2 was restricted to principal cells of collecting ducts, as previously described in normal rats (Fig. 2*A*) (Fushimi et al., 1993; Nielsen et al., 1993a). Intercalated cells were unstained. In the papilla of Brattleboro rats, the staining of principal cells was weaker than in the

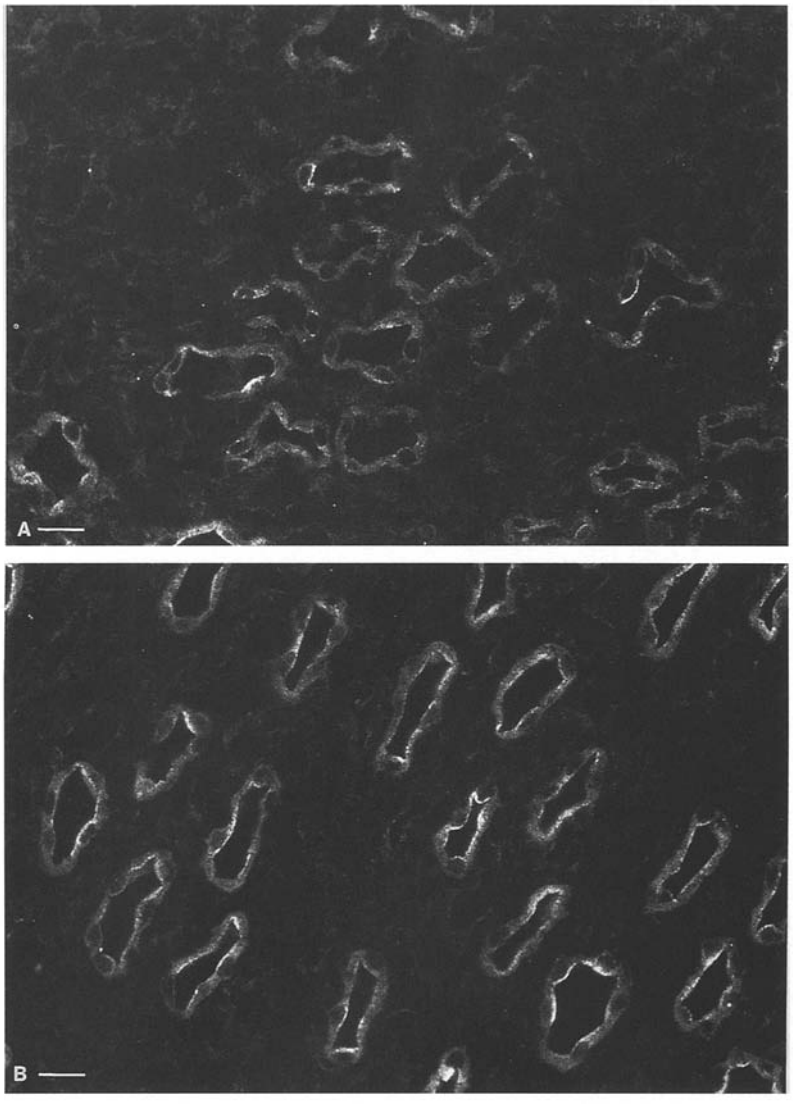

Fig. 2. Immunofluorescence localization of AQP2 in cryostat section of Brattleboro rat kidney papilla. In control rats, AQP2 had a weak and diffuse distribution in the cytoplasm of principal cells of papillary collecting ducts (*A*), whereas after vasopressin treatment, many cells showed a bright apical staining (*B*). In the absence of vasopressin, very few apically stained principal cells were seen (*A*). Bar = 20 μm.

medulla of Sprague-Dawley rats (compared Fig. 2*A* with Fig. 5*A*), and the staining pattern showed a diffuse punctate distribution throughout the cytoplasm. A few cells, however, had a heavier staining concentrated at the apical pole of the cell. In contrast, papillary principal cells from Brattleboro rats treated with vasopressin 30 min prior to fixation showed a marked increase in apical staining. In many cells, a sharp apical band was clearly visible in the principal cells (Fig. 2*B*). Rats treated for 15 min with vasopressin showed an intermediate pattern of labeling, with less fluorescence at the apical pole of principal cells than in rats treated for 30 min with vasopressin (*not shown*).

Immunogold Electron Microscopy

In control Brattleboro rats, most of the gold particle label was present in the cytoplasm, often clearly associated with vesicular structures (Fig. 3*A*). Only very few gold particles were found over the apical plasma membrane. In contrast, there was a sharp increase in the number of gold particles on the apical plasma membrane of principal cells from vasopressin-treated Brattleboro rats (Fig. 3*B*). The degree of apical staining varied somewhat from cell to cell, and cytoplasmic vesicles were still labeled in most cells. A small amount of label was seen over the basolateral plasma membrane, but this was considerably less than the apical membrane staining (Fig. 3*B*). Quantification of the apical gold labeling revealed a 7-fold increase in apical plasma membrane staining after vasopressin treatment for 30 min (Table).

By Western blotting (Fig. 4), a clear 28 kD band was detectable in papillary membranes from all rats. However, the intensity was much greater in membranes from normal rats compared to Brattleboro rats. Neither vasopressin treatment for 30 min, nor overnight dehydration

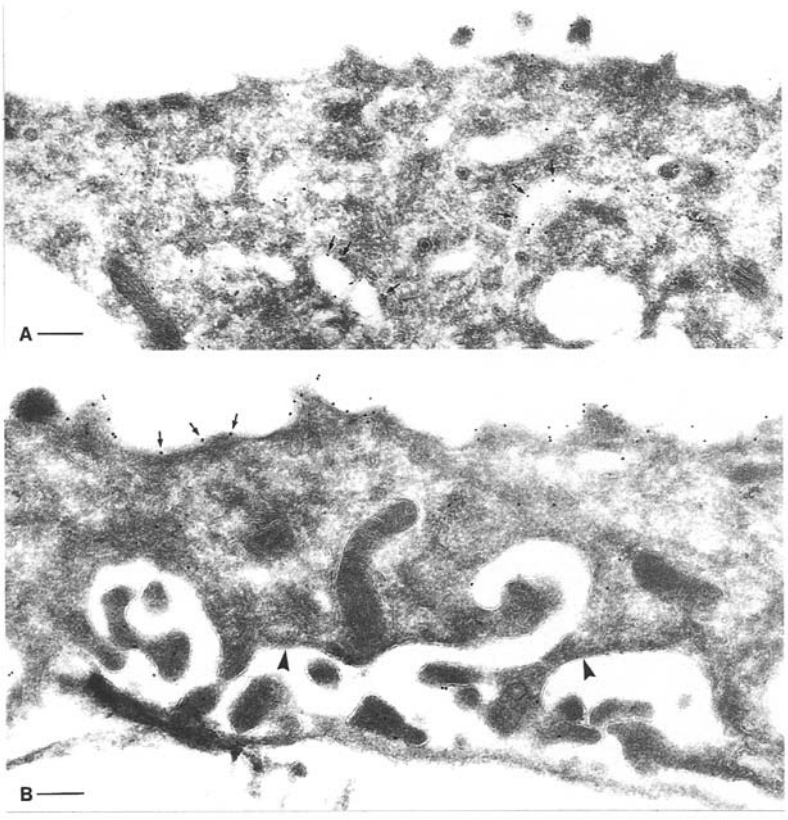

Fig. 3. Localization of AQP2 by immunogold electron microscopy on ultrathin frozen sections of rat papillary collecting ducts from a control Brattleboro rat (*A*), and a Brattleboro rat treated with vasopressin for 30 min (*B*). Under control conditions, intracellular vesicles are labeled with gold particles (arrows), but there is only a low level of apical plasma membrane labeling. In contrast, in the vasopressin treated rat, there is a marked increase in the number of gold particles associated with the apical plasma membrane (arrows). Note that the basolateral plasma membrane (arrowheads) is poorly labeled compared with the apical membrane (*B*). Bar = 0.25 μm (*A*) or 0.2 μm (*B*).

of Brattleboro rats noticeably increased the AQP2 signal of papillary membranes.

Effect of Colchicine on AQP2 Distribution

In contrast to the predominantly apical and subapical location of AQP2 in principal cells from control Sprague-Dawley rats (Fig. 5*A*), the immunoreactivity was distributed throughout the entire cytoplasm of colchicine-treated animals (Fig. 5*B*). Many of the stained vesicles were close to the basolateral plasma membrane of the principal cells.

AQP2 Expression in Neonatal Rat Kidneys

AQP2 was readily detectable in collecting duct principal cells at all times after birth. Collecting ducts in all regions of the kidney were stained, including those in the cortical subcapsular region (Fig. 6*A*). Axial variability in the staining of individual cortical tubules was observed, probably representing the transition between unstained distal tubules and stained connecting segments or cortical collecting tubules (Fig. 6*A*). Collecting ducts in the papilla were brightly stained, and the fluorescence was concentrated at the apical pole of the positive cells (Fig. 6*B*). In some cells, a punctate staining was also present throughout the cytoplasm, and towards the base of the cell. The papillary surface epithelium was unstained with anti-AQP2 antibodies. Negative cells were interspersed with positive cells in the papilla and outer medulla even in 1-day postnatal rats, whereas negative cells were rarely seen in cortical collecting ducts at this early age.

Proximal tubules, thick ascending limbs of Henle and distal convoluted tubules were negative for AQP2 (Fig. 6*C*) but were stained with antiproton pump antibodies (Fig. 6*D*). However, some tubule profiles in the cortex contained cells that showed an apical staining for both AQP2 and the proton pump (Fig. 6*C, D*). Examination of these cells with a BioRad 600 confocal micro-

Table. Quantification of AQP2 (gold particles per μm length) in principal cell apical plasma membranes of control and vasopressin-treated Brattleboro rats by immunogold electron microscopy

Brattleboro control	Brattleboro + VP	Thin limbs of Henle
0.36 ± 0.079 (n = 17)	2.45 ± 0.24 (n = 20)*	0.12 ± 0.06 (n = 7)

n = number of apical plasma membranes counted.
* Significantly different from Brattleboro control ($P < 0.001$).

scope confirmed that the proton pump and AQP2 fluorescence derived from the same cell, and was not an artifact produced by two superimposed cells within the plane of the 5 μm section. Because these tubules also contained a few cells that were much more intensely stained with antiproton ATPase antibodies (but that were not stained with anti-AQP2 antibodies), identifying them as intercalated cells, it is likely that these profiles represent developing connecting segments of the rat nephron.

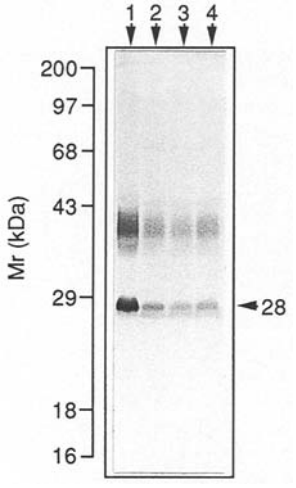

Fig. 4. Western blotting of AQP2 in vasopressin-treated and dehydrated rats. Total membrane proteins from normal rat papilla (1) and papillas of control Brattleboro rats (2), vasopressin-treated Brattleboro rats (3), and dehydrated Brattleboro rats (4) were blotted with the affinity purified anti-AQP2 antibodies, and stained. The protein bands that correspond to nonglycosylated (28 kDa) and glycosylated forms (40 kDa) of the AQP2 were much stronger in papillary membranes from the normal rat papilla than in papillary membranes form Brattleboro rats. Compared to controls (2), 30-min vasopressin treatment (3) and dehydration (4) did not elicit a significant change in density of the labeled bands. All membrane preparations had been denatured in a sample buffer at 65°C.

Discussion

The CHIP28 water channel is present in proximal tubules and thin descending limbs of Henle, segments that are constitutively permeable to water (Sabolić et al., 1992a; Nielsen et al., 1993c), and AQP2 is concentrated at the apical pole of collecting duct principal cells (Fushimi et al., 1993; Nielsen et al., 1993a). AQP2 is believed to be the vasopressin-induced water channel. In addition, a CHIP28-like protein(s) has also been demonstrated in the basolateral plasma membrane of kidney collecting duct principal cells (Valenti et al., 1994; Verbavatz et al., 1994), and a basolateral water channel that is possibly identical to this immunologically-detected basolateral intrinsic membrane protein (BLIP) has been sequenced and named aquaporin 3 (Ishibashi et al., 1994). Our present data, showing that vasopressin induces a marked apical polarization of AQP2 staining in principal cells of Brattleboro rats, supports the contention that AQP2 is the vasopressin-regulated channel. The vasopressin-induced change in AQP2 distribution occurs without any detectable increase in the amount of protein by Western blotting, indicating that it reflects a redistribution of preexisting protein. The short (30 min) time course of this process also argues in favor of a shuttling of stored AQP2 towards the apical pole of the cell in response to vasopressin. Recent work has shown that a patient with nephrogenic diabetes insipidus was a compound heterozygote for two mutations in the AQP2 gene, showing that AQP2 is essential for vasopressin-dependent urine concentration (Deen et al., 1994).

While no marked effect on protein content could be detected in Brattleboro rats after overnight dehydration, the amount of protein detected in papillary membranes of

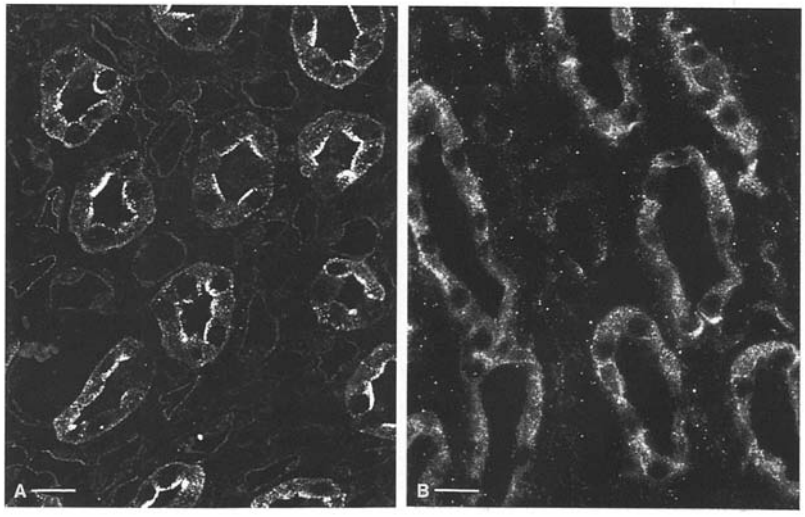

Fig. 5. Comparison of AQP2 immunofluorescence staining in cryostat sections of normal Sprague Dawley rat (*A*) and colchicine-treated Sprague-Dawley rat (*B*). Principal cells from the control animal show a bright staining at their apical pole. A few positive vesicles are present throughout the cytoplasm, some are close to the basolateral plasma membrane. A low level of nonspecific background staining is present in thin limbs of Henle in this preparation, which was stained using whole serum at a 1:400 dilution. This background was not present using affinity-purified antibodies—*see* Fig. 2. After colchicine treatment (*B*), the vesicles containing AQP2 are all diffusely scattered throughout the cytoplasm, and in some cells, they appear to be concentrated in the basal region of the cell. The apical membrane appears virtually unstained in the majority of cells. Bar = 20 μm.

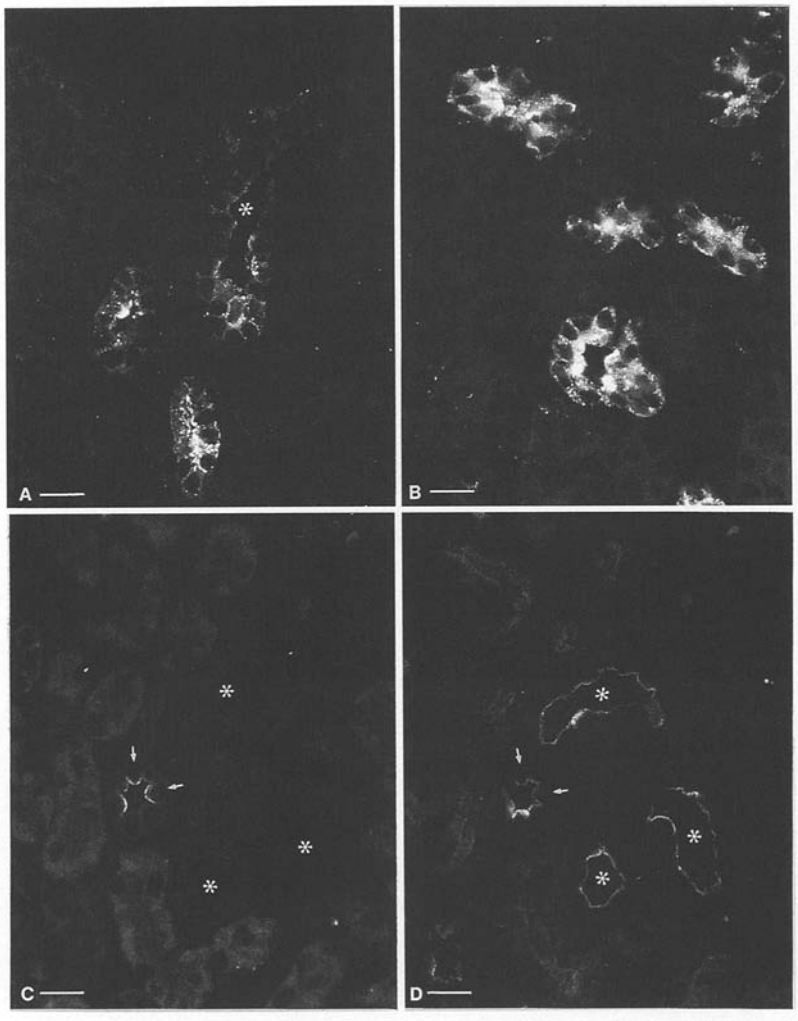

Fig. 6. Localization of AQP2 in cryostat sections of neonatal rat kidney. In 4-day postnatal kidney cortex (*A*), AQP2 is abundant in collecting ducts (CD). In one subcapsular tubule (asterisk), an axial variability in the level of staining is seen. This probably reflects a transition between a positive connecting segment and an unstained distal tubule. In the papilla of a 1-day postnatal rat (*B*) collecting duct principal cells show a heavy staining that is concentrated at the apical pole. In some cells, labeled vesicles are scattered throughout the cytoplasm. (*C*) and *D*) show a cryostat section of 11-day postnatal rat kidney cortex double stained to localize AQP2 (*C*) and the 31 kD subunit of the vacuolar proton pumping ATPase (*D*). AQP2 is present in some cells of a single tubule (*C*–arrows). Some of the cells that contain AQP2 in this tubule also contain the proton pump subunit (*D*–arrows). The cell that is more heavily stained with the proton pump antibody is probably an intercalated cell—it does not contain AQP2. Proximal tubules and distal convoluted tubules (asterisk) are unstained with anti-AQP2 antibodies, but do stain with the anti-proton pump antibody. Bar = 20 μm.

these animals was considerably lower than in normal Sprague-Dawley rats. This could also be seen by immunocytochemistry, where the labeling intensity of papillary principal cells from normal rats was much greater than that of Brattleboro rats. It has been reported that AQP2 message in normal rats is upregulated by dehydration (Nielsen et al., 1993a); the Western blotting technique used here may not be sufficiently sensitive to detect changes in protein level after only 16 h dehydration of Brattleboro rats. Alternatively, dehydration per se may not be sufficient to induce an upregulation of AQP2 in Brattleboro rats that lack vasopressin. Longer-term vasopressin treatment of these animals may be required.

The effect of vasopressin on urine concentration in Brattleboro rats can be partially inhibited by prior administration of the microtubule depolymerizing drugs colchicine or vinblastine (Dousa & Barnes, 1974). In addition, nocodazole, which also depolymerizes microtubules, partially inhibits the water permeability response to vasopressin in isolated perfused collecting ducts (Phillips & Taylor, 1989). Studies on toad bladder have shown that characteristic intramembranous particle aggregates that appear on the apical plasma membrane of granular cells in parallel with an increased water permeability, are reduced in number after colchicine treatment (Dratwa & Tisher, 1979; Kachadorian, Ellis & Muller, 1979; Valenti, Hugon & Bourguet, 1988). Since these IMP aggregates are inserted, as preformed structures, into the membrane by exocytotic fusion events, it follows that microtubules may be required for such fusion events to take place. Similar results have been obtained in many other systems in which stimulated secretory vesicle fusion occurs (Patzelt, Brown & Jeanrenaud, 1977; Malaisse-Lagae et al., 1979; Busson-Mabillot et al., 1982; Stetson & Steinmetz, 1983), and a general hypothesis is that microtubules are required to direct vesicles to the membrane domain at which fusion will occur (for example, the apical plasma membrane). In the absence of intact microtubules, vesicles become randomly distributed throughout the cytoplasm, and their local concentration beneath the apical plasma membrane is much less. Therefore, upon appropriate stimulation (e.g., with

vasopressin), fewer vesicles containing water channels are poised to insert into this membrane domain, and the biological response is significantly (but not completely) inhibited. This concept is fully supported by our data showing that vesicles containing AQP2 are randomly scattered in principal cells after colchicine treatment, and are no longer concentrated beneath the apical plasma membrane. Upon vasopressin stimulation, the number of vesicles in a position to fuse rapidly with the apical membrane would be considerably lower under these conditions, and the biological response would be reduced.

The neonatal kidney begins to concentrate urine at birth, but only progressively achieves maximum concentrating ability in the next few weeks. Several factors may combine to limit postnatal urine concentration. These include anatomical and biochemical constraints on the development of medullary interstitial hypertonicity (Spitzer & Schwartz, 1992), and a reduced response of collecting duct principal cells to the antidiuretic hormone, vasopressin, probably due to a decreased number of plasma membrane vasopressin receptors (Rajerison, Butlen & Jard, 1976). As a result, intracellular cAMP levels are elevated much less in neonatal than in adult kidneys in response to vasopressin (Schlondorff et al., 1978). A third factor that could limit concentrating ability would be a low or absent expression of water channel proteins in postnatal kidneys. It has recently been shown that the renal expression of CHIP28 is increased perinatally, which coincides with the onset of urine concentration that occurs in rat kidneys after birth (Smith et al., 1993).

The present study shows that the presumptive vasopressin-sensitive water channel, AQP2, is readily detectable in neonatal rat kidney, even during the first day after birth. The localization pattern is similar to that seen in adult rat kidneys, with most of the immunoreactive sites concentrated at the apical pole of collecting duct principal cells. Thus, absence of this collecting duct water channel does not account for the lower antidiuretic response to vasopressin that has been described in postnatal rats. As for adult rats, AQP2 was restricted to principal cells in both cortex and medullary collecting ducts. As previously reported for adult kidney (Nielsen et al., 1993b), intercalated cells, identified by strong proton pump staining, did not contain water channels. Both A- and B-intercalated cells in the cortex were unstained with antibodies against AQP2 (*not shown*). These immunocytochemical data confirm our previous studies in which we showed that intercalated cells do not recycle water channels in response to vasopressin (Lencer et al., 1990), and that collecting duct water channels are, therefore, restricted to principal cell endosomes. However, a few tubule profiles in the developing kidney cortex contained cells that were stained both with AQP2 antibodies, and antiproton pump antibodies. These tubules also contained some cells that had a staining pattern characteristic of intercalated cells (i.e., proton pump highly positive, AQP2 negative). They were distinct from other cortical tubules including distal convoluted tubules and thick ascending limbs (in which all epithelial cells were proton pump positive and AQP2 negative), and cortical collecting ducts (which had a highly characteristic mosaic pattern of staining indicating the presence of principal cells and intercalated cells). We conclude, therefore, that these tubules are developing connecting segments and that the cells showing staining for both antigens are connecting tubule cells. This staining pattern has not been observed in adult rats, and may indicate the presence of a transient population of "hybrid" cells in the developing nephron. It has previously been shown in cell culture that cells displaying some features of mature intercalated cells can give rise to cells having some principal cell features by asymmetric cell divisions (Fejes-Toth & Naray Fejes-Toth, 1992).

In summary, we have shown that the distribution of the collecting duct water channel AQP2 is modified by vasopressin treatment of Brattleboro rats, indicating that it is the vasopressin-regulated channel and that its apical distribution in principal cells is dependent upon intact microtubules. Finally, AQP2 is present in neonatal rat kidneys 1 day after birth, and its distribution is similar to that in adult rats.

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