Fourfold reduction of water permeability in inner medullary collecting duct of aquaporin-4 knockout mice

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Chou, C. L., Tonghui Ma, Baoxue Yang, Mark A. Knepper, and A. S. Verkman. Fourfold reduction of water permeability in inner medullary collecting duct of aquaporin-4 knockout mice. Am. J. Physiol. 274 (Cell Physiol. 43): C549–C554, 1998.—Aquaporin (AQP)-3 and AQP4 water channels are expressed at the basolateral membrane of mammalian collecting duct epithelium. To determine the contribution of AQP4 to water permeability in the initial inner medullary collecting duct (IMCD), osmotic water permeability (Pf) was compared in isolated perfused IMCD segments from wild-type and AQP4 knockout mice. The AQP4 knockout mice were previously found to have normal gross appearance, survival, growth, and kidney morphology and a mild urinary concentrating defect (T. Ma, B. Yang, A. Gillespie, E. J. Carlson, C. J. Epstein, and A. S. Verkman. J. Clin. Invest. 100: 957–962, 1997). Transepithelial Pf was measured in microdissected IMCDs after 18–48 h of water deprivation and in the presence of 0.1 nm arginine vasopressin (to make basolateral Pf rate limiting). Pf values (37°C; means ± SE in cm/s × 10–3) were 56.0 ± 8.5 for wild-type mice (n = 5) and 13.1 ± 3.7 for knockout mice (n = 6) (P < 0.001). Northern blot analysis of kidney showed that transcript expression of AQP1, AQP2, AQP3, and AQP6 were not affected by AQP4 deletion. Immunoblot analysis indicated no differences in protein expression of AQP1, AQP2, or AQP3, and immunoperoxidase showed no differences in staining patterns. Coexpression of AQP3 and AQP4 in Xenopus laevis oocytes showed additive water permeabilities, suggesting that AQP4 deletion does not affect AQP3 function. These results indicate that AQP4 is responsible for the majority of basolateral membrane water movement in IMCD but that its deletion is associated with a very mild defect in urinary concentrating ability.

The formation of a concentrated urine requires high collecting duct water permeability for osmotically driven movement of water from the duct lumen to the interstitium. Transepithelial water transport across the collecting duct epithelium is generally believed to occur by a transeellular route with serial passage across the apical and basolateral plasma membranes. Functional studies in isolated perfused inner medullary collecting ducts (IMCDs) have shown that transepithelial water permeability increases 8- to 10-fold in response to addition of vasopressin to the serosal surface (22). A substantial body of evidence indicates that water permeability of the basolateral plasma membrane is constitutively high, whereas water permeability of the apical plasma membrane is rate limiting for transepithelial transport and is regulated by vasopressin (reviewed in Refs. 14, 29). The regulation of water permeability involves the exocytic fusion and endocytic retrieval of water channel-containing vesicles with the apical plasma membrane (30).

Three aquaporin-type water channels have been identified in the water-transporting principal cells of collecting duct epithelium. Aquaporin-2 (AQP2) is the apical membrane water channel that undergoes regulated trafficking between an intracellular compartment and the cell plasma membrane (10, 19, 21, 23, 32). AQP3 and AQP4 colocalize at the basolateral membrane of collecting duct principal cells (8, 9), with somewhat more AQP3 in proximal segments and AQP4 in distal segments of the collecting duct (26). Functional studies in Xenopus laevis oocytes indicate that AQP4 is a water-selective channel (11), whereas AQP3 also transports small molecules including glycerol (6, 12, 17). Expression of epitope-tagged constructs in oocytes indicated that the single-channel water permeability of AQP4 is approximately sixfold higher than that of AQP3 (36), a result supported by stopped flow measurement of water permeability in proteoliposomes reconstituted with purified AQP4 (35). The reason(s) for coexpression of both AQP3 and AQP4 at the basolateral membrane of collecting duct is not known, and the contributions of these (and possibly other as yet unidentified) proteins to collecting duct water permeability have not been determined.

The purpose of this study was to determine the contribution of AQP4 to water permeability in the IMCD, the site of its greatest abundance along the collecting duct system. The strategy was to compare transepithelial water permeabilities in isolated perfused IMCDs of wild-type mice and homozygous AQP4 knockout mice that express no AQP4 protein (18). Measurements were carried out after water deprivation to upregulate AQP2 expression and at high vasopressin concentration to maximize AQP2 trafficking to the apical plasma membrane. Under these conditions, the basolateral membrane was predicted to be the rate-limiting barrier for transepithelial water transport. We found that, although the AQP4-deficient mice have grossly normal phenotype and only a mild urinary...
concentrating defect, IMCD water permeability in the knockout mice was remarkably reduced compared with that in wild-type mice. The knockout and wild-type mice had similar expression levels and localization of other kidney aquaporins, and water transport measurements in X. laevis oocytes showed that AQP4 expression did not affect AQP3 function. The results indicate that AQP4 provides the major route for water permeation through the basolateral plasma membrane of the rat IMCD. The relatively mild effect of reduced IMCD water permeability on urinary concentrating ability has important implications for the concentrating mechanism.

**EXPERIMENTAL PROCEDURES**

Transgenic mice. Transgenic knockout mice deficient in AQP4 protein were generated by homologous recombination as described previously (18). The knockout mice did not express detectable full-length AQP4 transcript or AQP4 protein in any organ. Measurements were done in tissues from litter-matched mice (6–8 wk of age) produced by intercrossing of CD1 heterozygotes. Genotype analysis of tail DNA was done by polymerase chain reaction at age 5 days. The investigators were blind to genotype information for tubule segments in other kidney aquaporins, and water transport measurements in X. laevis oocytes showed that AQP4 expression did not affect AQP3 function. The results indicate that AQP4 provides the major route for water permeation through the basolateral plasma membrane of the rat IMCD. The relatively mild effect of reduced IMCD water permeability on urinary concentrating ability has important implications for the concentrating mechanism.

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Isolated tubule microperfusion. IMCD segments were microdissected from the initial one-third of the distance along the inner medullary axis. The isolated tubules were mounted on glass pipettes and perfused in vitro at 37°C by the methods of Burg (2). Transepithelial water flux in response to a 200 mosmol/kgH2O bath-to-lumen osmolality gradient was measured to calculate the osmotic water permeability coefficient Ptf (30). The bath contained 0.1 mM arginine vasopressin for 30 min before and during the measurements. Fluorescein-5-(and-6)-sulfonic acid (1 mM) was used in the luminal perfusate as a volume marker. The fluorescence signal of perfusate and collected samples was measured by continuous flow fluorometry as described previously (31).

Electrophoresis and immunoblotting of membranes. Inner medullas from mouse kidneys were isolated, homogenized, and solubilized in Laemmli sample buffer after measurement of total protein concentration (bicinchoninic acid method; Pierce, Rockford, IL). Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 12% tris(hydroxymethyl)aminomethane-glycine gels (Novex, San Diego, CA) and transferred electrophoretically onto nitrocellulose membranes. Blots were blocked for 30 min with 5% nonfat dry milk in wash buffer (42 mM Na2HPO4, 8 mM NaH2PO4, 150 mM NaCl, and 0.05% Tween 20, pH 7.5), rinsed in wash buffer, and probed with affinity-purified anti-aquaporin antibodies (AQP1, AQP2, AQP3, AQP4) overnight at 4°C. The immune complexes were detected with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (1:5,000; Pierce). Sites of antibody-antigen reaction were visualized by enhanced chemiluminescence (Kirkegaard & Perry Lab, Gaithersburg, MD). Optical densities were quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis. Total RNAs from kidney cortex and inner medulla of wild-type and knockout mice were extracted using Trizol reagent (BRL). mRNAs were isolated from total RNAs using the Oligotex mRNA kit (Qiagen). RNAs (2 µg) were resolved on a formaldehyde-agarose gel and transferred to a Nylon+ membrane (Amersham). The blots were hybridized separately to 32P-labeled probes (Rediprime kit, Amersham) corresponding to the coding sequences of mouse AQP1, AQP2, AQP3, and AQP4 and of rat AQP6 in Rapid-hyb buffer (Amersham) under the following conditions: prehybridization at 67°C for 1 h, hybridization at 67°C with labeled probe for 1 h, and washing at 67°C twice in 2× saline sodium citrate (SSC)-1% SDS for 15 min each and twice in 0.2× SSC-1% SDS for 30 min each. Membranes were autoradiographed with Hyperfilm-MP (Amersham) overnight at room temperature.

Immunoperoxidase. Kidneys were removed, sliced, and fixed in 4% paraformaldehyde for 4 h. Samples were cryoprotected overnight with phosphate-buffered saline (PBS) containing 30% sucrose, embedded in OCT compound, and frozen in liquid N2. Cryostat sections (4–6 µm) were incubated for 10 min with PBS containing 1% bovine serum albumin (BSA) and then with antibodies for 1 h at 23°C in PBS containing 1% BSA as described previously (9). Slides were rinsed with 2.7% NaCl and then with PBS and incubated for 30 min with peroxidase-conjugated sheep anti-rabbit Fab fragment (1: 100; Amersham). Peroxidase activity was visualized by reaction with diaminobenzidine.

Oocyte expression experiments. Stage V and VI oocytes from X. laevis were isolated, defolliculated with collagenase, and microinjected with 50-nl samples of cRNAs (0–200 ng/µl) encoding rat AQP3 and/or AQP4 (36). After 24–27 h of incubation at 18°C, Ptf was measured from the time course of oocyte swelling at 10°C in response to a fivefold dilution of the extracellular Barth’s buffer with distilled water. Oocyte Ptf was calculated from the initial rate of swelling [d(V/Vo)/dt] by the relation Ptf = [d(V/Vo)/dt] [(S/Vo)(Osmout – Osmin)], where V is volume, Vo is initial volume, S is surface area, and Osmout and Osmin are internal and external osmolality, respectively, and where S/Vo = 50 cm−1, (Vw) = 18 cm3/mol, and Osmout – Osmin = 190 mosmol/kgH2O.

**RESULTS**

Figure 1A shows the Ptf values measured in isolated perfused IMCDs. We choose to carry out water permeability measurements in collecting ducts from the outer one-third of the inner medulla (“initial IMCD”) because previous studies in rats (9, 10, 26) indicated that this portion of the IMCD expresses the highest levels of AQP4 among collecting duct segments. Mice were water restricted for 18–48 h, and tubules were perfused with 100 µM vasopressin in the peritubular bath to maximize the water permeability of the apical plasma membrane, so that a defect in basolateral water permeability would produce a substantial decrease in transepithelial water permeability. Ptf (means ± SE in cm/s × 10−3; 37°C) was much lower (P < 0.001) in IMCD from AQP4 knockout mice (13.1 ± 3.7, n = 6) than in wild-type mice (56.0 ± 8.5, n = 5). For comparison, Fig. 1B summarizes averaged urine osmolalities from wild-type and knockout mice before vs. after a 36-h water deprivation period and after intraperitoneal administration of desmopressin (DDAVP) (18). There was a mild defect in urinary concentrating ability in the knockout mice with decreased urine osmolality after water deprivation both without and with DDAVP.

To determine whether deletion of AQP4 affects the expression of other kidney aquaporins, we did Northern and immunoblot analysis on kidneys from the wild-type and AQP4 knockout mice. Figure 2 shows representative Northern blots. By quantitative densitometry, averaged relative transcript expression levels
(normalized to 1.0 for wild-type mice; means ± SE; n = 3) were 1.03 ± 0.10 for AQP1, 0.94 ± 0.06 for AQP2, 0.98 ± 0.12 for AQP3, and 0.0 for AQP4. In addition, there were no significant differences in blots of mRNA prepared from separated renal cortex and medulla or in blots of total mRNA from kidneys of water-deprived mice.

Immunoblots for AQP4 were done on homogenates from the inner medullas of the right kidneys from the same mice used for the perfused tubule experiments. No detectable AQP4 protein was found in mice with the AQP4 knockout genotype (Fig. 3A). In contrast, AQP1, AQP2, and AQP3 were each expressed in inner medulla of wild-type and AQP4 knockout mice at approximately equal levels (Fig. 3B). AQP3 was seen mainly as a dimer under the conditions used here. A Coomassie-stained polyacrylamide gel loaded with the same samples (Fig. 3C) showed no obvious differences in the expression of major proteins present in the inner medulla. These results suggest that the low water permeability of initial IMCD is directly attributable to the absence of AQP4 rather than to a secondary effect of AQP4 gene deletion involving altered expression of other kidney aquaporins.

Immunoperoxidase localization of kidney AQP1, AQP2, AQP3, and AQP4 was done to investigate whether deletion of AQP4 changes the cellular distribution of the remaining aquaporins. Figure 4 shows localization of AQP1 to proximal tubule and thin descending limb of Henle and of AQP2 and AQP3 to the collecting duct. These results are consistent with previous data for expression of these aquaporins in mouse, rat, and human kidney (1, 9, 24, 26). There were no discernible differences in the expression patterns of these aquaporins for the wild-type vs. AQP4 knockout.
mice. AQP4 was seen at the basolateral membrane of collecting ducts of wild-type mice but was not detected in the knockout mice.

As found previously in rat and as above in mice, AQP4 colocalizes with AQP3 at the basolateral membrane of collecting duct epithelium. To investigate the possibility that the presence of AQP4 affects the function of AQP3, expression studies were carried out in \textit{X. laevis} oocytes. cRNAs encoding AQP4 and AQP3, alone and in combination, were injected into oocytes, and $P_f$ was measured. Figure 5 is a summary of the averaged oocyte $P_f$ values. After subtraction of background (channel-independent) water permeability measured in control water-injected oocytes, the incremental $P_f$ conferred by 2.5 ng of AQP4 cRNA and 5 ng of AQP3 cRNA were (in cm/s $\times 10^{-3}$) 6.5 $\pm$ 0.4 and 11.8 $\pm$ 0.8, respectively. The incremental $P_f$ value conferred by coexpression of AQP4 and AQP3 was 16 $\pm$ 1, not significantly different from the sum of the individual contributions (18 $\pm$ 1) from the two proteins. Similar measurements were done with relatively high AQP4 vs. AQP3 expression. Incremental $P_f$ values were 0.93 $\pm$ 0.04 (0.5 ng AQP3), 6.6 $\pm$ 0.3 (0.5 ng AQP4), and 7.7 $\pm$ 0.4 (AQP3 + AQP4). These results suggest that AQP4 and AQP3 are functionally independent, so that AQP4 knockout would not affect the function of the remaining AQP3.

\section*{DISCUSSION}

The kidney collecting duct is the target epithelium for the antidiuretic action of vasopressin. Three aquaporin-type water channels have been identified on membranes in collecting duct principal cells, the cell type that contains adenylyl cyclase-coupled vasopressin receptors and the cellular machinery to carry out CAMP-regulated trafficking of water channel-containing vesicles. There is a convincing body of evidence that AQP2 is a water channel that undergoes regulated vesicular trafficking between the principal cell apical plasma membrane and an intracellular compartment. The association of AQP2 mutations with non-X-linked congenital nephrogenic diabetes insipidus (4) suggests that AQP2 is the main water channel of principal cell apical membranes. In several immunocytochemistry studies, AQP2 staining was also found at the principal cell basolateral plasma membrane, raising the possibility that AQP2 may contribute to the constitutively high water permeability of the basolateral membrane. Furthermore, the water channels AQP3 and AQP4 are expressed in kidney exclusively at the basolateral membrane of collecting duct principal cells (9, 10). Thus all three of the collecting duct aquaporins could, in theory, contribute to the water permeability of the basolateral plasma membrane. The measurements reported here were carried out to assess the contribution of AQP4.

The principal finding was that IMCD water permeability was reduced by fourfold in tubule segments from AQP4 knockout mice compared with wild-type mice. The reduction in transepithelial water permeability was not related to differences in expression of AQP1, AQP2, and AQP3 in wild-type vs. knockout mice or to differences in their cellular localization in kidney. The lack of effect of AQP4 deletion on renal expression of the other aquaporins is consistent with the localization of the four respective genes on different chromosomes and with their unrelated upstream promoter sequences.
The permeability of the medullary collecting duct has been expected to result in only a limited decrease in water absorption in the cortical portion of the collecting duct far from the collecting duct, when increased to maximal levels by vasopressin, is considerably in excess of that necessary for osmotic equilibration across the collecting duct epithelium. Therefore, an 80% reduction in the P_{f} of the IMCD results in a disproportionately small decrease in percent osmotic equilibration.

In summary, the results here indicate that AQP4 is the major basolateral membrane water transporter in IMCD. Further studies in other tubule segments and in mice with multiple aquaporin deletions will be needed to quantify the significance of the known aquaporins and possibly of other unidentified aquaporins in the urine concentrating mechanism.

NOTE ADDED IN PROOF

Recent analysis of transgenic mice lacking AQP1 showed a major defect in urinary concentrating ability with severe dehydration and serum hyperosmolality after water deprivation (5, 6). The data indicate that at least 75% of the basolateral water permeability is due to AQP4. For example, if apical and basolateral membrane P_{f} values are equal in water-deprived wild-type mice after vasopressin stimulation, then a 75% reduction in transepithelial P_{f} would indicate an 86% reduction in basolateral membrane P_{f}. Assuming that the measured water permeability of the IMCD apical plasma membrane under the conditions of this study does not markedly exceed that of the basolateral plasma membrane, as was found in rat (7), AQP4 probably contributes much more than 75% of total basolateral membrane water permeability. Furthermore, permeation through the lipid phase of the basolateral plasma membrane is likely to account for a significant fraction of the non-AQP4-associated basolateral permeability. Thus we conclude that AQP3, AQP2, and any unidentified water channels are unlikely to make large contributions to the basolateral plasma membrane water permeability in the initial portion of the rat IMCD. In addition, the data indicate that paracellular water transport does not make a substantial contribution to transepithelial IMCD water permeability.

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