Aquaporin-4 is expressed in basolateral membranes of proximal tubule S3 segments in mouse kidney

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1Program in Membrane Biology/ Renal Unit and Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114; and 2Cardiovascular Research Institute, University of California, San Francisco, California 94143

Hoek, Alfred N. Van, Tonghui Ma, Baoxue Yang, A. S. Verkman, and Dennis Brown. Aquaporin-4 is expressed in basolateral membranes of proximal tubule S3 segments in mouse kidney. Am. J. Physiol. Renal Physiol. 278: F310–F316, 2000.—Because of the availability of knockout mouse models to examine renal transport mechanisms, it has become increasingly important to describe the cellular distribution of major renal transporters in mice. We have used immunocytochemistry and freeze-fracture electron microscopy to compare the renal distribution of aquaporin-4 (AQP4) with that previously described in rat. In rat kidney, AQP4 is present exclusively in basolateral membranes of collecting duct principal cells. In mice, however, AQP4 was also detected by immunocytochemistry in basolateral membranes of proximal tubule S3 segments, and not detected in S1 and S2 segments of proximal tubule. Freeze-fracture electron microscopy revealed orthogonal arrays of intramembrane particles (OAPs) on the basolateral membranes of the S3 segment. In AQP4-knockout mice, immunostaining was absent and OAPs were found neither in collecting ducts nor in the S3 segment of the proximal tubule. The urinary concentrating capacity after deletion of both AQP1 and AQP4 was further reduced compared with that of AQP1 or AQP4 null mice, suggesting an additive effect of AQP1 and AQP4 in the concentrating mechanism. The functional significance of the apparent species-dependent expression of AQP4 in proximal tubules is unknown, but may relate to physiological differences between rats and mice.

freeze-fracture electron microscopy; immunocytochemistry; orthogonal arrays; square array; aquaporin-4-knockout

THE AQUAPORINS ARE A FAMILY OF transmembrane water channel proteins the structure, localization, and function of which are the subjects of a considerable amount of research in a variety of organ systems (3, 14, 22, 23). These studies have been carried out mainly (but not exclusively) in the rat, and have involved the use of a variety of techniques to describe the cellular distribution of the aquaporins, including in situ hybridization, immunocytochemistry, and freeze-fracture electron microscopy. With the increasing use of knockout mice as models to study epithelial transport biology, it becomes important to provide a catalog of the distribution of the relevant transport proteins in mice. Aquaporin knockouts for AQP1, AQP4, and AQP5 have already been described (5, 6, 11). Several studies have shown that the cellular location of AQP1 and AQP2 in the kidneys of mice closely resembles that seen in other species. AQP1 is present in proximal tubules, thin descending limbs of Henle, and vasa recta (6), whereas AQP2 is located in collecting duct principal cells (2). Recent studies in the AQP1-knockout mouse reveal the dual importance of AQP1 in proximal tubule reabsorption and in the creation of a hypertonic medullary interstitium by countercurrent multiplication (5, 13, 17). The AQP4 null mice have a mild defect in urinary concentrating ability despite a fourfold reduction in water permeability in the inner medullary collecting duct (6, 12).

In this study, we have used immunocytochemistry and freeze-fracture electron microscopy to compare the renal distribution of AQP4 with that previously described in rats. AQP4 forms characteristic orthogonal arrays of intramembrane particles (OAPs) when expressed in transfected cells (20, 24), and similar OAPs are present in the plasma membranes of several cell types that express AQP4, including collecting duct principal cells (15). OAPs are absent from principal cell basolateral membranes in AQP4-knockout mice (21). We show here that AQP4 protein is expressed not only in collecting duct principal cells in mouse kidney but also on the basolateral plasma membranes of epithelial cells in the S3 segment of the proximal tubule.

MATERIALS AND METHODS

Transgenic mice. Transgenic knockout mice deficient in AQP1 and AQP4 protein were generated by targeted gene disruption as described previously (11, 12). Offspring were genotyped by PCR. AQP1 and AQP4-knockout mice were intercrossed to produce AQP1/AQP4 double-knockout mice. Urine osmolality measurements were done in tissues from litter-matched mice (6–8 wk of age) deprived of food and water for 36 h. Urine collections were done while the mice were being observed to transfer urine samples to small closed containers before evaporation occurred. The protocols for these studies were approved by the University of California, San Francisco, Animal Research Committee.

Immunocytochemistry. Kidneys from mice were removed, longitudinally sliced in 2–4 pieces, and fixed in 4% paraformaldehyde in PBS (10 mM sodium phosphate buffer containing...
0.9% NaCl, pH 7.4) for 4 h. Slices were washed and stored in PBS containing 0.01% NaN₃. For cryostat sectioning, slices were infiltrated with 30% sucrose in PBS, placed on a support in OTC compound (Sakura FineTek, Torrance, CA), and frozen with liquid nitrogen. Cryostat sections (5 µm) were mounted on Fisher Superfrost Plus (Fisher) microscope slides, air-dried, and stored at −20°C. For immunocytochemistry, sections were rehydrated in PBS for 5 min and blocked with 1% BSA in PBS for 20 min. In some cases, sections were subjected to a 4-min antigen-retrieval treatment with 1% SDS-PBS before blocking (4). Incubations with polyclonal AQP4 (1:100), AQP2 (1:100), and monoclonal gp330-megalin (1:200) primary antibodies, CY3-conjugated goat-anti-rabbit secondary antibodies (1:800), and/or FITC-conjugated donkey-anti-mouse secondary antibodies (1:60) in PBS were carried out at the indicated dilutions for 1 h at room temperature. Three wash/rinse steps of 5 min with PBS were included after antibody incubation. Immunostained sections were mounted by using a 1:1 mixture of Vectashield and 0.3M Tris·HCl (pH 8.9) before examination with a Nikon FXA epifluorescence microscope.

Images were collected either by conventional photography using Kodak TMx 400 film push-processed to 1,600 or by digital imaging. For this, images were collected by using an Optronics 3-bit color charge-coupled device camera, and in some cases up to 100 separate overlapping images taken with a ×20 objective were digitally merged into a mosaic by using IP Lab Spectrum software running on a Power Macintosh. The larger mosaics were reduced in size and printed from Adobe PhotoShop on a Tektronix Phaser 400 dye-sublimation printer.

Immunoblotting (SDS-PAGE and Western blotting). Mouse kidneys were perfused with PBS to remove blood. After removal from the animal, different regions of kidney (cortex, outer stripe, inner stripe, and papilla) were removed and frozen. Frozen samples were placed in 0.2 ml of lysis buffer [(in mM) 10 Tris (pH 8.0), 150 NaCl, 5 EDTA, 5 EGTA, and 0.2 phenylmethylsulfonyl fluoride, as well as 1% Triton X-100, 1% octyl-β-D-glucoside (Anatrace), and complete protease inhibitor (Boehringer Mannheim)]. The samples were homogenized by 10 strokes in a Dounce tissue grinder. Homogenates were spun at 12,000 g for 20 min. Protein concentration of the supernatants was determined by using the Bradford assay (Bio-Rad). SDS-gel electrophoresis and blotting were done with the Novex-XCell II Mini-Cell apparatus (Novex, San Diego, CA). Protein was added 2:1 to NuPAGE LDS sample buffer, separated through a 12% Laemmli SDS polyacrylamide minigel, using NuPAGE-Tris-Tricine (pH 8.23; Novex) as electrode/running buffer. With NuPAGE transfer buffer (pH 7.2) proteins were blotted onto poly(vinylidenefluoride (PVDF)-Immobilon membrane, 200-µm mesh, for 16 h at 10–25°C. The membranes were stained with Coomassie brilliant blue stain (0.2% Coomassie blue, 40% MeOH, 60% ddH₂O) then destained (60% MeOH, 40% ddH₂O) and photographed before stain was removed with 100% MeOH. Membranes were rewet in water and subjected to the “Western Breeze” protocol (Novex). Affinity-purified AQP4 antibody was diluted 1:1,000 in the manufacturer’s primary antibody solution, and the PVDF membrane was incubated 1.5 h at room temperature. Blots were detected by the chromogenic alkaline phosphatase assay.

Antibodies and controls. Antibodies against AQP4 were raised in rabbits against a COOH-terminal peptide (15 amino acids) derived from the rat AQP4 protein. The peptide was coupled to keyhole limpet hemocyanin. Whole serum was affinity purified against the immunizing peptide by using a Pierce affinity purification column kit (Pierce, Rockford, IL)
according to the manufacturer's instructions. The production and characterization of the rabbit polyclonal AQP1 and monoclonal anti-gp330-megalin antibodies have been described in previous publications (1, 16). Control procedures for immunocytochemistry included incubation of the tissues with preimmune serum, preabsorption of the antibodies with the immunizing peptide, and incubation of the AQP4 antibodies on tissues from knockout mice that do not express AQP4.

Freeze-fracture electron microscopy. Longitudinally sliced kidney pieces were fixed in 2% glutaraldehyde for 2 h and washed and stored in PBS containing 0.01% NaN₃. Fragments (0.5 × 0.5 cm × 1 mm in size) of inner/outer medulla were infiltrated with 30% glycerol in 0.1 M cacodylate buffer (pH 7.4) overnight, placed on flat copper specimen holders, and frozen in N₂-cooled Freon 22. Frozen samples were transferred into a freeze-fracture apparatus (Cressington Scientific Instruments, Watford, UK). At −145°C and a high vacuum (10⁻⁷ Torr), samples were shaved with the microtome blade until the thickness of the remaining tissue, adhering to the copper support, was −0.1 mm. A final knife pass was then carried out at a specimen temperature of −120°C to produce the final fractured surface. Samples were shadowed at an angle of 45° with platinum (−2 nm thickness). For mechanical support of the platinum cast, a 3-nm carbon coat perpendicular to the fracture plane was then applied. After floating off in water, replicas were digested with sodium hypochlorite solution for 2 h and then rinsed with distilled water. The replicas were picked up on EM grids and examined in a Philips CM10 electron microscope (Mahwah, NJ). The above procedure produced a single large replica from each sample that contained areas of both inner and outer stripes of the medulla, allowing an unequivocal identification of the S3 stripe.
RESULTS

Immunofluorescence. Indirect immunofluorescence staining of mouse kidney sections indicated localization of AQP4 in collecting ducts throughout the kidney as in the rat kidney. However, an additional pattern of staining that has not been described previously was detectable in the outer stripe of the outer medulla (Fig. 1). The staining was especially intense in tubules at the border between the outer and inner stripes, appearing to be S3 segments of proximal tubule. The tubules containing AQP4 were identified as proximal tubules by double staining with the proximal tubule-specific marker, gp330-megalin (Fig. 2). AQP4 (Fig. 2, A, C, and D) was localized to the basolateral plasma membranes of proximal tubule cells, whereas gp330-megalin was restricted to the apical brush-border membrane (1). In the collecting duct, principal cells showed a strong basolateral membrane staining for AQP4 as previously described in mouse (21) and rat kidney (9, 10, 19). Intercalated cells were negative. S1 and S2 proximal tubule segments were not stained for AQP4 (Fig. 1). To verify that the antibody was indeed recognizing AQP4, and not a closely related (but perhaps undiscovered) basolateral aquaporin, sections of AQP4 null (knockout) mice were examined. Although strong basolateral staining was seen in wild-type mice (Fig. 2D), no basolateral staining was seen in knockout mice, either in collecting ducts or in S3 proximal tubule segments (Fig. 2, B and E), which were clearly identified by a strong apical gp330-megalin staining (Fig. 2B). Preincubating the antibody with the specific AQP4 peptide (not shown) abolished AQP4 staining in wild-type mice.

Western blotting. Western blotting showed a strong AQP4 band at the appropriate molecular weight in samples from the papilla (Fig. 3, lane 4). The intensity of staining diminished in inner and outer stripe medullary samples (Fig. 3, lanes 2 and 3). The AQP4 band was very weak or absent in the cortical sample (Fig. 3, lane 1), as predicted from the relative lack of AQP4-containing cells in this kidney region. Specific staining was abolished by using peptide-preabsorbed AQP4 antiserum (not shown). There was no staining of kidney samples from AQP4 null mice (Fig. 3B, lanes 5–8).

Freeze-fracture electron microscopy. It was found previously that AQP4 forms characteristic OAPs in cell membranes when examined by freeze-fracture electron microscopy (15, 20, 24). Examination of proximal tubule plasma membranes (Fig. 4A) revealed the presence of OAPs in the basolateral membrane of S3 segments (Fig. 4, B–D). These structures were often located close to gap junctions that are an identifying feature of this membrane domain (Fig. 4B). Of epithelial cell types in the kidney, only proximal tubule cells express gap junctions on their plasma membranes (15). The number and size of the OAPs in the S3 segment of kidney were, however, considerably lower than in basolateral membranes of principal cells (Fig. 4E). OAPs were never found in principal cells (Fig. 4F) or in proximal tubules (not shown) of AQP4-knockout mice. As pointed out previously (21), a different type of IMP aggregate was found on basolateral membranes of principal cells (Fig. 4F). These clusters may represent AQP3 molecules, because a previous study from our laboratory showed similar-appearing IMP clusters on the plasma membranes of CHO cells that express AQP3 (20).

Urinary concentrating ability. Measurements of urinary concentrating ability were made by comparing wildtype mice with AQP1 null mice, AQP4 null mice, and AQP1/AQP4 double-knockout mice. The summarized data in Table 1 show a substantial decrease in the concentration capacity in AQP1 null mice, as reported (13), as well as a small but significant decrease in AQP4 null mice, as reported (12). By unpaired t-test, the data in Table 1 give P < 0.001 at 0 and 36 h (AQP1-knockout vs. AQP1/AQP4 double-knockout mice) and P < 0.05 at 18 h. The effect of AQP4 deletion on top of AQP1 deletion is highly significant, indicating a further decrease in the urinary concentration capacity, suggesting a functionally additive response.

DISCUSSION

AQP4 expression in the mouse kidney was studied by immunocytochemistry, immunoblotting, and freeze-fracture electron microscopy. Unexpectedly, AQP4 was detected in the basolateral membranes of proximal
Fig. 4. Freeze-fracture electron microscopy of mouse renal tissue. A: proximal tubule S3 segment with apical brush-border and lateral membrane. Note tight junction (t) that marks transition between apical and basolateral membranes, and note gap junctions (g) in lateral membrane. Bar = 350 nm. B: at higher magnification of a similar area, orthogonal or square arrays in vicinity of a gap junction are evident (arrows). Bar = 150 nm. C: orthogonal arrays of particles (OAPs; arrows) on basal membrane of proximal tubule. Bar = 150 nm. D: high magnification shows details of OAPs (arrows). Bar = 80 nm. E: basolateral membrane of principal cells (wild type) showing many OAPs. Bar = 150 nm. F: basolateral membrane of principal cells showing absence of OAPs in AQP4-knockout mice. Note remaining particles that are often arranged in clusters. Bar = 150 nm.
Table 1. Urine osmolality at indicated times after water deprivation

<table>
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<th>0 h</th>
<th>18 h</th>
<th>36 h</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>2,870</td>
<td>3,562</td>
<td>3,695</td>
</tr>
<tr>
<td>AQP1 (-/-)</td>
<td>636</td>
<td>870</td>
<td>816</td>
</tr>
<tr>
<td>AQP4 (-/-)</td>
<td>2,203</td>
<td>3,153</td>
<td>2,865</td>
</tr>
<tr>
<td>1/4 Double KO</td>
<td>547</td>
<td>773</td>
<td>625</td>
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Values are means ± SE in mosmol/kg H2O; n = 6 mice/group. AQP1 and AQP4, aquaporin-1 and -4, respectively; KO, knockout.

Table S3 segments in addition to its previously reported expression in the basolateral membranes of collecting duct principal cells. The relative contribution of the aquaporins to the renal concentrating mechanism in normal mice may be different from that in rats, and data derived from AQP-knockout mice should be interpreted with this differential AQP4 distribution in mind.

These results provide yet another example of different aquaporins coexisting within the same membrane domain. In mice, AQP4 not only colocalizes with AQP3 in the basolateral membrane of collecting duct principal cells (Van Hoek and Brown, unpublished observations) but also with AQP1 in proximal tubules. Functional studies of aquaporin-expressing Xenopus laevis oocytes have demonstrated two classes of water channels on the basis of their permeability characteristics. AQP1, AQP2, and AQP4 are examples of permselective aquaporins that transport water only, and AQP3 and AQP9 are nonselective channels because they transport certain small polar solutes in addition to water. Coexpression of two or more aquaporins in a single membrane domain (e.g., AQP3 and AQP4 in basolateral membranes of principal cells) might be explicable if one aquaporin allowed passage of water only and the other allowed passage of other molecules. However, the reason(s) are not clear for coexpression of two permselective water channels, AQP1 and AQP4, in basolateral membranes of the S3 segment of proximal tubules.

Functional studies in AQP4-knockout mice showed a mild urinary concentrating defect (Ref. 11; ~25% reduction), as confirmed in this study, whereas collecting duct permeability is reduced fourfold by deletion of AQP4 (6). AQP1 knockout mice have a low basal urinary osmolality and are unable to concentrate their urine further in response to water deprivation (5). The small effect of AQP4 deletion on urinary concentrating ability was explained on the basis of the predominant expression of AQP4 in inner medullary collecting duct, where relatively little water absorption occurs; AQP3 is predominantly expressed in cortical and outer medullary collecting duct, where the majority of water is reabsorbed during antiuresis. Indeed, AQP3 null mice have a substantially more severe urinary concentrating defect than do AQP4 null mice (Ma, Yang, and Verkman, unpublished observations). The functional advantage of coexpression of a permselective water channel AQP4 and a promiscuous AQP3 channel in the membrane is not clear but may be elucidated by functional studies in AQP3 null and AQP3/AQP4 double-knockout mice.

The interpretation of urinary concentrating data from AQP4 null mice was based on the premise that AQP4 is exclusively localized in collecting ducts (12). However, it is also possible that the absence of AQP4 from the proximal tubule of the S3 segment contributes to the ~25% reduction in urinary concentrating capacity found in AQP4 null mice. This assumes that the basolateral membrane permeability of principal cells may not be a limiting factor for the overall urinary concentration capacity after AQP4 deletion, despite the large permeability reduction seen in perfused collecting ducts. These issues will be addressed in future studies.

The relationship of orthogonal arrays of AQP4 to its water permeability properties has been previously discussed (25). It was proposed that water flow through OAPs is amplified by the close proximity of many AQP4 molecules, leading to the extremely high water permeability of membranes containing AQP4 (25). However, in the proximal tubule of mouse kidney, the number of detectable AQP4 arrays was small compared with those seen in collecting duct principal cells. In contrast, the fluorescence signal of proximal tubule membranes by immunocytochemistry was very strong and, in some S3 proximal tubules, was comparable to the fluorescence signal of many principal cells. The absence of OAPs and AQP4 immunostaining in the knockout mouse rule out the presence of a cross-reacting protein. The apparent discrepancy in immunostaining vs. freeze-fracture data raises the interesting possibility that AQP4 has less tendency to form OAPs in the proximal tubule.

It is intriguing that many of the OAPs were closely associated with gap junctions that are frequently found on the lateral membrane of proximal tubule epithelial cells (15). This may indicate that metabolic and ionic intracellular coupling through gap junctions is related to water transport through AQP4 in the mouse. It could also reflect the presence of localized membrane properties that, as discussed above, may be necessary for protein clustering to occur or be maintained in the plane of the membrane. Although the functional consequences are unclear, this close proximity of gap junctions and AQP4-OAPs in the proximal tubule is similar to the tight association of AQP0 (MIP26)-OAPs and gap junctions in lens fiber membranes (7). The observation that mice harboring a mutation in AQP0 have congenital cataracts suggests that AQP0 is required for lens transparency (18), but its physiology and relationship (if any) to intracellular coupling via gap junctions are not understood.

Finally, the species-dependent expression of AQP4 on the basolateral membrane of proximal tubule cells of the S3 segment may reflect differences in their urinary concentrating capacity/mechanisms. Rats concentrate urine less than mice and have relatively small papillae. Also, AQP4 expression levels in proximal tubule S3 segments may indicate the osmolality of the surrounding interstitium. In medullary rays close to glomeruli, the lack of AQP4 expression in S3 proximal tubule cells might be related to the lower local osmolality than in the outer medulla. It will be instructive to study AQP4 expression in proximal tubules in a variety of mammals to expand on this idea.
REFERENCES


