Expression and nephron segment-specific distribution of major renal aquaporins (AQP1–4) in *Equus caballus*, the domestic horse

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Floyd RV, Mason SL, Proudman CJ, German AJ, Marples D, Mobasheri A. Expression and nephron segment-specific distribution of major renal aquaporins (AQP1–4) in *Equus caballus*, the domestic horse. Am J Physiol Regul Integr Comp Physiol 293: R492–R503, 2007. First published April 18, 2007; doi:10.1152/ajpregu.00689.2005.—Aquaporins (AQPs) are a family of water channel proteins, which confer high intrinsic water permeability to epithelial cells of water transporting tissues (1). AQPs are important for facilitating near-isosmolar transepithelial fluid absorption and secretion and for rapid vectorial water movement driven by osmotic gradients (55). To date, 13 members have been identified in humans (AQP0-AQP12) (8), and numerous homologs exist in the plant and animal kingdoms. More than 200 members of the AQP family have been identified in plants (23, 24), microorganisms (7), invertebrates, and vertebrates (1).

AQPs belong to the ubiquitous major intrinsic protein superfamly of proteins, which incorporates bacterial channels known to exhibit specificity for water and/or neutral solutes. Evolutionary studies suggest that major intrinsic protein family proteins are derived from two divergent bacterial paralogues: a glycerol facilitator and an AQP (44). Current members of the mammalian AQP family have retained these or closely related physiological functions and can be split into two groups: those which are permeable to only water, the AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP11), and those permeable to water and small uncharged molecules, the aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) (1).

At least six (and possibly more) AQPs are expressed in the equine kidney (55). These renal AQPs, which include AQP1, AQP2, AQP3, AQP4, AQP6, and AQP7, are differentially and strategically distributed along the nephron (41–43) to facilitate water reabsorption and urine concentration. The cellular locations of four major AQPs (AQP1, AQP2, AQP3, and AQP4) have been fairly well established in rodent and human kidney. AQP1 is strongly expressed in apical and basolateral membranes of renal proximal tubules and descending thin limbs of the loop of Henle (2). AQP1 is also present within the glomerulus (podocytes) and in the visceral epithelium of the Bowman’s capsule and the vasa recta (51). AQP1 has been shown to be required for the formation of concentrated urine in mice, since AQP1 knockout mice become severely dehydrated after water deprivation and suffer from polyuria and polydipsia, suggesting a key role for AQP1 in urine concentration (30). Recent studies have shown that humans lacking the AQP1 gene also manifest a urinary concentrating defect (26). However, AQP1 deficiency in mice is more severe than in humans with regard to urinary concentrating ability and may indicate species-specific differences in the mechanisms of proximal tubule water reabsorption (27). In the collecting duct principal cell, AQP2 is the arginine-vasopressin-sensitive water channel, found in apical membranes and in subapical intracellular vesicles, which are inserted in apical plasma membranes when stimulated by arginine-vasopressin (11–13, 16, 28, 41); genetic defects in AQP2 or vasopressin type 2 receptors both result in severe nephrogenic diabetes insipidus (10, 11, 13, 40, 53). AQP3 and AQP4 both reside in the basolateral membranes of collecting duct principal cells, providing an exit pathway for the water entering the cells apically via AQP2 channels (22, 28, 42). AQP4 is also found in the proximal tubules (S3) of mouse.
but not of rat, kidney. Significantly less is known about AQP6 and AQP7 in the kidney (54). Recent studies suggest that AQP6 is present in intracellular vesicles in intercalated cells, and AQP7 has been localized to the brush border of cells in the third segment of the proximal convoluted tubule (42, 43).

Knockout mice lacking AQP3 also exhibit nephrogenic diabetes insipidus, suggesting that loss of AQP3 can produce a phenotype similar to loss of AQP2, resulting in polydipsia and polyuria as a result of a impaired urine concentration. Interestingly, mice lacking AQP3 also downregulate AQP2 expression, complicating interpretation of their urinary data (29). AQP1/AQP3 double knockout mice were recently shown to have a greater deficit in urinary concentrating capacity than AQP1 or AQP3 single knockout mice (57). Similarly, AQP3/AQP4 double knockout mice have a greater urinary concentrating defect than mice lacking AQP3 alone, indicating a dual role for these proteins in combination with apical AQP2 in urine concentration in the collecting duct (29). Studies of renal function in mice deficient in AQP6 and AQP7 will provide more information about their specific functions in the kidney.

Equus caballus, the domesticated horse, is a large mammal specifically bred for its superior athletic performance and workload. The total body water of a fully grown 600-kg equid is 360 liters, divided between intracellular (200 liters), extracellular (100 liters), and intravascular (60 liters) compartments. Despite the fact that the equine kidneys are responsible for filtering up to 30 liters of blood per hour, nothing is known about the distribution of major AQPs, sodium and potassium transporters, in the horse nephron, and comprehensive comparative studies of renal function in horses are still lacking. Furthermore, the maintenance of body fluid balance and the regulation of renal fluid loss are important physiological countermeasures in equine colic and endotoxemia. Accordingly, the aims of this study were to exploit a unique opportunity to 1) develop and test new polyclonal antibodies to the major renal AQPs (AQP1–4); 2) establish whether custom-designed polyclonal antibodies raised against major rat renal AQPs cross-react with their equine counterparts; 3) determine the expression and cellular localization of AQP1, AQP2, AQP3, and AQP4 in the equine kidney by immunohistochemistry and immunoblotting; and 4) compare the distribution pattern found in equine kidney with that found in rat kidney using custom-designed renal tissue microarrays (TMAs). The information presented in this paper provides, for the first time, evidence for the expression of major renal AQPs in distinct nephron segments of the equine kidney.

EXPERIMENTAL PROCEDURES

Chemicals. Unless otherwise stated, all chemicals were molecular biology grade and were purchased from Sigma-Aldrich (Poole, Dorset, UK).

Antibodies. Rabbit polyclonal antibodies against rat AQP1, AQP2, AQP3, and AQP4 were produced in partnership with Sigma Genosys (Poole, Dorset, UK). Antibodies to AQP1 and AQP2 were purified by affinity chromatography, but antibodies to AQP3 and AQP4 were not. The affinity-purified polyclonal antiserum to rat AQP1 (designated AQP1B) has recently been tested and found to cross-react with renal AQP1 in a number of mammalian species, including mouse, sheep (38), human (34, 36), dog, cat, and elephant. The AQP1 antibody was diluted 1:500 for Western blotting and 1:100 for immunohistochemistry; the concentration of the stock solution used for immunohistochemistry was 0.3 mg/ml. The affinity purified rabbit polyclonal antibody to rat AQP2 (designated AQP2D) has recently been used in immunohistochemical studies to establish the distribution of AQP2 in renal and extrarenal tissues (39). This affinity-purified antiserum was diluted 1:250 for Western blotting and 1:25 for immunohistochemistry; the concentration of the stock solution used for immunohistochemistry was 0.15 mg/ml. Rabbit polyclonal antibodies to rat AQP3 and rat AQP4 were also produced in partnership with Sigma-Genosys. Two different peptide sequences were used to generate antibodies against AQP3 and AQP4. The AQP3 peptide sequences were as follows: SGAQ3P3A, NH2–(c)ENVKALHMKHKEQI–COOH; and SGAQ3P3B, NH2–(c)LHRYRLLQLAEC–COOH. The AQP4 peptide sequences were as follows: SGAQ4PA, NH2–(c)DNRSQVETDDILK–COOH; and SGAQ4PB, NH2–(c)CRSQVETDELKPG–COOH. The SGAQ4PA and SGAQ4PB peptide sequences were designed to be heavily overlapped, as these are antigenic regions of the rat AQP4 protein, thus increasing the chances of getting a good immune response. A 77-day immunization protocol was chosen that consisted of preimmune serum collection and injection with 200-μg peptide conjugated to keyhole limpet hemocyanin in complete Freund's adjuvant on day 1. This was followed by five 100-μg booster injections in incomplete Freund's adjuvant on days 14, 28, 42, 56, and 70. The AQP3 and AQP4 antisera were diluted 1:250 for Western blotting and 1:500 for immunohistochemistry. A monoclonal antibody (designated α6F) raised against the α1-subunit of Na-K-ATPase, exhibiting broad species recognition (i.e., avian, amphibian, mammalian), was used as a renal-positive control to delineate the distribution of the sodium pump along the equine nephron. The α6F monoclonal (originally developed by D. Fambrough, Johns Hopkins University) was obtained as a hybridoma supernatant from the Developmental Studies Hybridoma Bank, under the auspices of the National Institute of Child Health and Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City. This hybridoma supernatant was diluted 1:20 for Western blotting and used undiluted for immunohistochemistry. We also used a polyclonal antibody (designated α620) raised against the α1-subunit of Na-K-ATPase (a generous gift from Dr. M. J. Caplan, Yale University School of Medicine, New Haven, CT).

Animals and renal tissues. All experiments were carried out according to international regulations for animal care and were approved by the Ethics Committee of the Faculty of Veterinary Science, University of Liverpool. Equine kidneys (n = 12) were obtained fresh (immediately after slaughter) from a local abattoir (Nantwich, Cheshire, UK). Kidneys were also obtained from animals euthanized at the Philip Leverhulme Large Animal Hospital (Leahurst Experimental Field Station, University of Liverpool, Neston, Cheshire) for unrelated clinical reasons (n = 6). Equine kidneys were transported to the laboratory on ice within 1 h and were carefully dissected into cortex, medulla, and papilla before fixing in 10% neutral buffered formalin for 24 h for immunohistochemical studies, or frozen in liquid nitrogen for sodium dodecyl sulfate (SDS)-PAGE and immunoblotting. Formalin fixation was not allowed to continue beyond 24 h to ensure adequate tissue fixation and to prevent drastic reduction of antigen recognition, which may occur for certain proteins exposed to formalin. Rats (Sprague-Dawley) were used in strict accordance with local ethical guidelines. They were fed standard chow and water ad libitum and were maintained under pathogen-free conditions. The animals were killed by rising CO2 levels confirmed by cervical dislocation, and the kidneys (n = 12) were dissected and either frozen immediately in liquid nitrogen or fixed for 24 h in 10% neutral buffered formalin and stored in 70% ethanol for subsequent immunohistochemical studies.

Renal tissue homogenization and extraction of total protein. Dissected renal samples were homogenized using a mechanical tissue homogenizer (T 25 Ultra-Turrax, Wilmington, NC) six times (30 s each) in RIPA buffer consisting of phosphate-buffered saline, 1% (vol/vol) Nonidet P-40, 0.5% (vol/vol) sodium deoxycholate, 0.1% (vol/vol) SDS, 1 mM sodium orthovanadate, and freshly added phosphatase and protease inhibitors. The homogenates were centrifuged at 12,000 g for 10 min at 4°C to remove debris and cellular debris. The supernatants were collected and stored at −80°C until use. The protein concentration of the supernatants was determined using a BCA assay (Pierce, Rockford, IL).
protease inhibitors: 4 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. Insoluble material was removed by two rounds of centrifugation at 10,000 g, and the supernatant containing total proteins was stored at −80°C until assayed for protein content.

**Protein determination.** Protein concentration of renal tissue homogenates was determined spectrophotometrically using a Bio-Rad detergent-compatible protein assay kit and a Pharmacia UV/Visible spectrophotometer. Bovine serum albumin was used as the protein standard.

**SDS-PAGE and Western blotting.** Protein extracts (30 μg/lane) were resuspended in RIPA buffer and solubilized in equal volumes of 2X concentrate Laemmli sample buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8, and heated at 95°C for a minimum of 3 min. For correct estimation of molecular weights, we used 2 μl of MagicMark XP (Invitrogen, Carlsbad, CA) mixed with 5 μl of SeeBluePlus2 (Invitrogen) in two lanes in each gel. This approach allowed us to monitor protein transfer during blotting (i.e., with SeeBlue) and to get a definitive chemiluminescent signal that accurately represents molecular weights on the radiographic film (i.e., Magic Mark). This strategy ensured that there were no errors in estimating the size of protein bands. Proteins were resolved on 12% SDS-PAGE minigels. Equal loads of total protein extracts from the renal cortex, medulla, and papilla were run on 12% SDS-PAGE minigels (Bio-Rad Protein II) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Pre- and post-blot gels were stained with Coomassie brilliant blue to confirm complete protein transfer. Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 0.01% sodium azide (pH 7.4), supplemented with 5% nonfat dried milk powder (Marvel, Premier Foods, UK). Primary antibodies were diluted in blocking solution and incubated with the blots overnight with agitation at 4°C. After three washes in TBS-T, membranes were incubated with a polymer of horseradish peroxidase conjugated to anti-rabbit IgG (DakoCytomation code no. K4065), diluted 1:3,000 in TBS-T containing 5% milk. Immunoreactive sites on PVDF membranes were revealed by enhanced chemiluminescence by exposure to SuperSignal West Pico chemiluminescent substrate (Pierce-Perbio Science, Northumberland, UK). Western blots were then exposed to Hyperfilm ECL film (Amersham Biosciences) and developed using a Kodak auto-developer (Kodak, Hemel Hempstead, UK).

**Peptide depletion experiments.** To demonstrate the specificity of primary AQP3 and AQP4 antibodies in immunological procedures, we carried out peptide blocking experiments following the antibody manufacturer’s recommendations (http://www.sigmaaldrich.com/Brands/Sigma_Genosys/Custom_Antisera/Key_Resources/Antisera Protocols.html). The AQP1 and AQP2 antibodies were already affinity purified and tested in immunohistochemical experiments in a previous study, so there was no need to carry out peptide depletion experiments for these antibodies. The prediluted primary AQP3 and AQP4 antibody (1.0 ml solution) was incubated with a 50-fold excess of the corresponding peptide antigen in TBS by adding 20 μl of reconstituted peptide (concentration: 1 mg/ml) and allowed to mix thoroughly overnight at 4°C, with gentle agitation. The mixture was centrifuged for 15 min at 4°C in a microcentrifuge (10,000 rpm) to pellet nonspecific immune complexes, and the supernatant containing the neutralized, peptide-absorbed antibody was carefully removed for incubation with PVDF membranes and tissue sections alongside nonabsorbed samples.

**Renal TMAs.** TMAs are an ordered array of fixed tissue cores on a charged glass slide (33). They permit high throughput immunohistochemical analysis of multiple tissue sections under identical experimental conditions, providing considerable savings on antibodies and significantly greater internal consistency compared with other standard low throughput techniques that rely on slide-by-slide comparisons. Renal TMAs containing samples of mouse, rat, feline, canine, human, equine, and elephant kidneys were prepared using an AbCam TMA builder (ab1802; Cambridge, UK). With the use of this basic TMA builder samples of equine cortex, medulla and papilla were carefully taken from wax-embedded blocks of formalin-fixed samples and placed alongside samples of renal cortex, medulla, and papilla from the above species (see Fig. 3A). A total of three TMAs were designed for this study and were coded named LUFVS TMA 24A/B/C. A veterinary pathologist was recruited to confirm that the tissues represented on these TMAs were normal and free from any microscopic signs of renal pathology. Sections from these TMAs will be made available to APS and FASEB members interested in comparative studies of kidneys from these mammalian species upon request. Microarrays were then cut (7-μm thickness) using a microtome and mounted on positively charged glass microscope glass slides and stained with Papanicolaou’s hematoxylin and eosin or immunostained with polyclonal AQP antibodies, as described below.

**Immunohistochemistry.** Immunohistochemical staining was performed on renal TMAs using a DAKOCytomation EnVision+ Dual Link System peroxidase (DAB+) kit, essentially as described in several recent papers (36–38, 51). This system is a two-step technique that takes advantage of the superior sensitivity of a horseradish peroxidase-labeled polymer, which is conjugated with secondary antibodies. The labeled polymer does not contain avidin or biotin, and, consequently, nonspecific staining resulting from endogenous avidin-biotin activity in tissues such as liver and kidney is eliminated. Briefly, slides were deparaffinized in xylene for 20 min to remove embedding media and washed in absolute ethanol for 3 min. The slides were gradually rehydrated in a series of alcohol baths (96, 85, and 50%) and placed in distilled water for 5 min. Endogenous peroxidase activity was blocked for 1 h in a 97% methanol solution containing 3% hydrogen peroxide and 0.01% sodium azide. The TMAs were then incubated for 30 min at room temperature with 2% protease-free bovine serum albumin (Sigma-Aldrich) in TBS-Tween to block nonspecific antibody binding. Slides were incubated overnight at 4°C with primary anti-AQP antibodies diluted as described earlier in TBS-Tween. The slides were then washed three times for 5 min each in TBS-Tween before incubation with horseradish peroxidase-labeled polymer conjugated to affinity-purified goat anti-rabbit and goat anti-mouse immunoglobulins for 30 min at room temperature. The sections were washed three times for 5 min in TBS-Tween before applying liquid DAB + Chromogen (DAKO; 3,3′-diaminobenzidine solution) for up to 10 min. The development of the brown-colored reaction was stopped by rinsing in distilled water. The stained slides were immersed for 5 min in a bath of Papanicolaou’s hematoxylin to counterstain cell nuclei. Finally the slides were washed for 5 min in running water and dehydrated in a series of graded ethanol baths before rinsing in three xylene baths and mounting in 1,3-diethyl-8-phenylxanthine (BDH Laboratories). Control experiments were performed by incubating slides with nonimmune rabbit serum, preimmune rabbit serum, by omitting primary antibody, or by peptide competition, as described above.

**Data acquisition and analysis.** The stained TMA microscope slides were examined using a Nikon Eclipse 80i microscope, and images were captured using a Nikon Digital Sight DS-5M camera connected to a PC running Eclipsenet imaging software (version 1.20, Laboratory Imaging for Nikon Instruments, Kingston-upon-Thames, UK).

**RESULTS**

**SDS-PAGE and Western blotting.** To determine whether our custom-designed antibodies raised against peptide sequences derived from the COOH termini of rat AQP1–4 cross-react with their equine counterparts, we performed standard SDS-PAGE to resolve total proteins derived from whole rat kidney and proteins isolated from cortical, medullary, and papillary regions of the equine kidney. This was followed by immuno-
blotting with the polyclonal antibodies to AQP1, AQP2, AQP3, and AQP4. Western blots were repeated a total of 16 times for consistency. Figure 1A shows a representative Coomassie brilliant blue stained gel in which equal amounts (30 μg, as assayed by the Bio-Rad detergent-compatible protein assay) of total protein isolated from various regions of the equine kidney were electrophoretically resolved. Expression of the α1-subunit of Na-K-ATPase, which is known to be expressed along the entire length of the nephron (35), was used as an internal positive control in all Western blot and immunohistochemical experiments to ensure that this abundantly expressed renal membrane protein was present in all of the total protein extracts prepared and that no degradation of its α1 had occurred during protein extraction. Figure 1B shows Western blots of the α1-subunit of Na-K-ATPase (98-110 kDa) in gels identical to that shown in Fig. 1A. Using two different anti-Na-K-ATPase antibodies, we were able to show that Na-K-ATPase was present in the total protein extract from rat kidney and in the cortex, medulla, and papilla of the equine kidney. Equine Na-K-ATPase was consistently detected as a doublet migrating at 98 and 110 kDa. These bands are likely to correspond to the plasma membrane and endoplasmic reticulum pools of Na-K-ATPase, which are differentially processed and glycosylated.

![Figure 1A: SDS-PAGE - Coomassie Blue](image)

![Figure 1B: Western Blotting - Na, K-ATPase](image)

Fig. 1. A: typical Coomassie brilliant blue stained SDS-PAGE gel containing equal amounts of protein (30 μg/lane) extracted from equine renal cortex (C), medulla (M), and papilla (P). B: Western blots for the α1-subunit of Na-K-ATPase using two different primary antibodies confirming abundant expression of this 98- to 110-kDa membrane protein throughout the equine kidney.
By running total protein extracts of rat kidney as well as proteins extracted from various regions of the equine kidney, we were able to confirm that antibodies to AQP1–4 recognize the rat proteins (Fig. 2E) and that the rabbit polyclonal antibodies raised against rat AQP1–4 recognize the equine AQPs as well as the rat AQPs. We are also able to demonstrate that AQP1–4 are present in distinct and specific regions of the equine kidney as they are in kidneys of other mammals (Fig. 2, A–D).

AQP1 is known to exist in two molecular forms, an unglycosylated 28-kDa protein and a 35- to 45-kDa glycosylated form (32, 47). However, the nonglycosylated 28-kDa protein was the main form expressed in the equine kidney cortex and medulla and also in the rat kidney. Expression of the 28-kDa form of AQP1 was lower in the papillary region of the equine kidney, as shown by Western blotting (Fig. 2A). Only one molecular mass form was detected in rat total kidney extract (Fig. 2E).

AQP2 was expressed in the cortical and medullary regions of the equine kidney and was detected as a single band migrating under 30 kDa (Fig. 2B). AQP2 was also detected in rat total kidney extract in these experiments (Fig. 2E). AQP2 was detected also in the equine papilla by Western blotting (Fig. 2B) and by immunohistochemistry (Fig. 4F). AQP3 was expressed in the equine cortex, medulla, and papilla mainly as a single band migrating slightly above 30 kDa (Fig. 2C) (14, 22). We also detected AQP3 in the rat total kidney extract as a major band over 30 kDa (Fig. 2E). AQP4 was detected in rat kidney as a major band at above 30 kDa (Fig. 2E). In the equine kidney, AQP4 was detected in the cortex, medulla, and papilla as bands just over 30 and 40 kDa, respectively (Fig. 2D). In early experiments using commercial AQP4 antibodies, we did not detect AQP4 in the equine papilla (results not shown). Our own antibodies revealed AQP4 expression in all regions of the horse kidney. Peptide-depleted antibodies either failed to produce any specific immunostaining on PVDF membranes (Fig. 2B; Western blot, panels labeled CP) or showed a significantly attenuated chemiluminescent signal compared with unadsorbed antibodies (Fig. 2, A, C, and D; Western blot panels labeled CP).

Microscopical morphology of the equine kidney. Histology was used to examine the structure of the equine kidneys in this study to ensure that the tissues did not display any morphological features associated with renal disease or autolytic tissue damage that may have occurred during transportation after the animals were slaughtered. Figure 3 shows the basic design of a representative renal TMA slide consisting of nineteen 2-mm-diameter spots of samples from the cortex, medulla, and papilla of seven mammals. Also included in this figure is an ensemble of fully annotated photomicrographs, which shows the major structures of the various nephron segments in the equine cortex, medulla, and papilla, and compares these regions with equivalent rat renal tissues. Some morphological differences were noted between the renal structures of the horse and those
of the rat. Although the basic morphological features of the equine kidney were similar to rodents and other domestic animal species (3), the equine kidney featured larger corpuscles and clearly defined distal tubules and macula densa. Equine medullary rays were abundant and clearly defined and additionally contained significantly longer and distended collecting ducts (Fig. 3, A, B, C, and D).

Comparative immunohistochemical localization of renal AQP1 in the equine and rat kidney. Immunohistochemical studies of the renal TMAs were performed to corroborate the immunoblotting results and determine the cell-specific expression and nephron segment localization of AQP1, AQP2, AQP3, and AQP4 in the equine kidney. All of the initial immunohistochemical experiments were done on renal TMAs, but subsequent experiments aimed at refining the methodology and improving the immunostaining were done with complete sections cut from the original wax-embedded equine renal tissues that were used to construct the tissue arrays. The renal TMAs simply provided us with a valuable tool for high throughput immunohistochemistry.

AQP1 was expressed predominantly in the equine renal cortex, particularly in apical and basolateral membranes of proximal convoluted and straight tubules (Fig. 4, A and B). Weak expression was also detected within the glomerulus, which may represent podocyte immunolabeling (Fig. 4A). AQP1 was detected in descending thin limbs of the loop of Henle and in the vasa recta in the equine renal medulla (Fig. 4B). Weaker AQP1 immunostaining was also observed in the loop of Henle and to a lesser extent in vasa recta of the inner medulla and papilla (Fig. 4C). In the rat, AQP1 was detected in identical nephron locations: apical and basolateral membranes of proximal convoluted and straight tubules (Fig. 5A). AQP1 was also seen in the descending loop of Henle and epithelium of the vasa recta in the rat renal medulla and papilla (Fig. 5, B and C).

AQP2, the arginine-vasopressin-regulated water channel, was weakly detected in the cortex by immunohistochemistry, a finding that was also reflected in Western blots (Fig. 2B). AQP2 expression in the medulla and papilla of the equine kidney was more robust (Fig. 4, D and E) and localized to apical plasma membranes of principal cells lining the cortical (Fig. 4D), medullary (Fig. 4E), and papillary (Fig. 4F) collecting ducts. In the rat kidney, AQP2 expression was detected in the cortex, medulla, and papilla. AQP2 expression was seen in apical membranes of principal cells in the distal convoluted tubule and collecting ducts (Fig. 5, D, E, and F). The intensity of AQP2 immunostaining in the equine kidney was significantly weaker compared with rat kidney (compare AQP2 staining in Fig. 4, D, E, and F with Fig. 5, D, E, and F).

AQP3 was strongly expressed in the equine kidney. The staining observed in distal convoluted tubules and collecting ducts was mainly basolateral (Fig. 4, G, H, and I). Original immunocytochemical and immunofluorescence studies of rat kidney revealed that AQP3 labeling is restricted to principal cells in the cortical, outer medullary, and inner medullary collecting ducts, with the labeling being confined to the basolateral domain (14). Similarly, our results show AQP3 in the rat cortex and medulla, in distal convoluted tubules, and in the
basolateral membranes of principal cells lining the cortical, medullary, and papillary collecting ducts (Fig. 5, G, H, and I). We were able to detect AQP4 expression in the equine renal cortex by immunohistochemical and Western blot methods. The immunostaining for AQP4 was seen throughout the kidney in the distal segment of the nephron in basolateral membranes of structures resembling distal convoluted tubules (Fig. 4J), including the medulla and papillary regions (Fig. 4, K and L). Immunostaining was also observed in some regions of the proximal convoluted tubule, which we presume could be similar to the S3 labeling reported in the mouse. In the rat kidney, AQP4 was detected in the cortex medulla (Fig. 5, J, K, and L). Original immunohistochemical studies of AQP4 expression in the rat kidney revealed AQP4 immunolabeling exclusively in the collecting duct principal cells, chiefly in the proximal two-thirds of the inner medullary collecting duct, with little or no expression in the outer medullary and cortical collecting ducts (48). In this study, AQP4 expression in rat kidney was found to be exclusively localized to the basolateral membranes of collecting duct principal cells.

Positive and negative controls. Immunostaining for the α1-subunit of Na-K-ATPase, which was used as a positive control, was detected throughout the equine and rat nephron. Immunohistochemical studies carried out in the early 1980s have firmly established that Na-K-ATPase is abundantly expressed in the distal convoluted tubules, thick ascending limbs of the loop of Henle, and collecting ducts (4). Proximal convoluted and straight tubules generally express lower levels and the thin portions of the loops of Henle, straight descending portions of proximal tubules, and do not stain with antibodies to Na-K-ATPase (4). Our data confirmed these earlier observations and showed Na-K-ATPase polarization to the basolateral membranes of cells lining the nephron in most regions of the equine kidney (Fig. 4, M, N, and O) and the cortical and medullary...
regions of the rat kidney (Fig. 5, M and N). In the equine kidney, Na-K-ATPase immunostaining in the papilla (Fig. 4O) was significantly diminished compared with the cortex and medulla (Fig. 4, M and N). In the rat kidney, Na-K-ATPase was present in both the cortex and medulla (Fig. 5, M and N), but was not detectable by immunohistochemistry in the papilla (Fig. 5O).

Negative control experiments were carried out at the same time as these experiments by incubating slides in the absence of primary antibody (Fig. 4, P, Q, and R; Fig. 5, P, Q, and R, bottom panels).

**DISCUSSION**

Interesting comparative studies of AQP water channels have recently focused on desert-dwelling rodents capable of high degrees of urine concentration (5, 19) and fecal dehydration by specialized colon water absorption mechanisms (17, 18). Studies on kidneys of Merriam’s desert kangaroo rat (Dipodomys merriami merriami), which produce extremely concentrated urine, have shown that AQP4 is undetectable in any region of the kidney (as confirmed by Western blotting and immunohistochemistry). The absence of AQP4 expression in kidneys of the kangaroo rat has led to the suggestion that this is a physiological adaptation that may be critical for the extreme urinary concentration that occurs in this species (21). These findings suggest that the expression or lack of expression of AQPs (especially AQP4) may be related to the ability to concentrate urine (21). Aside from these published studies on desert-dwelling rodents, little is known about the expression of AQPs in several domestic animal species. For example, to our

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Fig. 5. Immunohistochemical localization of AQP1, AQP2, AQP3, and AQP4 in the rat renal cortex, medulla, and medulla/papilla junction. AQP1 was present in apical and basolateral membrane of proximal convoluted tubule cells and in squamous epithelial cells of the descending loop of Henle (A, B, and C). AQP2 expression was restricted to cells of the cortical and medullary collecting ducts and distal convoluted tubules (D, E, and F). AQP3 immunostaining was observed in distal convoluted tubules and collecting ducts (G, H, and I). AQP4 was expressed in the basolateral membrane of distal convoluted tubules and collecting ducts (J, K, and L). Na-K-ATPase was abundantly expressed in distal convoluted tubules, thick ascending limbs of the loop of Henle, and collecting ducts of the rat kidney (M, N, and O). Na-K-ATPase expression was significantly lower in rat proximal tubules (M), and its immunostaining in the papilla (O) was significantly diminished compared with the cortex (M) and medulla (N). Negative controls are shown in panels P, Q, and R. Glomeruli are labeled G; bars represent 10 μm.
knowledge, there are no published reports that have studied the distribution of AQPs in the equine kidney and compared the renal expression of AQPs in the horse and rodent species. Thus far, nothing is known about molecular mechanisms involved in fluid reabsorption in the equine colon and kidney. The molecular and immunohistochemical evidence presented in this paper demonstrates that the major renal AQPs (AQP1, AQP2, AQP3, and AQP4) are expressed at strategic sites along the nephron in kidneys of Equus caballus, the domestic horse. The major findings of this study are summarized in Fig. 6. In terms of abundance and cellular distribution, AQP1, AQP2, AQP3, and AQP4 immunostaining was very similar to that found in the human and rat kidney (36, 39).

**Expression of AQP water channels in different nephron segments governs water permeability.** Different segments of the nephron exhibit differential permeabilities to water and solutes, since the nephron is lined with a plethora of different cell types exhibiting diverse morphologies, metabolic properties, and permeabilities. It is well established that the proximal nephron is the site of active sodium and nutrient reabsorption. The proximal tubule is also the primary and most important region of the nephron for water reabsorption, with ~65% of filtered water being reabsorbed here. Consequently, AQP1 is abundantly expressed in apical and basolateral membranes of the proximal part of the nephron, allowing water to pass across the brush border, through the cells, and back into blood. Original studies in human and sheep kidney have shown that AQP1 immunolabeling is uninterrupted from the proximal convoluted tubule to the proximal straight tubule (the latter displaying the most intense labeling) (32) and continues into descending thin limbs in the outer medulla (6). Abrupt transition from heavy AQP1 labeling to no AQP1 labeling of descending thin limbs in the outer medulla (6). AQP1 plays a vital role in maintaining the water permeability and the functional transition that occurs in terms of water permeability involves loss of AQP1 expression. Therefore, AQP1 plays a vital role in maintaining the water permeability of the proximal nephron, allowing water to be reabsorbed in this segment. The functional significance of AQP1 in this process is further highlighted by the fact that loss of the AQP1 gene results in impaired urinary concentration in both mice and humans (26, 30). The results presented in this study are strongly suggestive of a similar role for AQP1 in water reabsorption in the proximal equine nephron. It is interesting that the equine cortex and medulla express mainly the nonglycosylated form of AQP1.

Abundance of AQP2 in the apical membranes of collecting duct epithelial cells is regulated by the hormone arginine-vasopressin (11, 15). Binding of vasopressin to V2R receptors results in production of cAMP and exocytosis of subapical vesicles preloaded with AQP2 channels, resulting in insertion of AQP2 channels to the apical membrane (20, 31). AQP2 thus controls the permeability of apical membranes of collecting duct epithelial cells. In this study, AQP2 was detected in the apical membranes of equine cortical and medullary collecting duct cells, as well as subapical vesicles. This observation is identical to other species (13, 20, 31). Western blotting experiments of protein extracts from different regions of the equine kidney confirmed the data obtained by immunohistochemistry. Labeling for AQP2 was surprisingly weak in both blots and in immunohistochemistry: it is not clear if this reflects low levels of AQP2 protein in the horse, or limited cross reaction of the antibody raised against rat AQP2 with the equine AQP2 protein.

Abundant AQP3 was found to be expressed mainly in the cortical region of the equine kidney, in basolateral cell membranes (Fig. 4G). In the rat, we also detected AQP3 in the cortex and medulla. Previous studies in the rat have localized AQP3 to collecting duct cells (22). Knockout studies suggest that AQP3 is also important for the regulation of urine concentration (56). Different animals will have different requirements for concentrating urine. This may depend on their size, weight, diet, water intake, habitat, and lifestyle of the animal. In terms of water homeostasis and renal function, the require-

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**Figure 6.** Schematic depicting the segment-specific expression of renal AQP channels in the equine kidney. AQP1 is specifically expressed in apical and basolateral membranes of proximal convoluted tubules in the renal cortex and in cells lining the descending loops of Henle in the equine renal medulla and papilla. AQP2 is expressed only in apical membranes of cells lining the cortical, medullary, and papillary regions of the equine kidney, mainly in basolateral membranes of cells lining the distal tubule and collecting ducts. Basolateral Na-K-ATPase is expressed along the entire length of the nephron (however, immunohistochemical studies presented here confirm that expression of Na-K-ATPase is significantly lower in the papilla and is almost undetectable using this technique).
ments of a 600-kg horse will clearly be different from a 500-g rodent. There are no studies that suggest horses have a high urinary concentrating capacity. Thus the observed differences in equine and rat AQ3 protein expression (i.e., the lower expression of AQ3 in the equine medulla) may be as a result of genuine anatomical and physiological differences in the osmoregulatory requirements of the two species.

AQ4 immunostaining showed specific expression of this AQ protein in collecting ducts in the rat kidney. AQ4 expression in the equine kidney was similar to rat, although very weak expression was noted in medullary collecting ducts (Fig. 4K). In Western blots, we detected AQ4 expression in the equine cortex, medulla, and papilla. The equine AQ4 immunostaining results in the medulla contrast sharply with rat kidney, where AQ4 is found primarily in the basolateral plasma membranes of principal cells and inner medullary collecting ducts (25, 42), and in mouse kidney, where AQ4 immunoreactivity appears in the S3 segment of the proximal tubule (25, 52). This difference in AQ4 expression may be indicative of variation in the urinary concentrating capacity between the two species.

In conclusion, we have shown that AQ1, AQ2, AQ3, and AQ4 are expressed in distinct regions of the equine kidney, and in particular subcellular locations in specific cell types along the nephron. These water and small solute channels are likely to play a critical role in the physiology of the equine kidney by participating in renal water reabsorption and urinary concentration. The presence of AQ1 in proximal convoluted tubules, vasa recta, and descending loop of Henle supports a role for AQ1 in water reabsorption in the proximal part of the equine nephron. The presence of AQ2 in apical membranes of collecting duct principal cells is identical to all other species studied to date and suggests that this vasopressin-regulated water channel plays a central similar role in regulating the apical permeability of the collecting duct to water in response to vasopressin release. The low level of AQ2 seen in the equine collecting ducts may well be consistent with a limited urinary concentrating capacity in this species. Expression of AQ3 in the distal equine nephron suggests that this channel may be involved in the transport of water and small solutes across the basolateral membrane of the equine collecting duct principal cells. Antibodies to rat AQ4 revealed that expression of equine renal AQ4 is similar to that of rat, except in the medulla where equine collecting ducts show weak AQ4 staining. The apparent difference in AQ4 expression may be as a result of real physiological differences between the kidney or the animal’s water intake, diet, and metabolism. Therefore, this apparent difference in AQ4 expression in the rat and horse may be suggestive of a special role for AQ4 in water homeostasis in the horse.

Disorders of water balance in the equine species. While nephrogenic diabetes insipidus and urinary obstruction are rare conditions in horses, they do occur, and, in particular, male racehorses are more susceptible to urinary obstruction due to the frequent use of nonsteroidal anti-inflammatory drugs and a narrow urethra. The most common urinary disorder in equines is psychotic polyuria, and, in animals which have had this long term, the ability to concentrate urine is lost. Fluid balance and the ability to regulate renal fluid loss have important equine welfare implications. Millions of equids are used as working animals throughout the world, often in countries with hot, dry climates. Dehydration is recognized as a major threat to the welfare of such animals, and differences in susceptibility between horses, mules, and donkeys have been reported (45). The single most important cause of equine mortality is intestinal disease (49, 50); dehydration arising from intestinal obstruction and endotoxemia is usually the terminal event in such cases. Equine athletes are often required to compete in endurance events or in testing climatic conditions. The efficiency of fluid homeostasis may be an important determinant of performance (9, 46). Further research into the roles of AQPs in the equine kidney and other organ systems (e.g., gastrointestinal tissues) are likely to lead to a better understanding of water balance disorders in horses. Future studies will need to focus on profiling of these and other specific pathophysiological conditions in which the expression of members of the AQP gene family might be altered in the horse.

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