Rat Vasopressin V_2 Receptor

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Abstract

The arcades are long, branched renal tubules which connect deep and mid-cortical nephrons to cortical collecting ducts in the renal cortex. Because they are inaccessible by standard physiological techniques, their functions are poorly understood. In this paper, we demonstrate that the arcades are a site of expression of two proteins, aquaporin-2 (the vasopressin-regulated water channel) and the V_2 vasopressin receptor, that are important to regulated water transport in the kidney. Using a peptide-derived polyclonal antibody to aquaporin-2, quantitative ELISA in microdissected segments showed that aquaporin-2 is highly expressed in arcades and that the expression is increased in response to restriction of fluid intake. Immunocytochemistry revealed abundant aquaporin-2 labeling of structures in the cortical labyrinth in a pattern similar to that of the Na^+-Ca^{2+} exchanger and kallikrein, marker proteins expressed in arcades but not in cortical collecting ducts. RT-PCR experiments demonstrated substantial aquaporin-2 and V_2 receptor mRNA in microdissected arcades. In situ hybridization, using ^{35}S-labeled antisense cRNA probes for the V_2 receptor demonstrated strong labeling of both arcades and cortical collecting ducts. Thus, these results indicate that the arcades contain the specific proteins associated with vasopressin-regulated water transport, and may be a heretofore unrecognized site of free water absorption. (*J. Clin. Invest.* 1996. 97:2763–2771.) Key words: ELISA • RT-PCR • immunocytochemistry • arcades • kidney

Introduction

Vasopressin increases water permeability in collecting ducts by triggering exocytic insertion of intracellular vesicles containing aquaporin-2 (AQP2; previously called WCH-CD or AQP-CD)[1] water channels into the apical plasma membrane (1–3). While this response is generally thought to be associated only with collecting ducts, it is clear from micropuncture studies (4, 5) that vasopressin induces an increase in water absorption proximal to the beginning of the collecting duct system. This response has been attributed to the "initial collecting tubule" (6), i.e., the final portion of the distal tubule in the cortical labyrinth which contains collecting duct principal cells and intercalated cells. The potential roles of other cortical distal tubule segments have not been fully evaluated, however.

One potential site of water permeability regulation in the renal cortex is the arcades. Arcades are highly branched renal tubule segments that ascend through the cortical labyrinth in association with the interlobular vessels, and connect distal convoluted tubules from mid-cortical and deep nephrons to the origins of the cortical collecting ducts in the superficial cortex (see Fig. 1). Within the arcades, approximately 70–80% of cells are connecting tubule cells and 20–30% are intercalated cells (7). The epithelial surface area of the arcades is roughly equal to that of the cortical collecting ducts in rodent species and thus, if the arcades do exhibit a substantial water permeability in response to vasopressin, they could be an important site of water absorption. However, relatively little information is available regarding the role of arcades, if any, in urinary concentrating mechanism. This is because they are inaccessible to micropuncture and are difficult to study by in vitro microperfusion due to their highly branched nature.

The recent cloning of cDNAs for the vasopressin V_2 receptor (8, 9) and the vasopressin-regulated water channel, AQP2 (10), has provided new tools for the study of vasopressin action at the single tubule level. In this study, we use immunochemical methods with a polyclonal antibody to aquaporin-2 (single-tubule ELISA and immunocytochemistry), RT-PCR amplification of mRNAs for AQP-2 and the V_2 vasopressin receptor as well as in situ hybridization histochemistry for V_2 receptor to investigate the possible role of the arcades in vasopressin-regulated water transport.

Methods

Experimental animals

Pathogen-free Sprague-Dawley rats of either sex (Taconic Farms Inc., Germantown, NY) were used. The animals were maintained in pathogen-free state and were fed ad libitum with autoclaved commercial rodent diet (Ziegler Brothers Inc., Gardner, PA), and had free access to sterile drinking water. Unless otherwise mentioned, the animals were thirsted for 24 h before experimentation.

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1. *Abbreviations used in this paper:* AQP2, aquaporin-2; ARC, arcade; CCD, cortical collecting duct; MTAL, medullary thick ascending limb; PCT, proximal convoluted tubule; RT-PCR, reverse transcription-polymerase chain reaction.

Thirsting protocol

To study the effect of water deprivation on AQP2 expression, we followed a protocol reported earlier from our laboratory (11). Specifically, six male rats were initially water loaded by providing 600 mM sucrose solution as the sole drinking fluid for the first 48-h equilibration period. The animals consumed large volumes of this sweetened water, establishing a moderate diuresis, which was the basal state for all animals. At the end of the first 48-h period, half the animals were randomly chosen for a thirsted group and deprived of water for the next 48 h while the second half continued to receive the sucrose water to ensure that they were water loaded. To minimize variation, kidneys from all the animals in this experiment were dissected on two consecutive days. Rats from both groups were included on each day.

Fluorescence-based ELISA for AQP2 on microdissected tubules

To provide a means for absolute quantification of aquaporin proteins at single tubule level, we developed and improved an ultramicro fluorescence-based ELISA sufficiently sensitive to measure the aquaporins in 1–4-mm microdissected renal tubule segments (12, 13). For this, male rats weighing between 120–150 grams were used. The kidneys were prepared for microdissection as reported previously (12). Cortical collecting ducts (CCD), arcade segments and medullary thick ascending limbs (MTAL) were dissected. Arcade segments were identified based on their location in cortical labyrinth between medullary rays and by the presence of multiple branches (Fig. 1). The length of each microdissected segment was measured with an ocular micrometer. Fig. 2 shows microphotographs of representative microdissected arcade and CCD.

The ELISA procedure is described in detail elsewhere (12, 13). Briefly, samples of microdissected tubule segments (2–3 mm total length per sample) were permeabilized and membrane proteins were solubilized by exposing to osmotic shock and low concentration of non-ionic detergent (0.5% Triton X-100) in the wells of a micro-titer plate fitted with a Durapore low protein binding membrane filters (MultiScreen-HV; Millipore Corp., Bedford, MA). These plates allow for rapid washing and exchange of solutions through the use of a vacuum manifold (MultiScreen Vacuum Manifold; Millipore Corp.).

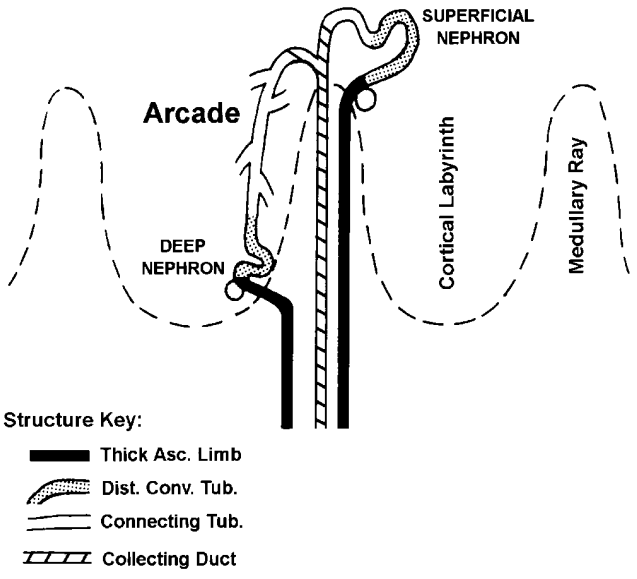

Figure 1. Diagram showing the organization of distal nephron. The superficial connecting tubules connect the distal convoluted tubules of superficial nephrons to the cortical collecting ducts. The arcade segments are long, branched segments consisting chiefly of connecting tubule epithelium which connect distal convoluted tubules of several mid-cortical and deep nephrons to the origin of a collecting duct.

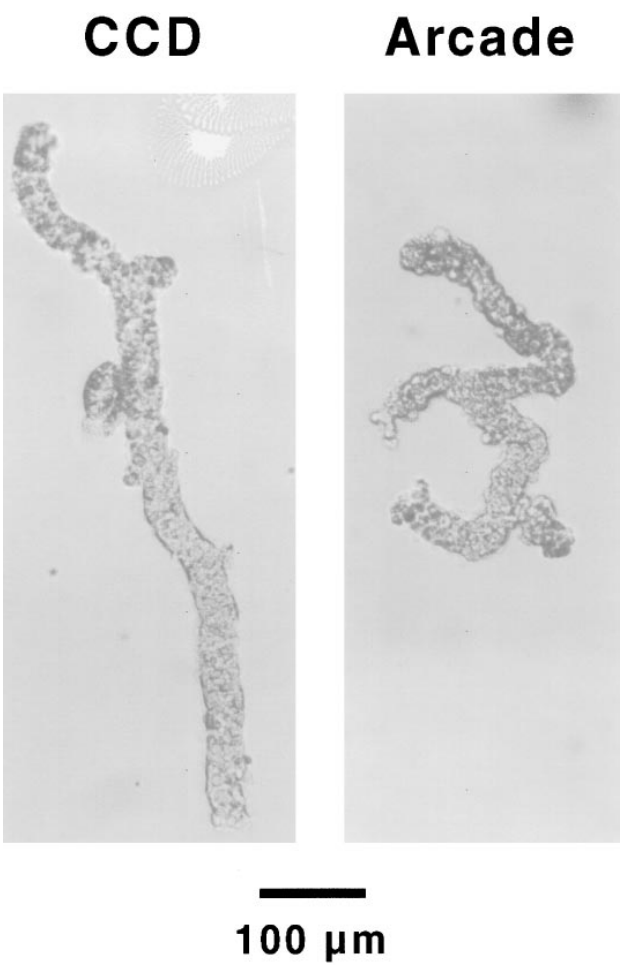

Figure 2. Profiles of microdissected arcade segments and CCD. Cortical collecting duct (CCD) and arcade segment (ARC) microdissected from a collagenase-digested rat kidney. Note the typical branched and arched appearance of arcade segments in contrast to the straight profile of the CCD. Compare with Fig. 1.

AQP2 standards (0–100 fmoles per well; see below) were also loaded in different wells. The solubilized proteins and the AQP2 standards were covalently coupled to the pre-loaded epoxy-activated Sepharose 6B beads (Pharmacia Biotech, Uppsala, Sweden) in the wells.

After the immobilization of proteins, the beads lying at bottom of the wells were processed through an ELISA procedure. The antibody against AQP2 was raised by immunizing rabbits with a synthetic peptide corresponding to COOH-terminal sequence (amino acids 250–271; mol wt 2618). It was affinity purified and previously characterized by immunoblotting and immunocytochemistry (14, 15). AQP2 standards were prepared by conjugating this immunizing peptide to maleimide-activated bovine serum albumin (Pierce, Rockford, IL) in a molar ratio of 1.28:1. The construction and characterization of this conjugate were described in detail elsewhere (13). The secondary antibody was donkey anti-rabbit IgG conjugated to β-galactosidase (Amersham Corp., Arlington Heights, IL). 4-methylumbelliferyl-β-galactopyranoside (MUG) was used as the fluorogenic substrate for β-galactosidase. Fluorescence intensity (photon counts per second, cps) was read on a SPEX-FluoroMax Spectrofluorometer (SPEX Industries, Inc., Edison, NJ) with excitation set at 360 nm and emission set at 445 nm. The blank values generated by carrying out the MUG incubation in the absence of the secondary antibody were subtracted from the measured fluorescence values of the samples. The mean±

SEM of blank values for the assays reported here were 0.30±0.18 $\times 10^6$ cps (n = 17). AQP2 standards were run in parallel to the samples in each plate and the quantity of AQP2 in the renal tubule segments was calculated from the standard curve.

Immunolocalization of AQP2 in arcades

Male rats weighing 150–200 grams were anesthetized with ketamine-pentobarbital. The kidneys were fixed in situ for immunolocalization by retrograde perfusion through the abdominal aorta. An isotonic phosphate buffer (16) was perfused briefly to clear the kidneys of blood followed by perfusion with paraformaldehyde. Initial studies used a periodate-lysine-3% paraformaldehyde solution (17) and a fixation time of 1 h, but higher levels of immunoreactivity were observed when fixation was limited to a 5 min exposure to 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed kidneys were rinsed with PBS and sliced into 2–4-mm-thick pieces before incubation for 1 h in a solution containing 10% EDTA as a cryoprotectant in 0.1 M Tris buffer at 4°C. Each slice was then placed on an aluminum foil platform, plunge-frozen into propane-isopentane cooled by liquid nitrogen, and stored at −80°C in an air-tight container.

The frozen slices of kidney were sectioned on a cryostat microtome. 6–10-μm-thick sections were picked up on coverslips subbed with 1% fish gelatin that had been cross-linked by 2% glutaraldehyde. Sections were treated with 6 M guanidine for 10 min to expose antigenic sites (18), rinsed twice in high salt wash solution (500 mM NaCl, 1% BSA in phosphate buffer), and exposed to 5% H_2O_2 in PBS for 10 min to block endogenous peroxidase activity. Samples were then washed three times with wash solution and incubated with 50 mM glycine, 1% BSA in PBS for 20 min. Anti-AQP2 antibody was the same as described above under ELISA procedure. Anti-rat urinary kallikrein was that reported by Oza et al (19). The antibody to Na^+-Ca^{2+} exchanger was kindly provided by Dr. George Lindenmayer (20). These primary antibodies were diluted to 10 μg/ml with PBS containing 0.1% BSA and 0.02% sodium azide, and incubated with sections overnight at 4°C. The following day, sections were washed five times over 1 h with high salt wash followed by incubation in goat anti–rabbit antibody for 2 h at room temperature. After washing, sections were then incubated in peroxidase anti-peroxidase for another 2-h period followed by incubation in 3,3′-diaminobenzidine with H_2O_2 for 20 min and mounted in glycerol.

RT-PCR detection of mRNA for AQP2 and vasopressin V_2 receptor

PCR primers. For AQP2, the specific primers used were: antisense, 5′ TAA GCA CAG TCC CCC AGA AGG 3′ corresponding to bases 1111–1131 from ATG, and the sense primer 5′ TCC AGC AGT TGT CAC TGG C 3′ corresponding to bases 570–588 from ATG on the cDNA sequence reported by Fushimi et al. (10). The anticipated PCR product is 562 bp in length.

For the V_2 receptor, the specific primers used were: antisense, 5′ TGA GGC ATC TGT CCC AGT TGC TTC C 3′ corresponding to bases 1314–1338, and the sense 5′ ATC CGG AAG CTC CTC TGG AAA GAC C 3′ corresponding to bases 887–911 from ATG on the cDNA sequence reported by Lolait et al (8). The anticipated PCR product is 452 bp in length.

Microdissection, reverse transcription and polymerase chain reaction. The microdissection, reverse transcription, and PCR were carried out essentially as described previously (21), which is a modified version of the procedure described by Terada et al. (1993). Glomeruli, proximal convoluted tubules (S1+S2), cortical collecting ducts and arcade segments were microdissected. The length of each microdissected tubule segment was measured. 1–2 mm of tubule segments or 4 glomeruli were transferred into the PCR tubes containing 6.7 μl of Triton X-100 solution (a mixture of 96 μl 2% Triton X-100, 3.5 μl placental RNase inhibitor, 0.5 ml 1 M DTT) and frozen on dry ice to improve permeabilization. Reverse transcription was done at 42°C for 60 min, followed by inactivation of the reverse transcriptase at 95°C for 5 min. For the PCR, after the initial melt step (94°C for 3 min), 28 cycles were carried out as follows: melt step, 94°C for 1 min; annealing for 1 min at 60°C; extension for 1 min at 72°C. A final extension at 72°C was carried out for 7 min. Controls without the addition of reverse transcriptase were run in parallel to rule out genomic amplification. A water blank was included in each assay to detect any contamination. 10 μl of each PCR product was mixed with 3 μl of loading buffer (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF in water), and was electrophoresed on a 2% agarose gel. The PCR products separated on the gels were visualized by staining with ethidium bromide and photographed. The specificity of the PCR-amplified products were further verified by Southern hybridization. The blots were probed with ^{32}P end-labeled oligonucleotides which were directed to sequences that were flanked by the two PCR primers. The oligonucleotide probes for AQP2 and V_2 receptor were 5′ TGC AGT GAG GCT CAA GAA GGG 3′ and 5′AGG GTT CTT TTA CAG CCC TGG 3′ respectively.

In situ hybridization histochemistry for V_2 receptor mRNA

This method for the detection of vasopressin V_2 receptor mRNA was similar to that described previously (22). Male and female rats were killed by decapitation under CO_2 anesthesia. Kidneys were rapidly removed, frozen on dry ice, and stored at −80°C. 24-μm-thick frozen sections were thaw-mounted on to gelatin/chrome alum subbed slides. Probes were transcribed from a PstI–PvuII fragment (bases 398–911) of the rat V_2 receptor cDNA cloned into pGEM3z. Antisense and sense probes were labeled with [^{35}S] UTP (New England Nuclear, Boston, MA) according to methods described by Promega, the manufacturer of the T7 and SP6 polymerases. Specificity of the probes has been described previously (22, 23). Tissue sections were thawed, immersed in 4% formaldehyde in PBS, treated with acetic anhydride, and delipidated in a graded series of alcohols followed by chloroform. Sections were incubated for 22 h at 55°C with $\sim 1.5 \times 10^6$ cpm/slide/80 μl of hybridization buffer. Tissue sections were rinsed in 4× saline sodium citrate buffer (SSC), incubated with 20 mg/ml RNase-A at 37°C and washed in decreasing concentrations of SSC with dithiothreitol. After a final 30 min wash in 0.1× SSC at 67.5°C, slides were dehydrated through a series of alcohols containing 300 mM ammonium acetate, dried, exposed to film, and subsequently dipped in Ilford K5D nuclear emulsion. Slides were developed in Kodak D-19 and stained with hemotoxylin and eosin.

Statistical methods

All quantitative data are expressed as mean±SEM. Where appropriate, differences between the means were assessed by unpaired t test and P values < 0.05 were considered significant.

Results

Quantification of AQP2 protein in microdissected arcades by ELISA. We applied the highly sensitive fluorescence-based ELISA on 2–3-mm samples of microdissected arcade segments and CCD (Fig. 2). The AQP2 standards (immunizing peptide-BSA conjugate) were run on each ELISA plate in parallel to the microdissected arcades and CCD. Fig. 3 shows that the standards were linear in the range of 0–100 fmoles of AQP2 peptide equivalents per sample. In additional studies the standards were found to be linear up to 200 fmoles per sample (not shown here; 13). The lower limit of detection (defined as 3 × SEM above the mean background signal value) was 2.3 fmoles (13). As shown in Fig. 4 the CCD had 17.3±1.8 fmoles of AQP2 per mm length (n = 14), and the arcade segments had 64% of this mean value (11.2±1.4 fmoles/mm, n = 9). When expressed as the number of molecules per unit length, these values represent 10.4±1.1 $\times 10^9$ and 6.7±0.9 $\times 10^9$ copies per millimeter for CCD and arcades respectively. On the other hand, medullary thick ascending limbs (MTAL), which are im-

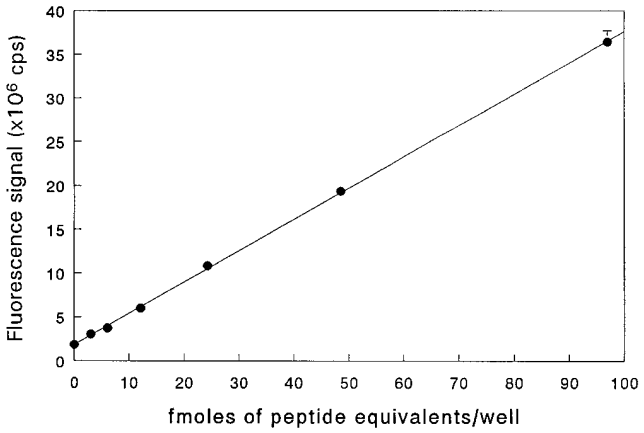

Figure 3. Fluorescence-based ELISA for AQP2. Typical calibration curve for fluorescence-based ELISA using AQP2 peptide-BSA conjugate. The fluorescence intensity was measured in photon counts per second (cps). The blanks (omission of secondary antibody) in this particular assay gave a mean value of $0.207 \pm 0.050 \times 10^6$ cps. Each point is mean±SEM of triplicate samples.

permeable to water, had < 0.4 fmoles/mm AQP2. Thus, these data demonstrate the presence of substantial quantities of AQP2 water channel protein in the arcade segment.

Effect of water deprivation on the expression of AQP2 in arcades. To examine if water deprivation has any effect on the expression of AQP2 in arcades, we measured AQP2 levels in arcade segments dissected from water loaded and 48h thirsted animals. Fig. 5 shows that CCD from thirsted animals had an approximately 70% increase in the AQP2 content as compared to the water-loaded group (16.4±1.6 fmoles/mm, $n = 6$ vs. 9.6±1.3 fmoles/mm, $n = 6$, $P < 0.01$). Thirsting also increased the AQP2 content of arcade segments to a comparable extent (90%; 10.0±1.2 fmoles/mm, $n = 8$ vs. 5.3±1.7 fmoles/mm, $n = 7$, $P < 0.05$). Thus, these observations demonstrate that arcades function as a site of long-term regulation of water channel expression in response to water deprivation.

Immunocytochemical localization of AQP2. We also demonstrated that the AQP2 water channel is expressed in arcade segments using immunocytochemistry (Fig. 6). In cryosections through the cortical labyrinth, distinct immunolabeling for AQP2 was consistently observed in segments near cortical radial vessels where arcades are located (7). To positively identify these segments as arcades, antibodies to kallikrein and the Na^+-Ca^{2+} exchanger were utilized on serial sections of the cortex adjacent to a section utilized for localization of AQP2. Kallikrein and Na^+-Ca^{2+} exchanger serve as markers for the connecting tubule cells, which are the majority cell type of the

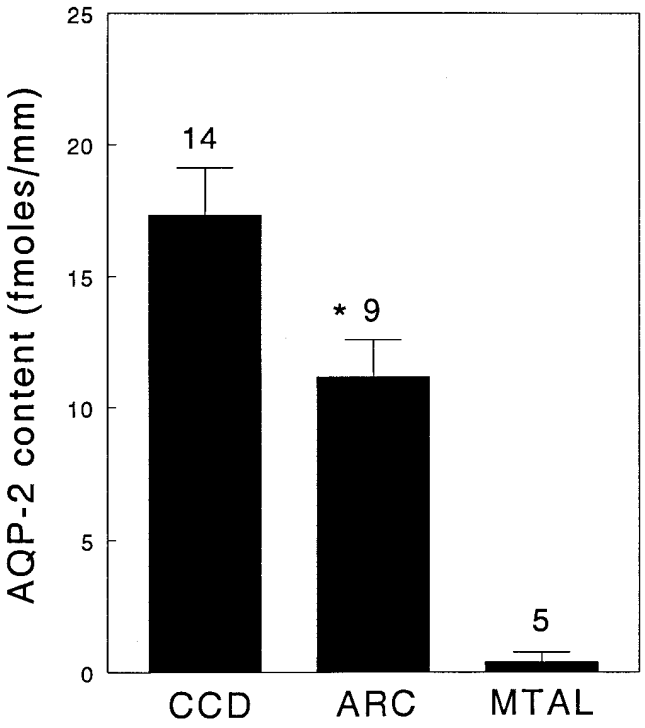

Figure 4. Quantification of AQP2 protein in microdissected arcades and CCD. Cortical collecting ducts (CCD) or arcade segments (ARC) or medullary thick ascending limbs (MTAL) were dissected from collagenase-digested kidneys from rats that were thirsted for 24 h and assayed for AQP2 protein by ELISA. Data obtained from four rats. The number of samples assayed is indicated above each bar. Each sample used 2–3 mm total length of the dissected renal tubules. *$P < 0.03$ vs. CCD by unpaired t test.

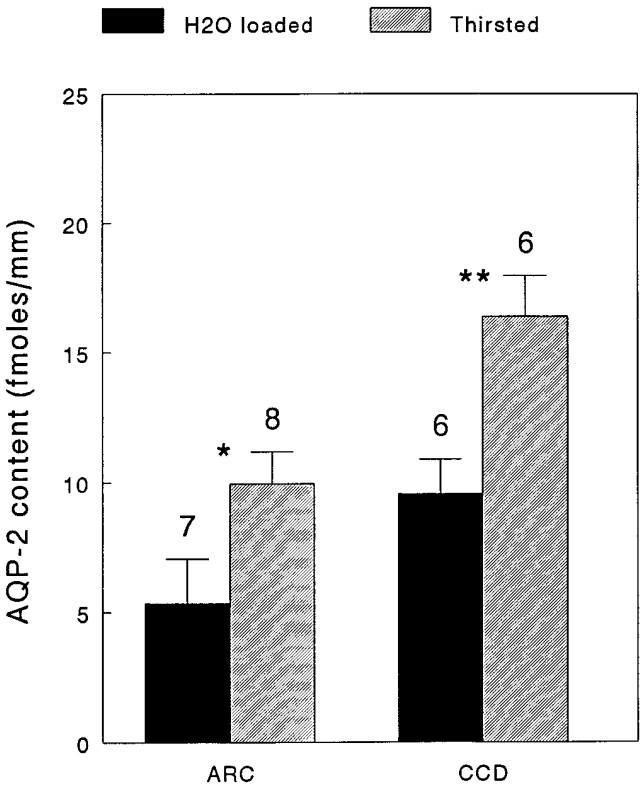

Figure 5. Effect of thirsting on the expression of AQP2 protein in arcades and CCD. Arcade (ARC) segments and cortical collecting ducts (CCD) dissected from collagenase-digested kidneys of rats that had been either water-loaded by sucrose solution intake for 4 d ($n = 3$) or thirsted for 2 d after initial water loading for 2 d by sucrose solution intake ($n = 3$) were assayed for AQP2 protein by ELISA. The number of samples assayed is indicated above each bar. Each sample used 2–3 mm of total dissected tubules. *$P < 0.05$ and **$P < 0.01$ vs. corresponding water-loaded groups by unpaired t test.

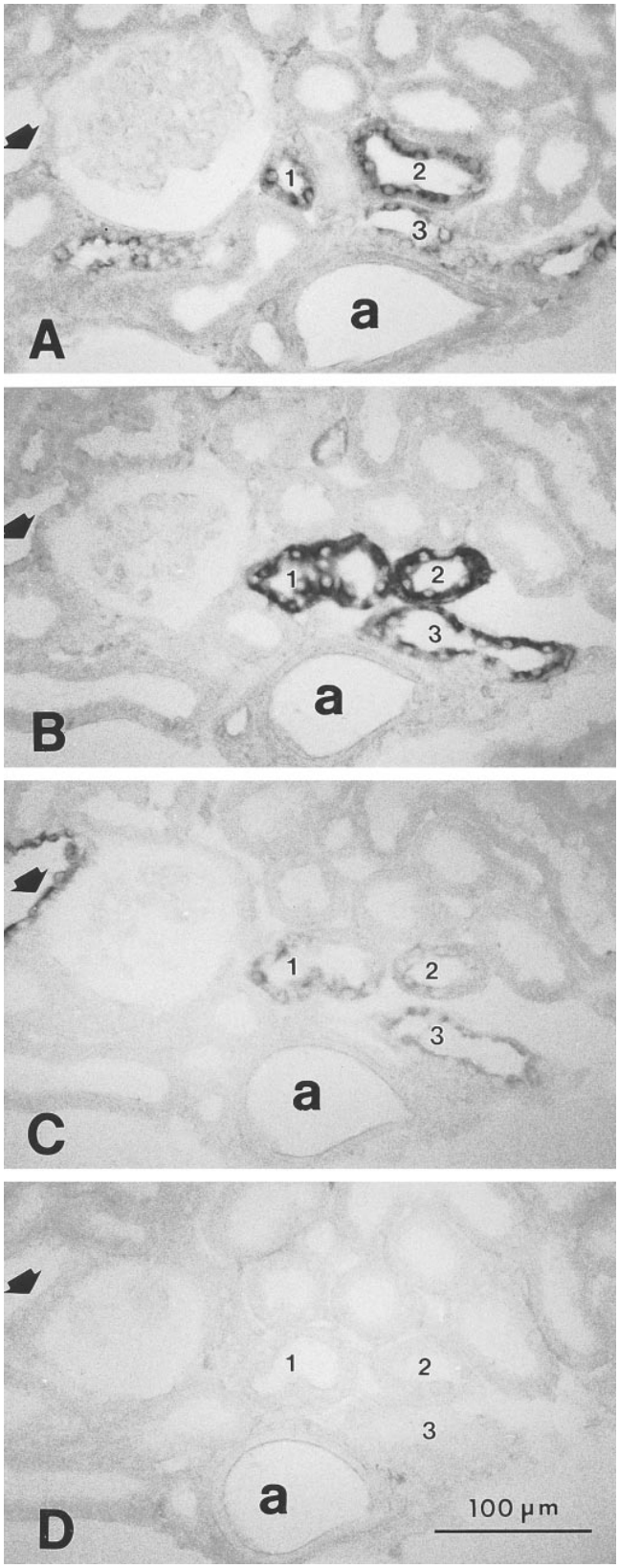

Figure 6. Immunocytochemical co-localization of AQP2 with kallikrein and Na^+-Ca^{2+} exchanger. Serial cryosections through the cortical labyrinth were probed with specific antibodies either to kallikrein (*A*) or Na^+-Ca^{2+} exchanger (*B*) or AQP2 (*C*). *D* shows a preabsorption control for AQP2 antibody. Three loops (numbered 1–3) of an arcade can be seen in each section, adjacent to an interlobular artery (*a*). Arrows on left margin of the panels point to a collecting duct segment.

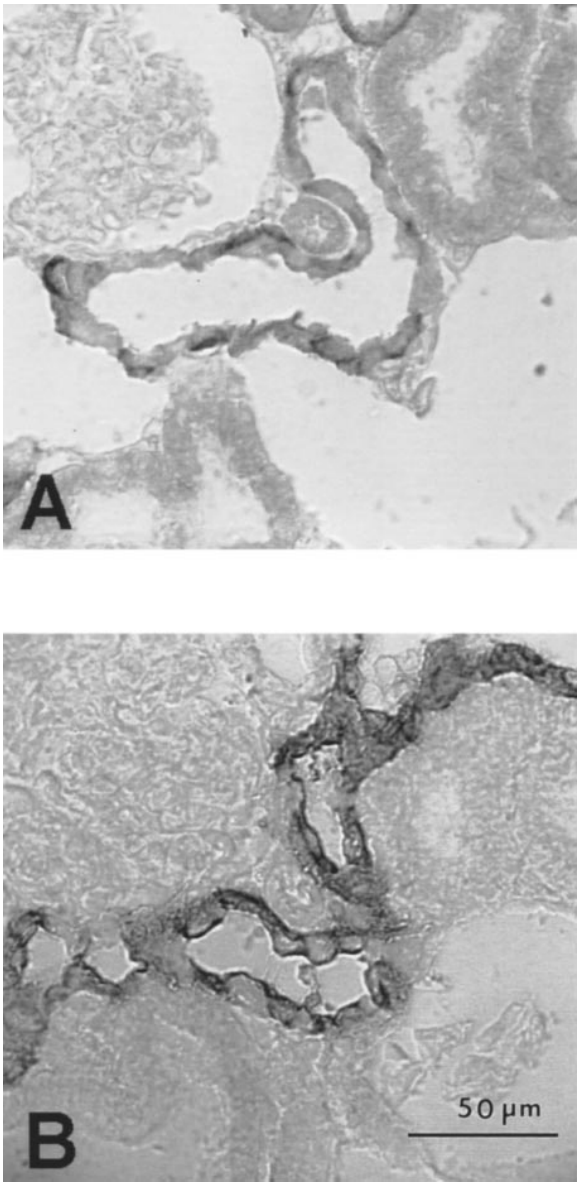

Figure 7. High resolution immunocytochemical localization of AQP2 protein in thirsted rat: Serial cryosections through the cortical labyrinth were probed with specific antibodies to either Na^+-Ca^{2+} exchanger (*A*) or AQP2 (*B*). *A* shows a typical arcade segment in which most of the cells were labeled for the recognized connecting tubule marker. *B* shows that the same segment is heavily labeled with the specific antibody to AQP2 demonstrating the overlap in labeling with the two antibodies. Note that both antibodies labeled more than 50% of the cells in the profile. In addition, the labeling of arcade segment for AQP2 was markedly enhanced in thirsted rats (compare with Fig. 6 *C*).

arcades (7). Fig. 6 shows three loops (numbered 1–3) of an arcade. Specific antibodies to kallikrein (Fig. 6 *A*) and to Na^+-Ca^{2+} exchanger (Fig. 6 *B*) each labeled most of the cells of these loops but not the nearby cortical collecting ducts (Fig. 6,

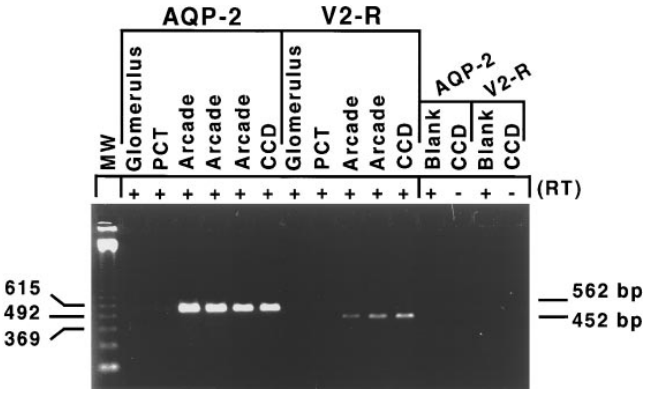

Figure 8. Detection of AQP2 and V_2 receptor mRNA in the arcades by RT-PCR. RT-PCR was performed on arcade segments (1–2 mm/tube). Cortical collecting ducts (CCD; 1–2 mm/tube) were run in parallel as positive controls. Proximal convoluted tubules (PCT; 1–2 mm/tube) and glomeruli (4/tube) served as negative controls. Assays were carried out in the presence (+) or in the absence (−) of reverse transcriptase (RT) to detect any amplification of genomic DNA. Blanks refer to assays without the samples (glomeruli or tubules). After 28 cycles of polymerase chain reaction, 10 μl of the PCR products were electrophoresed on 2% agarose gel, and the separated DNA fragments were visualized under UV light after staining with ethidium bromide. Left lane, molecular size markers (123 bp DNA ladder). The expected length of DNA fragments are 562 and 452 base pairs for the AQP2 and V_2 receptor respectively.

A and *B*; arrows). The antibody to AQP2 also labeled most of the cells in these loops (Fig. 6 *C*) as well as the cortical collecting duct (Fig. 6 *C*; *arrow*). The magnitude of the labeling for the arcade segments tends to be weaker than in the collecting duct, consistent with the ELISA findings. A certain degree of tubule to tubule variation in AQP2 labeling was found with some regions showing relatively little labeling (Fig. 6 *C*, loop2). A section exposed to AQP2 antibody that had been pre-adsorbed with an excess of the immunizing peptide showed no labeling (Fig. 6 *D*).

Fig. 7 shows two adjacent sections from the mid-cortex of a thirsted rat labeled with the Na^+-Ca^{2+} exchanger antibody (*A*) and the antibody to aquaporin-2 (*B*). Panel A shows a typical arcade segment in the cortical labyrinth labeled with the anti Na^+-Ca^{2+} exchanger antibody. Most of these cells are labeled. This same segment is heavily labeled with the antibody to aquaporin-2 (*B*), demonstrating the overlap in labeling with the two antibodies. In general, consistent with the findings of the ELISA assay, labeling of arcade segments was markedly enhanced after thirsting of the rats (compare with Fig. 6 *C*).

Detection of AQP2 and V_2 receptor mRNA in arcades by RT-PCR. Reverse transcription and polymerase chain reaction (RT-PCR) were performed directly on tubules dissected from collagenase-digested kidneys. Figure 8 shows the PCR products separated on a 2% agarose gel and visualized after staining with ethidium bromide. As seen in the Fig. 8, when amplified using specific primers for AQP2, clear and prominent bands could be visualized in the arcade samples. The intensities of these bands were comparable to those seen with CCD sample. These PCR products were of the expected molecular mass (562 bp) for the primers used. The specificity of the PCR products was further confirmed by Southern hybridization using a probe complimentary to the mid-portion of the target sequence, which demonstrated a single band at 562 bp (data not shown). Furthermore, neither glomeruli nor proximal convoluted tubules (PCT) showed the bands.

Similarly, when RT-PCR was run using specific primers for V_2 receptor, each arcade sample showed a single clear band that was slightly less intense than those seen with CCD samples. These bands were of the molecular mass (452 bp) expected for the primers used, and Southern blots using a probe complimentary to the mid-portion of the target sequence had confirmed that the correct target in the collecting ducts is amplified (data not shown; 11). Again, neither glomeruli nor PCT showed these bands, as expected.

In addition, CCD samples amplified in the presence of AQP2 or V_2 receptor primers without prior reverse transcription failed to show any PCR products, thus ruling out the possibility that products were due to the amplification of genomic DNA. Samples without tubules (blanks) also did not reveal any PCR products indicating that there was no cDNA contamination of samples (Fig. 8).

Demonstration of expression of V_2 receptor mRNA in arcades by in situ hybridization histochemistry. In situ hybridization was performed on kidney sections from male and female rats using specific probes to detect V_2 receptor mRNA. Fig. 9 shows a representative composite of the hybridization pictures. In the bright field images (Fig. 9, *A* and *C*) branches of interlobular arteries could be seen in the mid-cortical region, around which the branches of arcade segments intertwined (Fig. 9, *A* and *C*). A diffuse labeling (silver grains) could be seen on these arcade segments around the arteries (Fig. 9, *A* and *C, arrows*). In the corresponding dark field images (Fig. 9, *B* and *D*), V_2 mRNA was labeled in multiple short tubular segments around the arteries, characteristic of branches of an arcade (Fig. 9, *B* and *D, arrows*). Interestingly, the distribution of the silver grains on the arcade segments was often diffuse, but not patchy, covering almost all the segmental profile, which indicates that the V_2 receptor mRNA is probably expressed in a large percentage of the cell population. The expression of V_2 receptor mRNA is denser in cortical collecting ducts than in arcade segments (Fig. 9 *B, ccd*).

Discussion

Renal water excretion is regulated by the peptide hormone vasopressin. It is generally accepted that vasopressin acts on the renal collecting duct to increase its water permeability and thus altering water absorption. However, early micropuncture studies revealed that vasopressin induces an increase in water absorption in the distal nephron proximal to the collecting duct system (4, 5). In fact, during antidiuresis, quantitatively more water is absorbed before the collecting duct system to raise tubule fluid to isotonicity than in the medullary collecting ducts to produce a hypertonic urine (24). Thus, an identification of the sites and mechanism of vasopressin-stimulated water absorption proximal to the collecting duct is of considerable importance.

The results reported here point to the renal arcades as a likely site of vasopressin-regulated water absorption proximal to the collecting duct. We demonstrate that the vasopressin-regulated water channel, aquaporin-2, is abundantly expressed in the arcade segment at both the mRNA level (Fig. 8) and protein level (Fig. 4–7). Importantly, we show that the expression of aquaporin-2 is regulated in the arcades in response to

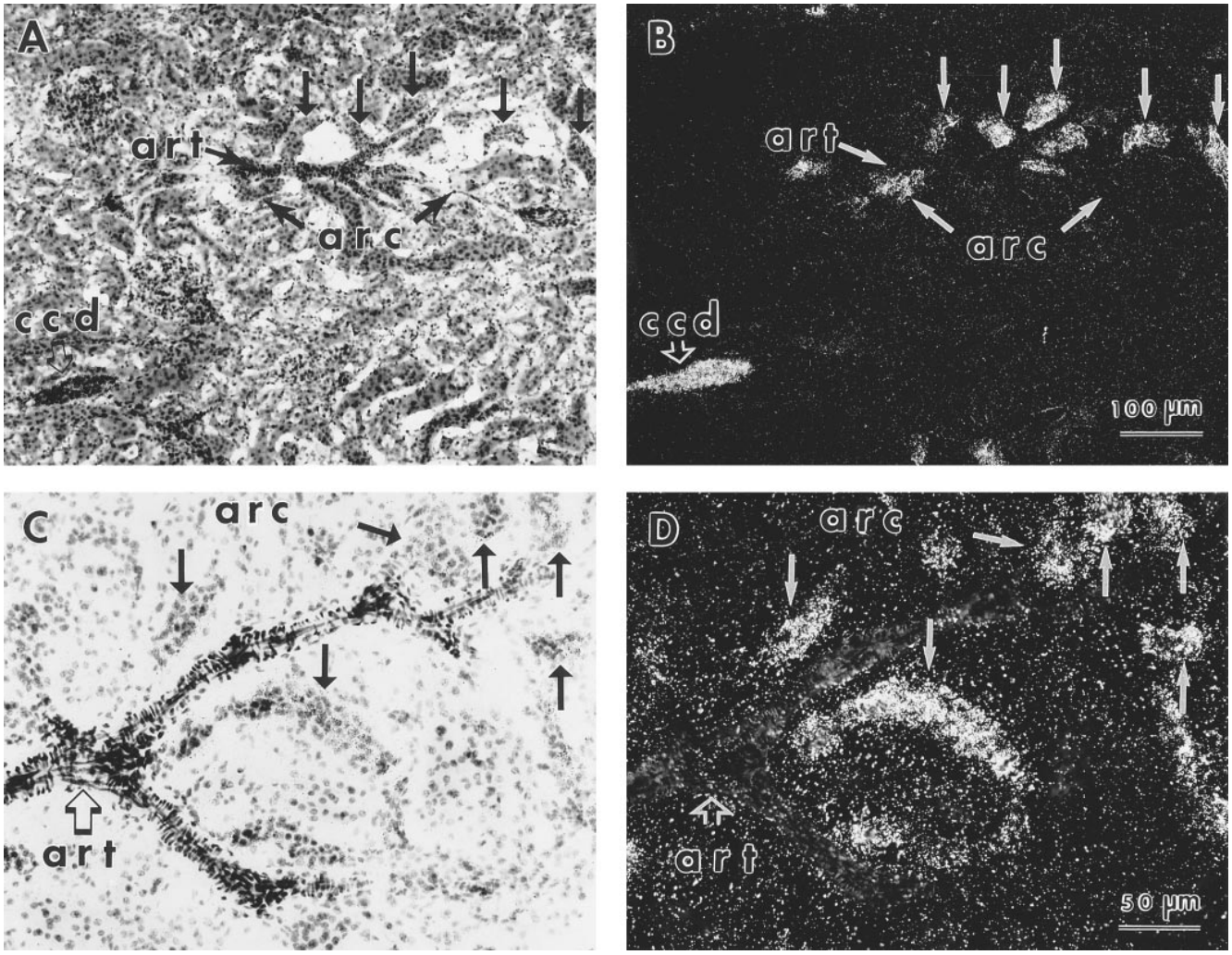

Figure 9. V_2 receptor mRNA labeling in renal cortex by in situ hybridization. (*A*) Bright field photomicrograph showing an arcade (arc) in close proximity to an interlobular artery (art) as it courses toward the renal capsule (at right, not shown), and a segment of cortical collecting duct (ccd). (*B*) the corresponding darkfield image shows V_2 receptor mRNA expression in tubular elements (*arrows*) in close proximity to the interlobular artery (art). Silver grains, which appear white, labeled multiple, short tubular segments in a pattern characteristic of the branches of an arcade (arc). Note the difference in the pattern and density of V_2 mRNA expression in the cortical collecting duct (ccd; bottom left) and the tubular segments in the arcade. (*C*) A higher magnification bright field photomicrograph of branching point of another interlobular artery (*open arrow*) and the associated curved tubular elements (*arrows*) comprising a portion of the arcade (arc). (*D*) the corresponding darkfield image shows the pattern of expression of V_2 mRNA in the curved tubular segments which correspond to branching segments of an arcade.

water restriction (Figs. 5 and 7), a process that we have previously demonstrated in collecting ducts is mediated by changes in circulating vasopressin levels (15). This suggests a regulatory role of vasopressin in the arcades. Consistent with such a role for vasopressin, we have demonstrated that the mRNA that encodes V_2 subtype of vasopressin receptor is expressed in the arcade, a result also consistent with the prior reports of vasopressin-regulated adenylyl cyclase activity in microdissected arcades (25, 26). Vasopressin-regulated water absorption in the arcades can potentially account for the large amounts of free-water absorption proximal to the collecting duct during antidiuresis owing to the very large aggregate epithelial surface area of the arcades which is roughly equivalent to that of the cortical collecting ducts. Furthermore, the propinquity of the arcades to the major blood vessels of the renal cortex, viz. the cortical radial artery and vein (also called "interlobular artery and vein") (7), is likely to provide a means for efficient return of the large amounts of absorbed free water to the general circulation. In the following discussion, we amplify these conclusions and discuss them in the context of the foregoing literature.

Aquaporin-2 is expressed in the renal arcade segment. In this paper, we present extensive evidence that the vasopressin-regulated water channel (aquaporin-2) is expressed in the arcade segment of the rat renal cortical labyrinth. We have applied three independent localization techniques: (*a*) Fluorescence-enhanced ELISA, applied to microdissected arcade and collecting duct segments, demonstrated that aquaporin-2 is nearly as abundant in arcade segments as in cortical collecting ducts, a major site of vasopressin-regulated water absorption (Fig. 4). (*b*) Immunocytochemistry (immunoperoxidase) using our highly specific antibody to aquaporin-2 demonstrates abundant labeling of numerous renal tubule segments present in the cortical labyrinth (Figs. 6 and 7). These segments were

also labeled with antibodies to both kallikrein and the Na^+-Ca^{2+} exchanger, recognized markers for connecting tubule cells, the predominant cell type of the arcades. (*c*) RT-PCR applied to microdissected arcade segments demonstrated the presence of aquaporin-2 mRNA.

What cell type contains aquaporin-2 in the arcades? According to Kaissling and Kriz (7), in the rat ~ 70–80% of cells in the arcade segment are connecting tubule cells, while 20–30% are intercalated cells. They also point out that there are often a few interspersed distal convoluted tubular cells and, near the distal transition of the arcade to the collecting duct, there is some intermingling of collecting duct principal cells (7). Among these cell types, it appears unlikely that intercalated cells contain aquaporin-2 because, throughout the collecting duct system, intercalated cells are devoid of aquaporin-2 (14). Based on our immunocytochemistry, it is clear that numerous segments in the cortical labyrinth contain more than 50% connecting tubule cells, based on abundant labeling with antibodies to two connecting tubule cell markers, viz. kallikrein and the Na^+-Ca^{2+} exchanger (Figs. 6 and 7). Since these tissue sections are from the mid-cortex, most of the labeled segments can be identified as renal arcades. As demonstrated in Fig. 7, a majority of cells in these segments label with the antibody to aquaporin-2. Hence, it appears likely that, within the mid-portion of arcades, connecting tubule cells contain aquaporin-2. Presumably, if principal cells were also present in our microdissected arcades, they could also have contributed to the aquaporin-2 measured in these segments by ELISA. However, since these arcades are dissected primarily from the mid-cortex, they are unlikely to contain a sufficient number of principal cells to account for the high level of aquaporin-2 measured. This conclusion is bolstered by the immunocytochemical observations showing aquaporin-2 in arcades in the mid-cortex in the vicinity of the cortical radial vessels (Fig. 6), rather than in the superficial cortex where most of the initial collecting tubules connect to cortical collecting ducts. Furthermore, in the RT-PCR experiments using specific primers for AQP2, prominent signals from the arcade samples were seen which were comparable in intensity to the signals seen with similar lengths of cortical collecting ducts, segments known to be rich in AQP2 mRNA (Fig. 8). This suggests that a substantial proportion of the cells in arcade segments also express the AQP2 mRNA.

Vasopressin V_2 receptor is expressed in arcades. Both RT-PCR experiments in microdissected segments and in situ hybridization histochemistry unequivocally demonstrated the presence of mRNA coding for vasopressin V_2 receptor in arcades. Morel and associates measured the AVP-dependent adenylate cylcase activity in single pieces of different tubular segments from collagenase treated rabbit kidneys and showed that the connecting tubule and arcade segments (corresponding to the DCTg and CCTg in their terminology, respectively) do not respond significantly to 10^{-6} M AVP (25–27). On the other hand, in response to AVP, the connecting tubule segments (i.e., DCTg) of mouse showed substantial increases in cAMP formation that are comparable to the responses seen in CCD (26, 27). They could not study in detail the CNT segments from rat due to limitations in the microdissection. Nevertheless, studies on a limited number of rat connecting tubule segments showed a 16-fold increase in cAMP formation as compared to a 23-fold increase in cortical collecting tubule (CCT) upon exposure to AVP (cited in 28). Thus, from the studies of Morel and associates there appears to be species differences between rabbits and rodents in the sensitivity of connecting tubule and arcades to AVP. These findings also are consistent with the observations of Imai (29) that the rabbit arcade does not respond to vasopressin with an increase in water permeability (vide infra).

Role of arcades in urinary concentrating process. Despite extensive anatomical and histological characterization, little direct information is available on the role of arcades in urinary concentrating mechanism in rodents. This is because they are not readily accessible by conventional physiological techniques such as micropuncture or in vitro microperfusion. However, Imai (29) succeeded in microperfusing rabbit arcade segments and reported a low basal water permeability that was insensitive to vasopressin exposure. Similar isolated perfused tubule studies have not been possible in rat due to the difficulty of microdissecting arcade segments from noncollagenase digested tissue. Nevertheless, based on the observations limited to rabbit, the view that connecting tubules and arcades are completely unresponsive to vasopressin and thus do not participate in the renal concentrating mechanism, has been widely accepted. However, in the present work using multiple approaches that involve modern molecular techniques we demonstrate that the rat renal arcades may be a heretofore unrecognized site of free water transport during antidiuresis. Absorption of large quantities of free water in the cortex of the concentrating kidney will, in theory, enhance maximal urinary osmolality and water conservation by allowing the water to be absorbed in the cortical labyrinth where blood flow is very rapid. If this large amount of water were absorbed in the medulla or medullary rays, the consequences would be interstitial dilution and impairment of concentrating capacity (30).

The absence of vasopressin-regulated water transport in arcades dissected from rabbits is seemingly at odds with the results reported here. Certainly the most likely explanation for the difference in conclusions lies in possible differences in the distal nephron of rabbits vs. rats. It is well known that rabbits are not capable of concentrating their urine nearly as well as rats. This difference in concentrating capacity could be owing in part to the lack of the ability of the rabbits to absorb free water in the cortical labyrinth. It is also possible that the short tubule segments (< 500 μm) dissected by Imai (29) might not be representative of the entire arcade region in the rabbit. Unfortunately, our antibody to aquaporin-2 does not recognize aquaporin-2 in the rabbit (unpublished observations) so we are unable to test for the presence of aquaporin-2 in the rabbit arcades.

Effect of water deprivation on the expression of AQP2 in arcades. Maximal concentrating capacity in response to acute administration of vasopressin is markedly enhanced when the experimental subject is pre-conditioned by restriction of water intake (31, 32). We have demonstrated that this conditioning phenomenon is due in part to long-term upregulation of water permeability in the collecting duct (33), which in turn is due to a marked elevation in the level of AQP2 expression in the medullary collecting duct (15). The increase in AQP2 expression is thought to be due to a direct action of vasopressin on collecting duct cells (15), most likely by cAMP-mediated transcriptional activation (34, 35). In this paper we extend these observations to demonstrate thirsting-induced upregulation of AQP2 expression in the arcade segment. The presence of a thirsting-induced upregulation of AQP2 in the arcade segment

provides definitive evidence that the increase is not dependent on local changes in interstitial osmolality, which are prevented in the cortical labyrinth by the extremely rapid blood flow there (30).

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References

1. Nielsen, S., C.-L. Chou, D. Marples, E.I. Christensen, B.K. Kishore, and M.A. Knepper. 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc. Natl. Acad. Sci. USA.* 92:1023–1017.

2. Yamamoto, T., S. Sasaki, K. Fushimi, K. Ishibashi, E. Yaoita, K. Kawasaki, F. Marumo, and I. Kihara. 1995. Vasopressin increases AQP-CD water channel in apical membrane of collecting duct cells in Brattleboro rats. *Am. J. Physiol.* 268:C1546–C1551.

3. Sabolic, I., T. Katsura, J.-M. Verbavatz, and D. Brown. 1995. The AQP2 water channel: effect of vasopressin treatment, microtubule disruption, and distribution in neonatal rats. *J. Membrane Biol.* 143:165–175.

4. Wirz, H. 1956. Der osmotische Druck in den corticalin Tubuli der Rattenniere. *Helv. Physiol. Pharmacol. Acta.* 14:353–362.

5. Gottschalk, C.W., and M. Mylle. 1959. Micropuncture study of the mammalian urinary concentrating mechanism: Evidence for the countercurrent hypothesis. *Am. J. Physiol.* 196:927–936.

6. Woodhall, P.B., and C.C. Tisher. 1973. Response of the distal tubule and cortical collecting duct to vasopressin in the rat. *J. Clin. Invest.* 52:3095–3108.

7. Kaissling, B., and W. Kriz. 1992. Morphology of the loop of Henle, distal tubule, and collecting duct. *In* Handbook of Physiology, Section 8: Renal Physiology. Vol I. E. E. Windhager, editor. Oxford University Press, Oxford. 109–167.

8. Lolait, S.J., A.-M. O'Caroll, O.W. McBride, M. Konig, A. Morel, and M.J. Brownstein. 1992. Cloning and characterization of a vasopressin V_2 receptor and possible link to nephrogenic diabetes insipidus. *Nature (Lond.).* 357: 336–339.

9. Birnbaumer, M., A. Seibold, S. Gilbert, M. Ishdo, C. Barberis, A. Anataramian, P. Brabet, and W. Rosenthal. 1992. Molecular cloning of the receptor for human antidiuretic hormone. *Nature (Lond.).* 357:333–335.

10. Fushimi, K., S. Uchida, Y. Hirata, F. Marumo, and S. Sasaki. 1993. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature (Lond.).* 361:549–552.

11. Ecelbarger, C., C.-L. Chou, S.J. Lolait, M.A. Knepper, and S.R. DiGiovanni. 1996. Evidence for dual signalling pathways for V_2 vasopressin receptor in rat inner medullary collecting duct. *Am. J. Physiol.* 270:F623–F633.

12. Maeda, Y., B.L. Smith, P. Agre, and M.A. Knepper. 1995. Quantification of aquaporin-CHIP water channel protein in microdissected renal tubules by fluorescence-based ELISA. *J. Clin. Invest.* 95:422–428.

13. Kishore, B.K., J.M. Terris, and M.A. Knepper. 1996. Quantitation of aquaporin-2 abundance in microdissected collecting ducts: Axial distribution and control by AVP. *Am. J. Physiol.* In press.

14. Nielsen, S., S.R. DiGiovanni, E.I. Christensen, M.A. Knepper, and H.W. Harris. 1993. Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. *Proc. Natl. Acad. Sci. USA.* 90:11663–11667.

15. DiGiovanni, S.R., S. Nielsen, E.I. Christensen, and M.A. Knepper. 1994. Regulation of collecting duct water channel expression by vasopressin in Brattleboro rats. *Proc. Natl. Acad. Sci. USA.* 91:8984–8988.

16. Andrews, P.M., and A.K. Coffey. 1984. A technique to reduce fixation artifacts to kidney proximal tubules. *Kidney Int.* 25:964–968.

17. McLean, I.W., and P.K. Nakane. 1979. Periodate-lysine-paraformaldehyde fixative for immunoelectron microscopy. *Histochem. Cytochem.* 22:1077–1083.

18. Peranen, J., M. Rikkonen, and L. Kaarianen. 1993. A method for exposing hidden antigenic sites in paraformaldehyde-fixed cultured cells, applied to initially unreactive antibodies. *J. Histochem. Cytochem.* 41:447–454.

19. Oza, N.B., V.M. Amin, R.K. McGregor, A.G. Scicli, and O.A. Carretero. 1976. Isolation of rat urinary kallikrein and properties of its antibodies. *Biochem. Pharmacol.* 25:1607–1612.

20. Juhaszova, M., A. Ambesi, G.E. Lindenmayer, R.J. Bloch, and M.P. Blaustein. 1994. Na^+-Ca^{2+} exchanger in arteries: identification by immunoblotting and immunofluorescence microscopy. *Am. J. Physiol.* 266:C234–242.

21. Terada, Y., K. Tomita, H. Nonoguchi, and T. Yang. 1993. Different localization and regulation of two types of vasopressin receptor messenger RNA in microdissected rat nephron segments using reverse transcription polymerase chain reaction. *J. Clin. Invest.* 92: 2339–2345.

22. Ostrowski, N.L., W.S. Young III, M.A. Knepper, and S.L. Lolait. 1993. Expression of vasopressin V1a and V_2 receptor messenger ribonucleic acid in the liver and kidney of embryonic, developing, and adult rats. *Endocrinology.* 133:1849–1859.

23. Ostrowski, N.L., S.J. Lolait, D.J. Bradley, A-M. O'Carroll, M.J. Brownstein, and W.S. Young III. 1992. Distribution of V1a and V_2 vasopressin receptor messenger ribonucleic acids in rat liver, kidney, pituitary and brain. *Endocrinology.* 131:533–535.

24. Knepper, M.A., and F.C. Rector. 1996. Urine concentration and dilution. *In* Brenner and Rector's The Kidney, B.M. Brenner, editor. Vol I, W.B. Saunders Company, Philadelphia, PA. 532–570.

25. Morel, F., D. Chabardès, and M. Imbert. 1976. Functional segmentation of the rabbit distal tubule by microdetermination of hormone-dependent adenylate cyclase activity. *Kidney Int.* 9:264–277.

26. Chabardès, D., M. Imbert-Teboul, M. Gagnan-Brunette, and F. Morel. 1978. Different hormonal target sites along the mouse and rabbit nephrons. *In* Biochemical Nephrology. W.G. Guder and U. Schmidt, editors. Hans Huber Publishers, Bern. 447–454.

27. Imbert-Teboul, M., D. Chabardès, and F. Morel. 1980. Vasopressin and catecholamines sites of action along rabbit, mouse and rat nephron. *Contr. Nephrol.* 21:41–47.

28. Morel, F., and A. Doucet. 1986. Hormonal control of kidney functions at the cell level. *Physiol. Reviews.* 66:377–468.

29. Imai, M. 1979. The connecting tubule: A functional subdivision of the rabbit distal nephron segments. *Kidney Int.* 15:346–356.

30. Knepper, M.A., and M. Burg. 1983. Organization of nephron function. *Am. J. Physiol.* 244:F579–F589.

31. Jones, R.V.H., and H.E. de Wardener. 1956. Urine concentration after fluid deprivation or pitressin tannate in oil. *Br. Med. J.* 1:271–274.

32. Epstein, F.H., C.R. Kleeman, and A. Hendrikx. 1957. The influence of bodily hydration on the renal concentrating process. *J. Clin. Invest.* 36:629–634.

33. Lankford, S.P., C-L. Chou, Y. Terada, S.M. Wall, J.B. Wade, and M.A. Knepper. 1991. Regulation of collecting duct water permeability independent of cAMP-mediated AVP response. *Am. J. Physiol.* 261: F554–F556.

34. Ma, T., H. Hasegawa, W.R. Skach, A. Frigeri, and A.S. Verkman. 1994. Expression, functional analysis, and in situ hybridization of a cloned rat kidney collecting duct water channel. *Am. J. Physiol.* 266:C189–C197.

35. Hayashi, M., S. Sasaki, H. Tsuganezawa, T. Monkawa, W. Kitajima, K. Konishi, K. Fushimi, F. Marumo, and T. Saruta. 1994. Expression and distribution of aquaporin of collecting duct are regulated by vasopressin V_2 receptor in kidney. *J. Clin. Invest.* 94:1778–1783.