Regulation of Aquaporin-2 Trafficking by Vasopressin in the Renal Collecting Duct

ROLES OF RYANODINE-SENSITIVE Ca\(^{2+}\) STORES AND CALMODULIN*

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In the renal collecting duct, vasopressin increases osmotic water permeability (Pf) by triggering trafficking of aquaporin-2 vesicles to the apical plasma membrane. We investigated the role of vasopressin-induced intracellular Ca\(^{2+}\) mobilization in this process. In isolated inner medullary collecting ducts (IMCDs), vasopressin (0.1 nm) and 8-(4-chlorophenylthio)-cAMP (0.1 mM) elicited marked increases in [Ca\(^{2+}\)](fluor-4). Vasopressin-induced Ca\(^{2+}\) mobilization was completely blocked by preloading with the Ca\(^{2+}\) chelator BAPTA. In parallel experiments, BAPTA completely blocked the vasopressin-induced increase in Pf without affecting adenosine 3’,5’-cyclic monophosphate (cAMP) production. Previously, we demonstrated the lack of activation of the phosphoinositide-signaling pathway by vasopressin in IMCD, suggesting an inositol 1,4,5-trisphosphate-independent mechanism of Ca\(^{2+}\) release. Evidence for expression of the type 1 ryanodine receptor (RyR1) in IMCD was obtained by immunofluorescence, immunoblotting, and reverse transcription-polymerase chain reaction. Ryanodine (100 μM), a ryanodine receptor antagonist, blocked the arginine vasopressin-mediated increase in Pf and blocked vasopressin-stimulated redistribution of aquaporin-2 to the plasma membrane domain in primary cultures of IMCD cells, as assessed by immunofluorescence immunocytochemistry. Calmodulin inhibitors (W7 and trifluoperazine) blocked the Pf response to vasopressin and the vasopressin-stimulated redistribution of aquaporin-2. The results suggest that Ca\(^{2+}\) release from ryanodine-sensitive stores plays an essential role in vasopressin-mediated aquaporin-2 trafficking via a calmodulin-dependent mechanism.

Arginine vasopressin (AVP)\(^{1}\) regulates water transport across the epithelium of the renal collecting duct, allowing precise control of water excretion. Water transport across the collecting duct is mediated by molecular water channels, the aquaporins (1, 2). Aquaporin-2 provides the water transport pathway across the apical plasma membrane of the collecting duct principal cells, whereas aquaporins-3 and -4 facilitate water transport across the basolateral plasma membrane. AVP increases the osmotic water permeability (Pf) of the collecting duct cells by triggering translocation of intracellular vesicles containing aquaporin-2 to the apical plasma membrane (3), thus increasing the number of water channels in the rate-limiting barrier for transepithelial water transport. This response depends on the binding of AVP to V2 vasopressin receptors in the basolateral plasma membrane. These receptors couple to the heterotrimeric G protein, Ga, which activates the effector enzyme adenyl cyclase type VI (4) and increases cyclic AMP levels in the cells. Vasopressin, acting via the V2 receptor, also causes a transient increase in intracellular Ca\(^{2+}\) (5–8). Little is known about the mechanism of the vasopressin-induced increase in intracellular Ca\(^{2+}\), although previous studies establish that it occurs in the absence of activation of the phosphoinositide signaling pathway (9). Little is known also about the physiological role of the vasopressin-induced increase in intracellular Ca\(^{2+}\) in the regulation of aquaporin-2 trafficking. However, studies of a wide variety of vesicular-trafficking processes have pointed to a key role for localized increases in intracellular Ca\(^{2+}\) in triggering the fusion of vesicles with their target membranes (10), raising the possibility that the same could be true for aquaporin-2 vesicle trafficking. One calcium-dependent mediator that has been suggested to play a role in water permeability regulation in the vasopressin-responsive toad bladder epithelium is calmodulin (11). Based on recent studies of homotypic fusion of yeast vacuoles, Peters and Mayer conclude that a critical final step in the process of vesicle fusion is dependent on calmodulin (12), and calmodulin actions can be postulated at other steps involved in vasopressin signaling or aquaporin-2 trafficking. In the present study, we investigate the role of intracellular Ca\(^{2+}\) and calmodulin in the AVP-mediated regulation of aquaporin-2 trafficking, assessed through the measurement of osmotic water permeability (Pf) in isolated perfused inner medullary collecting duct (IMCD) segments and through immunofluorescence localization of aquaporin-2 in cultured IMCD cells. The results support the view that stimulation of aquaporin-2 vesicle trafficking to the plasma membrane by AVP requires the AVP-induced rise in intracellular Ca\(^{2+}\) and is dependent on calmodulin. In addition, they show that the rise in intracellular Ca\(^{2+}\) can be induced by an exogenous cyclic AMP analog. Furthermore, the results demonstrate that AVP-stimulated aquaporin-2 trafficking is

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‡ The abbreviations used are: AVP, arginine vasopressin; Pf, osmotic water permeability; IMCD, inner medullary collecting duct; IBMX, 3-isobutyl-1-methylxanthine. BAPTA-AM, 1,2-bis(o-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid, tetraacetoxyethyl ester; CPT-cAMP, 8-(4-chlorophenylthio)-cyclic AMP; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; RT-PCR, reverse transcription-polymerase chain reaction. RyR, ryanodine receptor.
dependent on ryanodine-sensitive Ca\(^2+\) stores and that the type 1 ryanodine receptor (RyR1) is expressed in IMCD cells in a distribution similar to that of aquaporin-2.

**EXPERIMENTAL PROCEDURES**

**Perfusion of Microdissected IMCD Segments**—IMCD segments were dissected from inner medullas of pathogen-free male Sprague-Dawley rats (80–120 g; Taconic Farms, Germantown, NY) by free-hand dissection with Dumont number 5 forceps without enzymatic pretreatment using a physiological perfusate solution containing 120 mM NaCl, 2 mM KH2PO4, 5 mM KCl, 25 mM NaHCO3, 2 mM CaCl2, 1.2 mM MgSO4, 5.5 mM glucose (290 mosmol). The tubes were placed on miniature glass pipettes and perfused in vitro at 37 °C by the method described originally by Burg et al. (13).

**Measurement of Intracellular Ca\(^2+\)**—The intracellular calcium concentration, [Ca\(^{2+}\)], in IMCD cells was determined from the confocal fluorescence images of fluo-4-loaded isolated, perfused IMCD segments using techniques previously described for similar measurements in renal microvessels (14). The IMCDs were incubated with 5 μM fluo-4/AM (Molecular Probes, Eugene, OR) in perfusate solution at room temperature for 15 min. The tubules were washed, and perfused in vitro with the same perfusate solution without fluo-4/AM at 37 °C for another 30 min to allow de-esterification. Changes in fluo-4 fluorescence intensity were monitored in control mouse-to-mouse intraglomerular segments with excitation at 488 nm. Fluo-4 exhibits an increase in green fluorescence (525 nm) upon binding of Ca\(^{2+}\). Confocal fluorescence images were acquired with a Zeiss 40× plan-apochromat objective (numerical aperture 1.2, water immersion) at a zoom factor around 3, which covers the field of 6–8 IMCD cells. Residence time of the laser on the IMCD for each image was 0.4 s. Emitted light was filtered with a bandpass filter (522–535 nm). Images were sampled at 0.5 Hz and stored digitally. The temporal variations of fluo-4 emission were monitored in individual IMCD cells during playback of the stored fluorescence image using the specialized software (Bio-Rad time course/ratiometric software module).

**Osmotic Water Permeability Measurements in Isolated, Perfused IMCD Segments**—To measure osmotic water permeability, an osmotic gradient was created across the epithelium, and the rate of fluid movement was measured. The lumens were perfused with the physiological perfusate solution described above, and the peritubular bath solution was the same as perfuse except that an additional 111 mM NaCl was added to raise the osmolality to 490 mosmol. 1 mM fluorescein sulphonate (Molecular Probes) was added to the luminal perfusate as an impermeant luminal marker that is concentrated when water moves from the lumen to the peritubular bath. Fluorescein sulphonate concentrations in perfusate and collected fluid were measured by continuous-flow fluorometer (15), allowing calculation of transepithelial water flux and osmotic water permeability (Pf) according to the equation of Al-Zaheh et al. (16).

**Cyclic AMP Measurements**—Cyclic AMP production was measured in IMCD segments dissected from collagenase-treated inner medullas as described previously (17). Two-mm lengths of IMCD segments were microdissected for each sample. All measurements were made in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma) to inhibit cyclic nucleotide phosphodiesterases. After a 10-min incubation with 1 mM IBMX, various agents (described in Table I) were added for an additional 5 min, with continued presence of IBMX. The incubations were then terminated by the addition of 10% trichloroacetic acid. Cyclic AMP content of the samples was measured using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

**Immunofluorescence Studies in Primary Cultures of IMCD Cells**—Primary cultures enriched in inner medullary collecting duct cells were prepared as follows. Freshly resected rat kidneys were aseptically transferred into Dulbecco’s modified phosphate buffer, pH 7.4 (Life Technologies, Inc.) supplemented with 50 μM ultra pure urea (Life Technologies, Inc.) and 130 mM NaCl (640 mosmol). The inner medullas were quickly dissected, minced (1–2 mm), and digested in enzyme solution: 50 ml Dulbecco’s modified Eagle’s medium/F12 without phenol red (Life Technologies, Inc.), 100 mg of collagenase B (Roche Molecular Biochemicals), 35 mg of hyaluronidase (Worthington Biochemical, Lakewood, NJ), 450 μl of 10 mM Na2HPO4, 570 mg of NaCl. Tissues were incubated for 30 min at 37 °C with continuous stirring (5% CO2, 95% O2) in perfusate solution at room temperature for 15 min. The tubules were then washed and perfused in prewarmed Dulbecco’s modified Eagle’s medium/F12 medium without enzymes (640 mosmol) three times. The cell pellet was resuspended in 50% Dulbecco’s modified Eagle’s medium low glucose (Irvine Scientific, Santa Ana, CA), 50% Coon’s Improved F12 (Cellgro, Mediatech, Herndon, VA) hypertonic medium (640 mosmol) with urea (80 mmol/liter), and NaCl (130 mmol/liter), 10 mM HEPES, 2 mM t-glutetamine, penicillin G (10,000 units/ml), streptomycin sulfate (10,000 units/ml), 50 μM hydrocortisone, 5 μg 3,3,5-triiodo-L-thyronine, 1 mM sodium selenate, 5 μl 100 marine serum-free hypertonic medium and used for the experiments 48 h later.

Cells in each chamber were fixed with 500 μl of 4% paraformaldehyde in Dulbecco’s modified phosphate buffer for 15 min at room temperature. Cells were washed 3 times (5 min each) with 500 μl of TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) at room temperature and incubated in 5% bovine serum albumin (fraction V, Sigma) in TBST for 1 h. The cells were then incubated with rabbit polyclonal anti-aquaporin-2 (L217, affinity-purified) at an IgG concentration of 0.90 μg/ml in 3% bovine serum albumin in TBST. After washing with TBST (3 times, 5 min each) the cells were incubated for 1 h with 1:200 dilution of goat-antirabbit IgG antibody linked to Alexa 488 (Molecular Probes), counterstained with propidium iodide (1 μg/ml; Molecular Probes) to allow visualization of the nuclei. After incubations, the samples were washed with 0.5% A (0.5 μg/ml; Sigma), then coated with SlowFade anti-fade solution (Molecular Probes) and covered with a coverslip. Cells were examined on the epifluorescence microscope (Olympus, Melville, NY) of a laser-scanning cytomter (Compucyte, Cambridge, MA), and digital images were acquired with a Kodak Digital Science DC 120 zoom digital camera.

**RT-PCR Amplification of Ryanodine Receptor mRNA**—Total RNA samples were extracted from rat tissues by the guanidinium thiocyanate method of Chomczynski and Sacchi (18). Tissues were homogenized in RNAzol B (Tel-Test Inc., Friendswood, TX). RNA was extracted using chloroform, purified by isopropanol precipitation, and washed with 70% ethanol. The RNA pellets were resuspended in Tris/EDTA buffer and stored at −80 °C until used for RT-PCR. In RT-PCR, RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and random hexamer primer to initiate cDNA synthesis for 60 min at 42 °C and 5 min at 95 °C. After completion of RT, the temperature was raised to 95 °C for 5 min to inactivate the enzyme and denature the RNA-DNA hybrids and then lowered to 4 °C. PCR was initiated by adding 50 μl of a mixture containing the PCR buffer, Taq polymerase (PerkinElmer), and RyR2 gene-specific primers (19). The samples were overlaid with mineral oil and processed for 30 cycles (94 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s). At the end of the last cycle, the elongation time at 72 °C was extended to 7 min. 10 μl of each PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide, destained, and photographed.

**Immunoblotting**—To detect ryanodine receptor protein in IMCD cells, we prepared IMCD suspensions as described previously (20). Cytosolic, and detergent fractions by incubation in dissection fluid containing collagenase B and hyaluronidase. One-third of the inner medullary suspension was collected without centrifugation (see Fig. 8, whole IM). The remaining two-thirds of the inner medullary suspension was subjected to three low speed centrifugations (each at 80 × g, 30 s) to enrich IMCD fragments in the pellets (see Fig. 8, IMCD pellet) from the lighter non-IMCD structures in the supernatants (non-IMCD). Samples were homogenized and solubilized in Laemmli buffer (10 mM Tris, pH 6.8, 15% SDS, 6% glycerol, 0.05% bromphenol blue, 40 mM dithiothreitol) before loading for SDS-PAGE. Sarcolasmatic reticulum was isolated from rat skeletal muscle by the method of Saito et al. (21) and solubilized in Laemmli sample buffer.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 4–12% gradient polyacrylamide gels (NOVEX, San Diego, CA) and transferred electrophoretically onto nitrocellulose membranes. Blots were blocked for 30 min with 5% nonfat dry milk in wash buffer (42 mM NaH2PO4, 8 mM Na2HPO4, 150 mM NaCl, and 0.05% Tween 20, pH 7.5), rinsed, and probed with the respective primary antibodies overnight at 4 °C. The primary antibodies were mice monoclonal antibodies to type 1 (Upstate Biotechnology number 0-5269, Lake Placid, NY), type 2 (Upstate Biotechnology number 06-269), and type 3 (Upstate Biotechnology number 06-416) ryanodine receptor, and rabbit polyclonal antibodies to aquaporin-1 (22) and aquaporin-2 (23). The immune complexes were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (1:5000 dilution; Pierce). Sites of antibody-antigen reaction were revealed using enhanced chemiluminescence.
**Fig. 1.** Mean normalized time course of changes in fluo-4 emission intensity measured from single cells of perfused IMCD tubules. A, effect of 0.1 nM AVP on [Ca$^{2+}$]$_i$, of IMCD cells measured from control tubules. $n = 58$ cells/9 IMCDs. B, effect of 0.1 nM AVP on [Ca$^{2+}$]$_i$, of IMCD cells from IMCD tubules preincubated with 50 $\mu$M BAPTA-AM for 30 min. $n = 31$ cells/4 IMCDs. C, effect of 0.1 mM CPT-cAMP on [Ca$^{2+}$]$_i$, measured in control tubules $n = 21$ cells/4 IMCDs. AVP or CPT-cAMP was introduced to the peritubular bath at time = 0. Filled circles represent data point that has normalized fluorescence intensity statistically significantly greater than 1.0. Dotted lines are S.E.

**RESULTS**

**Effect of Arginine Vasopressin on [Ca$^{2+}$]$_i$ and Osmotic Water Permeability**—Previous studies demonstrated that vasopressin increases [Ca$^{2+}$]$_i$ in the IMCD (5–8). Fig. 1A confirms this finding, showing that a physiological concentration of AVP (0.1 nM, Peninsula Laboratories, Belmont, CA) added to the peritubular fluid of isolated perfused IMCD segments transiently increases [Ca$^{2+}$]$_i$. In contrast, IMCDs preincubated for 30 min with BAPTA (Biomol, Plymouth Meeting, PA), an intracellular Ca$^{2+}$ chelator, AVP, caused no significant change in [Ca$^{2+}$]$_i$ (Fig. 1B). Fig. 1C shows that 0.1 mM CPT-cAMP (Research Biochemicals, Natick, MA) also induced an intracellular Ca$^{2+}$ increase.

**Fig. 2.** Effect of intracellular Ca$^{2+}$ chelator BAPTA on AVP (A) or CPT-cAMP (B) stimulated osmotic water permeability ($P_f$) in isolated perfused IMCD segments. Time on the horizontal axis depicts minutes after the perfused tubules were warmed to 37°C for experiments. In BAPTA group (filled circles), IMCDs were treated with 50 $\mu$M BAPTA-AM added to the peritubular bath for 30 min before the addition of 0.1 nM AVP (A, $n = 4$) or 0.1 mM CPT-cAMP (B, $n = 3$). In the control group (open circles), tubules were treated with equal amounts of Me$_2$SO (vehicle for BAPTA-AM) for 30 min before the addition of 0.1 nM AVP or 0.1 mM CPT-cAMP. Data are mean ± S.E. averaged from $n$ tubules. *, statistically significant.

**Effects of Calmodulin Inhibitors on AVP-stimulated $P_f$**—We tested whether AVP-stimulated $P_f$ is dependent on calmodulin by using the calmodulin inhibitors, W7 and trifluoperazine. Fig. 3A shows when 0.1 nM AVP was added first to stimulate $P_f$, subsequent addition of W7 caused a rapid decrease in $P_f$ to the basal level. Similarly, we found that trifluoperazine (30 $\mu$M;
The presence of antagonist and then a 5-min incubation with AVP in the peritubular bath, whereas upon washing W7 out of the peritubular bath, a rapid and continued presence of IBMX). In the antagonist-treated group, IMCDs were preincubated with antagonist (BAPTA-AM or ryanodine) for 20 min followed by another 10-min incubation with 1 mM IBMX in the presence of antagonist and then a 5-min incubation with AVP in the presence of antagonist and IBMX.

### Table I

|               | cAMP production, fmol/mm length |
|---------------|---------------------------------|
|               | n  | Mean ± S.E. | P    |
| Control group |    |             |      |
| Basal         | 7  | 4.0 ± 0.5   |      |
| 0.1 nM AVP    | 9  | 12.7 ± 1.1  | <0.0001 |
| 100 μM AVP    | 5  | 48.9 ± 10.7 | <0.0001 |
| BAPTA-treated IMCD |  |           |      |
| Basal         | 6  | 5.3 ± 0.6   |      |
| 0.1 nM AVP    | 6  | 14.5 ± 2.1  | <0.001 |
| Ryanoanide-treated IMCD |  |           |      |
| Basal         | 4  | 6.3 ± 0.7   |      |
| 0.1 nM AVP    | 4  | 12.3 ± 2.2  | <0.05  |

![Fig. 3. Effect of calmodulin inhibitor (W7) on AVP-stimulated osmotic water permeability (P).
](image)

Calbiochem), inhibited CPT-cAMP-stimulated $P_f$ (data not shown). Fig. 3B shows the results that when IMCD segments were pretreated with 25 μM W7 for 20 min before the addition of 0.1 nM AVP, $P_f$ was not significantly increased by AVP, whereas upon washing W7 out of the peritubular bath, a rapid increase in $P_f$ was seen. The inhibitory effect of W7 on $P_f$ presumably represents an effect on aquaporin-2 trafficking in the IMCD cells. To address this directly, we tested the effect of W7 on AVP-induced aquaporin-2 trafficking using immunofluorescence in primary cultures of IMCD cells (Fig. 4). As shown by a comparison of Fig. 4A (no AVP) with Fig. 4B (AVP alone), AVP induced a redistribution of aquaporin-2-labeling to the periphery of the cells, consistent with the previously demonstrated AVP-induced trafficking to the plasma membrane (3).

When the calmodulin inhibitor W7 was added in addition to AVP (Fig. 4C), aquaporin-2-labeling shifted into a cytoplasmic localization, with the greatest labeling in the perinuclear region. Therefore, these results suggest that Ca$^{2+}$/calmodulin plays a critical role in the action of AVP to increase $P_f$ in IMCD cells by regulation of aquaporin-2 trafficking to or from the plasma membrane. Fig. 4D shows the lack of effect of W7 alone (compare with 4A). When AVP was added with W7 after exposure to W7 alone (Fig. 4E), it did not trigger a redistribution of aquaporin-2 to the cell surface (compare with Fig. 4B). However, washout of W7 without washout of AVP resulted in a redistribution of aquaporin-2 to the cell periphery (Fig. 4F), similar to that seen in Fig. 4B. Thus, we conclude that the effect of calmodulin inhibitors on water permeability is due to a reversible inhibition of aquaporin-2 trafficking to the cell periphery.

### Role of Extracellular Ca$^{2+}$ and Ryanodine-sensitive Ca$^{2+}$ Stores in the Action of Vasopressin—As shown in Fig. 5A, with a nominally zero extracellular Ca$^{2+}$ concentration, 0.1 nM AVP increased $P_f$ from 38 ± 4 to 381 ± 42 μl/s (n = 4, p < 0.001). We conclude from this that the water permeability response to AVP does not depend critically on extracellular Ca$^{2+}$ and that the Ca$^{2+}$ required for the AVP-simulated $P_f$ increase is likely to originate from intracellular stores. Previously, we demonstrated a lack of effect of AVP on phosphoinositide hydrolysis in IMCD cell suspensions (9), suggesting that AVP-induced Ca$^{2+}$ mobilization in the IMCD is not due to Ca$^{2+}$ release from ryanodine-sensitive Ca$^{2+}$ channels in AVP-stimulated P. Fig. 5B shows that in the presence of 100 μM ryanodine (and in the absence of extracellular Ca$^{2+}$), 0.1 nM AVP did not significantly increase the $P_f$ of IMCD segments (compare $P_f$ response in control tubules in Fig. 5A). Similar results were obtained with another antagonist, procaine (1–0.1 mM; Sigma) (data not shown). The lack of a $P_f$ response in ryanodine-treated tubules was not due to impairment of cAMP production by ryanodine treatment (Table I). In additional studies, caffeine (10 mM; Calbiochem), a ryanodine receptor agonist, was found to increase intracellular calcium concentration as measured by fluo-4 fluorescence (data not shown).

Fig. 6 confirms the data in Fig. 4 showing that vasopressin stimulates trafficking of aquaporin-2 in primary cultures of IMCD cells from the cytoplasm (Fig. 6A) to the cell surface (Fig. 6B) and shows that ryanodine blocks the vasopressin-induced aquaporin-2 trafficking (Fig. 6D). Furthermore, ryanodine had an AVP-independent effect on aquaporin-2 localization (Fig. 6C) as compared with the control cells (Fig. 6A), suggesting that calcium release from ryanodine-sensitive stores may provide calcium required for base-line aquaporin-2 trafficking in the unstimulated state.

### RT-PCR and Immunohistochemical Detection of RyR1 in Inner Medulla—RT-PCR experiments were carried out in total RNA samples from renal cortex, outer medulla, and inner medulla to assess which ryanodine receptor isoforms are expressed. As shown in Fig. 7, both RyR1 and RyR2 are expressed in all three major regions of the kidney. However, RyR3 was undetectable in kidney but present in brain total RNA.

To investigate whether ryanodine receptor protein is present in IMCD, we performed immunohistochemical experiments (Fig. 8) and immunofluorescence localization (Fig. 9) using anti-ryanodine receptor antibodies. Fig. 8A shows immunoblot with a monoclonal anti-RyR1 antibody in whole inner medulla and in two inner medullary cell fractions obtained by low speed cen-
Regulation of Aquaporin-2 Trafficking by Vasopressin

Intracellular calcium mobilization is known to play a critical role in triggering exocytosis in neurotransmitter release from the presynaptic region of axons (25), in catecholamine release from adrenal chromaffin cells (26), and in insulin release from pancreatic β cells (27). Vasopressin-stimulated calcium release from the sarcoplasmic reticulum is known to be mediated by the ryanodine receptor (RyR1), which is a calcium-release channel sensitive to ryanodine (3). In inner medullary collecting duct (IMCD) cells, the RyR1 has been shown to be localized to the collecting duct (Fig. 8A), measured by immunoblotting in Fig. 8B, confirming the presence of immunoreactive RyR3 protein in brain while yielding no evidence for RyR3 in IMCD.

Fig. 9 shows immunofluorescence localization of type 1 ryanodine receptor in inner medulla of rat kidney. The type 1 ryanodine receptor was localized to the collecting duct (Fig. 9A), i.e. in the same cells as the aquaporin-2 water channel (Fig. 9B), although type 1 ryanodine receptor labeling also appears in thin limb of Henle in inner medulla. Fig. 9C shows that the RyR1 receptor labeling was ablated with preadsorption of the antibody with sarcoplasmic reticulum membranes.

**DISCUSSION**

In the renal collecting duct, vasopressin increases water permeability by increasing the number of aquaporin-2 water channels in the apical plasma membrane via regulated exocytosis of aquaporin-2 vesicles (3). This process depends on a rise in intracellular Ca²⁺, although the mechanism by which cyclic AMP triggers exocytosis is not well understood. It has long been recognized that AVP, acting through the V₂ receptor, increases [Ca²⁺] in the IMCD (5–8). However, the role of this AVP-induced Ca²⁺ increase in aquaporin-2 trafficking has not been investigated until now. In the present study, we provide evidence that the AVP-induced Ca²⁺ increase is necessary for the water permeability response. Furthermore, we have demonstrated that vasopressin-induced trafficking of aquaporin-2 to the cell surface is dependent on calmodulin, suggesting that the role of calcium could be through calmodulin activation. The results also implicate ryanodine-sensitive calcium stores in aquaporin-2 trafficking and demonstrate the presence of type 1 ryanodine receptors in inner medullary collecting duct cells.

The fact that the AVP-induced Ca²⁺ increase is mimicked by a cyclic AMP analogue suggests that the calcium release may be triggered by cyclic AMP, possibly acting through protein kinase A. In the remainder of this discussion we analyze these conclusions from the perspective of the foregoing literature.

A role for intracellular calcium mobilization in regulated exocytosis has been previously established in a variety of tissues. For example, increases in intracellular Ca²⁺ are known to play a critical role in triggering exocytosis in neurotransmitter release from the presynaptic region of axons (25), in catecholamine release from adrenal chromaffin cells (26), and in insulin release from pancreatic β cells (27). Vasopressin-stimulated calcium release from the sarcoplasmic reticulum is known to be mediated by the ryanodine receptor (RyR1), which is a calcium-release channel sensitive to ryanodine (3). In IMCD cells, the RyR1 has been shown to be localized to the collecting duct (Fig. 8A), measured by immunoblotting in Fig. 8B, confirming the presence of immunoreactive RyR3 protein in brain while yielding no evidence for RyR3 in IMCD.

**Fig. 4.** Effect of calmodulin inhibitor (W7) on AVP-induced aquaporin-2 trafficking in primary cultures of IMCD cells. Cells were counterstained with propidium iodide (red, nuclear labeling). Aquaporin-2-labeling is shown in green. A, control cells. B, cells after 30 min of incubation with 1 nM AVP. C, cells after 30 min of incubation of 1 nM AVP followed by another 30 min of incubation of 50 μM W7 in the continued presence of 1 nM AVP. D, cells treated with 50 μM W7 only. E, cells treated with 50 μM W7 for 30 min followed by 1 nM AVP for 30 min with the continued presence of W7. F, cells treated with 50 μM W7 for 30 min followed by 30 min with W7 plus 1 nM AVP followed by 30 min with 1 nM AVP alone.

**Fig. 5.** A, AVP-stimulated osmotic water permeability (P₁) measured at zero extracellular Ca²⁺ conditions. IMCD tubules were dissected in perfusate solution containing 2 mM CaCl₂ as described under “Experimental Procedures.” After cannulation, each tubule was perfused through its lumen with a perfusate solution containing zero added CaCl₂ and 2 mM EGTA throughout the experiment. The tubules were bathed initially in a bath solution containing 2 mM CaCl₂ (40 min), allowing IMCD equilibration at 37 °C after microdissection. The bath solution was then changed to solution containing zero added CaCl₂ and 2 mM EGTA to achieve the zero extracellular Ca²⁺ condition. The tubules were incubated in Ca²⁺-free solutions for 30 min before the addition of 0.1 nM AVP. n = 4. B, effect of ryanodine-sensitive Ca²⁺ channel antagonist, ryanodine, on AVP-stimulated P₁. IMCD tubules were perfused in zero extracellular Ca²⁺ conditions as described above except that 100 μM ryanodine was added to the peritubular bath solution 30 min before the addition of 0.1 nM AVP. n = 6.
regulated exocytosis of aquaporin-2-containing vesicles in collecting duct cells is generally recognized to be dependent on increases in intracellular cyclic AMP (2), and recent studies indicate that the trafficking is dependent on protein kinase A-mediated phosphorylation of the aquaporin-2 channel itself (28). However, the present findings point to a critical role for calcium as an intracellular mediator of the vasopressin-induced water permeability response. Specifically, we showed that buffering of intracellular calcium levels with BAPTA or the addition of calmodulin inhibitors can completely block the water permeability response to vasopressin. Thus, we conclude that vasopressin-mediated trafficking of aquaporin-2 is dependent both on increases in intracellular cyclic AMP and increases in intracellular calcium. It should be emphasized that the observed rise in intracellular Ca\(^{2+}\) occurred in response to a physiological level of vasopressin, 0.1 nM, a level that elicits a half-maximal increase in cyclic AMP production in the IMCD (20).

The finding in the present study that calmodulin inhibitors block the water permeability response to vasopressin in the IMCD was predated by similar findings in the toad bladder, a vasopressin-responsive collecting duct analogue (11), and in rabbit cortical collecting duct (29). The availability of antibodies to aquaporin-2 have now allowed us to demonstrate in primary cultures of IMCD cells that the blockade of the water permeability response is associated with a failure of aquaporin-2 to redistribute to the cell periphery in response to vasopressin. Calmodulin is a ubiquitous 17-kDa protein that is involved in a host of regulatory processes including activation of calmodulin-dependent protein kinases, activation of myosin light chain kinase, regulation of type 1 cyclic nucleotide phosphatase, regulation of calcineurin and other protein phosphatases, and stimulation of types I, III, and VIII adenyl cyclase (30). Because calmodulin has so many regulatory targets, it would be fruitless to speculate extensively at this point regarding the specific role it plays in aquaporin-2 trafficking. However, some clues arise from the immunofluorescence localization of aquaporin-2 after treatment with calmodulin inhibitors. Aquaporin-2 was redistributed to a perinuclear location (Fig. 4C). In a previous study in aquaporin-2-transfected LLC-PK1 cells, a similar perinuclear localization of aquaporin-2 was seen in response to reduced temperature or treatment with the vacuolar protein pump inhibitor bafilomycin (31). The authors concluded that aquaporin-2 recycles continuously between the trans-Golgi network and the plasma membrane and that the effect of vasopressin is to alter the steady-state rates of exocytic and endocytic translocation between these compartments in favor of increased aquaporin-2 in the plasma membrane. The effect of low temperature, bafilomycin, and putatively calmodulin inhibitors would be to decrease markedly the exocytic translocation from the trans-Golgi network to the plasma membrane or (less likely) to markedly accelerate the endocytic rate. Assuming the former, it would appear that the block is not at a late step in this translocation process, e.g. at the level of docking and fusion of the aquaporin-2 vesicles with the plasma membrane, which would be expected to arrest the aquaporin-2 vesicles in the vicinity of the plasma membrane. Rather, the

**Fig. 6. Effect of ryanodine on AVP-induced aquaporin-2 localization in primary cultures of IMCD.** To mimic the experimental condition in Fig. 5, the culture medium was changed to zero Ca\(^{2+}\) 30 min before the experiment. Cells were counterstained with propidium iodide (red nuclear labeling). Aquaporin-2 labeling is shown in green. A, control cells. B, cells after a 30-min incubation with 1 nM AVP. C, cells after a 30-min incubation with 100 \(\mu M\) ryanodine alone. D, cells with a 30-min incubation with 100 \(\mu M\) ryanodine followed by another 30-min incubation with 1 nM AVP in the continued presence of 100 \(\mu M\) ryanodine.

**Fig. 7. RT-PCR detection of RyR mRNA in rat kidney.** RNA was reverse-transcribed using random hexamer primers, and the cDNA was amplified by PCR for 30 cycles. For kidney samples, 4 \(\mu g\) of total RNA was loaded in each individual PCR tube; for skeletal muscle, heart, and brain, 2 \(\mu g\) of total RNA was loaded in individual tubes. Previously published primer sets for all three RyR receptors were used in PCR (19). Amplified fragments corresponding to PCR target regions of RyR1 (435 bp), RyR2 (635 bp), but not RyR3 (505 bp), are found in all three major regions of the kidney. In control experiments, RT-PCR products without reverse transcriptase produced no bands (not shown).
Regulation of Aquaporin-2 Trafficking by Vasopressin

blockade is likely to involve an early step such as vesicle budding from the trans-Golgi or cytoskeleton-dependent translocation of aquaporin-2-bearing vesicles toward the plasma membrane. Previous studies have demonstrated a role for microtubules in the vasopressin-induced water permeability increase (32).

A previous study from our laboratory demonstrated that, although the muscarinic agonist carbachol markedly stimulated inositol 1,4,5-trisphosphate production in the inner medullary collecting duct, vasopressin did not have such an effect (9). These findings suggested that vasopressin (at physiological concentrations) does not activate the phosphoinositide-signaling pathway in the IMCD. This result led us to conclude that it is unlikely that vasopressin-induced Ca$^{2+}$ mobilization in the IMCD is mediated by inositol 1,4,5-trisphosphate receptors. The results of the present study point instead to a likely role for ryanodine receptors. RT-PCR studies demonstrated the presence of mRNA for both type 1 and type 2 ryanodine receptors in the renal inner medulla. However, antibody localization studies suggested that type 1 ryanodine receptor, characteristic of skeletal muscle, is expressed in IMCD cells. Measurements using the intracellular Ca$^{2+}$ indicator fluo-4 demonstrated that caffeine, a ryanodine receptor agonist, induced a rapid increase in intracellular calcium. A physiological role for the collecting duct ryanodine receptor was suggested both by water permeability measurements and aquaporin-2 immunofluorescence in cultured IMCD cells. These studies support the view that ryanodine blocks the ability of vasopressin to stimulate aquaporin-2 trafficking to the plasma membrane.

A direct physiological agonist for RyR-mediated Ca$^{2+}$ release has not been identified in the present studies. In cardiac and skeletal muscle cells, the ryanodine receptors mediate Ca$^{2+}$-
induced Ca\(^{2+}\) release in which Ca\(^{2+}\) ions activate the Ca\(^{2+}\) conductance (33). Other regulators act to alter the sensitivity of ryanodine receptors to calcium. Such mediators include cyclic adenosine 5’-diphosphate ribose (cADPR-ribose) and nicotinic acid adenine dinucleotide phosphate (NAADP\(^{+}\)), a metabolite of NADP (34). However, a direct effect of AVP on intracellular cADP-ribose or NAADP\(^{+}\) level has not been documented in these cells. Another possibility is that cyclic AMP itself could regulate the sensitivity of RyR1 in IMCD cell to calcium, possibly via the action of protein kinase A. This possibility has support from several previous studies. For example, cAMP, which functions as an intracellular messenger stimulating salivary amylase secretion in rat parotid gland acinar cells, was reported to induce a ryanodine-sensitive Ca\(^{2+}\) release that could be inhibited by a protein kinase A inhibitor (35). Similarly in glucose-stimulated pancreatic β cells, the effect of caffeine on calcium release via ryanodine receptors was enhanced by forskolin, an activator of adenylyl cyclase (36). Also in pancreatic β cells, glucagon-like peptide-1 was found to induce a protein kinase A-dependent sensitization of ryanodine receptors (37). Sensitization of ryanodine receptors by cyclic AMP has also been reported in HEK293 cells (38).

Ryanodine receptors are intracellular Ca\(^{2+}\) release channel proteins, which exist as tetrameric complexes of large polypeptide monomers (approximately 5000 amino acid residues) (39). To date, different ryanodine receptor isoforms have been identified, mainly in the excitable cells. Type 1 is expressed predominantly in skeletal muscle cells, type 2 is expressed predominantly in cardiac muscle cells, and type 3 is expressed predominantly in brain. In rabbit kidney cortex and in the rabbit kidney epithelial cell line LLC-RK1, Tunwell and Lai (40) detected both mRNA and protein of type 2, but not type 1, ryanodine receptors. In human embryonic kidney cells (HEK293 cells), Querfurth et al. (41) demonstrate the expression of both type 1 and type 2, but not type 3 ryanodine receptor mRNA. However, there were no data prior to this study regarding the expression of ryanodine receptors in rat kidney or in individual renal tubule segments. Our RT-PCR experiments have demonstrated the presence of mRNA for both RyR1 (the skeletal muscle isoform) and RyR2 (the cardiac muscle isoform) in the renal inner medulla. The use of monoclonal antibodies to these two receptors in immunoblotting experiments indicates the presence of RyR1 but not RyR2 in inner medullary collecting duct cells (Fig. 7). Immunocytochemistry using double-labeling with the RyR1 antibody and an antibody to aquaporin-2 indicates that RyR1 is distributed in the IMCD cell cytoplasm predominantly in the apical region of the cells.

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