Vasopressin Increases Plasma Membrane Accumulation of Urea Transporter UT-A1 in Rat Inner Medullary Collecting Ducts

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Urea transport, mediated by the urea transporter A1 (UT-A1) and/or UT-A3, is important for the production of concentrated urine. Vasopressin rapidly increases urea transport in rat terminal inner medullary collecting ducts (IMCD). A previous study showed that one mechanism for rapid regulation of urea transport is a vasopressin-induced increase in UT-A1 phosphorylation. This study tests whether vasopressin or directly activating adenylyl cyclase with forskolin also increases UT-A1 accumulation in the plasma membrane of rat IMCD. Inner medullas were harvested from rats 45 min after injection with vasopressin or vehicle. UT-A1 abundance in the plasma membrane was significantly increased in the membrane fraction after differential centrifugation and in the biotinylated protein population. Vasopressin and forskolin each increased the amount of biotinylated UT-A1 in rat IMCD suspensions that were treated ex vivo. The observed changes in the plasma membrane are specific, as the amount of biotinylated UT-A1 but not the calcium-sensing receptor was increased by forskolin. Next, whether forskolin or the V2-selective agonist dDAVP would increase apical membrane expression of UT-A1 in MDCK cells that were stably transfected with UT-A1 (UT-A1-MDCK cells) was tested. Forskolin and dDAVP significantly increased UT-A1 abundance in the apical membrane in UT-A1-MDCK cells. It is concluded that vasopressin and forskolin increase UT-A1 accumulation in the plasma membrane in rat IMCD and in the apical plasma membrane of UT-A1-MDCK cells. These findings suggest that vasopressin regulates urea transport by increasing UT-A1 accumulation in the plasma membrane and/or UT-A1 phosphorylation.


Urea plays a critical role in the urinary concentrating mechanism and hence in the regulation of water homeostasis. Urea's importance to the generation of concentrated urine has been appreciated since at least 1934 (1). Several studies have shown that maximal urine-concentrating ability is decreased in protein-deprived animals and humans and is restored by urea infusion (reviewed in references [2,3]). Urea is transported by the products of mammalian genes: UT-A (Slc14a2) and UT-B (Slc14a1). The UT-A family of urea transporters currently consists of six protein isoforms, four of which (UT-A1, UT-A2, UT-A3, and UT-A4) are expressed in kidney medulla (reviewed in references [2,3]). UT-A1 is the largest protein and is expressed in the apical plasma membrane of the inner medullary collecting duct (IMCD) (4–6). UT-A3 is also expressed in the IMCD (reviewed in references [2,3]). A UT-A1/UT-A3 knockout mouse (7,8) lacks urea transport across its IMCD and has a severe urine-concentrating defect, indicating that urea transport, mediated by UT-A1 and/or UT-A3, is important for the production of concentrated urine.

Adding arginine vasopressin (AVP; also known as antidiuretic hormone) to the bath of a perfused rat terminal IMCD results in binding to V2 receptors, stimulating adenylyl cyclase and cAMP production and increasing urea transport (9–12). We showed that one mechanism for rapid regulation is that vasopressin increases UT-A1 phosphorylation (13). Vasopressin (and forskolin) also increases urea flux and UT-A1 phosphorylation in MDCK cells that we stably transfected with UT-A1 (UT-A1-MDCK cells) (14).

Another mechanism by which vasopressin could rapidly increase urea transport is by increasing UT-A1 accumulation in the apical plasma membrane. Vasopressin-regulated trafficking of aquaporin-2 (AQP2) between subapical vesicles and the apical plasma membrane is the major mechanism for acute regulation of water absorption by vasopressin in the collecting duct (reviewed in reference [15]). One previous study tested whether vasopressin regulates the trafficking of UT-A1 in the rat IMCD but did not find evidence for regulated trafficking in Brattleboro rats, which lack endogenous vasopressin (16). The collecting duct of Brattleboro rats is chronically conditioned (17) and may not be representative of collecting duct of normal rats. In this study, we reexamined this question by studying normal (basal), vasopressin-replete Sprague-Dawley rats. We found...
that exogenous vasopressin does increase UT-A1 accumulation in the plasma membrane of the IMCD from basal rats. In addition, forskolin, which directly activates the catalytic subunit of adenylcyclase (18), increases UT-A1 accumulation in the plasma membrane of the IMCD from basal rats and from rats that were made water diuretic for 2 wk and in our stably transfected UT-A1-MDCK cells (14).

Materials and Methods

Animal Preparation

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) that weighed 125 to 200 g received free access to water and standard rat chow (Test Diet 5001; Purina Mills, Brentwood, MO) that contained 23% protein. Some rats were made water diuretic for 2 wk by feeding them 10% glucose water (19).

Basal or water diuretic rats were given a misoscapular subcutaneous injection of vasopressin (5 nmol in 100 μl of normal saline; American Regent, Shirley, NY) or vehicle, then after 45 min were killed by decapsulation. The kidneys were removed and placed on ice immediately, and the inner medullas were separated. The inner medullary tissue was placed into ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose [pH 7.6], 1 μg/ml leupeptin, and 2 mg/ml PMSF) and homogenized, then SDS was added to a final concentration of 1% for Western analysis of the total cell lysate (19–21). Total protein in each sample was measured by a modified Lowry method (DC protein assay reagent; Bio-Rad, Richmond, CA). In some cases, inner medullary tissue lysate was subjected to differential centrifugation to obtain a 17,000 × g (low-speed, high-density) plasma membrane fraction and a 200,000 × g (high-speed, low-density) intracellular vesicle fraction as described previously (16,22–26).

Fresh suspensions of rat IMCD (from rats that were not pretreated with vasopressin) were prepared as described previously (13,27). Briefly, rat inner medullas were minced in 1 ml of suspension buffer (in mM: 118 NaCl, 5 KCl, 25 NaHCO3, 1.2 MgSO4, 2 CaCl2, 5.5 glucose, 5 Na-acetate, and 4 Na2PO4) that contained 2 mg/ml collagenase and 0.65 mg/ml hyaluronidase, then incubated for 30 min at 37°C. Next, DNase (5 μl of 1 mg/ml) was added and incubated for another 30 min. Finally, the IMCD were washed free of enzymes by centrifugation three times with exchanges of suspension buffer, then resuspended in 1 ml of this buffer. Rat IMCD suspensions were pretreated with 10 mM vasopressin or 10 μM forskolin for 15 min at 37°C, then vasopressin or forskolin was left resident during the biotinylation incubation (described next).

UT-A1-MDCK Cells

Construction and selection of the UT-A1-MDCK cells were described previously (14). We plated 2 × 105 cells per Costar Transwell insert (Corning, Acton, MA; 1 cm2 growth surface area) and studied confluent high-resistance (>800 Ω cm2) polarized epithelial monolayers 4 d later. Transepithelial resistance was measured daily using an epithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL). Insets in which there was not an orderly increase in the transepithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL). In-transmembrane resistance was measured daily using an epithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL).

Transmembrane resistance was measured daily using an epithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL). In-transmembrane resistance was measured daily using an epithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL).

Briefly, rat inner medullary tissue, IMCD suspensions, or UT-A1-MDCK cells were homogenized in ice-cold isolation buffer and brought to 1% SDS for Western analysis of total cell lysate (25,29,32). Proteins were size-separated by SDS-PAGE on Laemmli gels, electrophobted to polyvinylidene difluoride membranes, and incubated with primary antibody overnight at 4°C. After washing, the blot was incubated with fluorescently labeled secondary antibody and visualized with the Odyssey infrared imaging scanner. Parallel gels were stained with Coomassie blue to verify uniformity of gel loading (LICOR, Lincoln, NE). Laser densitometry was used to quantify the intensity of the resulting bands on Western blot. Antibodies to anti–UT-A1 and anti-AQP2 were prepared by our laboratory; anti–calcium-sensing receptor (33,34) was a gift of Dr. Tyler Miller (Case Western Reserve University, Cleveland, OH).

Biotinylation of UT-A1

Rat inner medullary tissue, IMCD suspensions, or UT-A1-MDCK cells were biotinylated using a modification of the method described previously (16). Suspensions were prepared by enzyme digest as described previously (13). In the studies in which the rat received a subcutaneous injection of vasopressin, the inner medullary tissue was not treated with vasopressin ex vivo. In the studies of fresh suspensions of rat IMCD (from rats that were not pretreated with vasopressin) that were subsequently treated ex vivo or in the UT-A1-MDCK cell studies, the treatments (vasopressin, forskolin, or dDAVP) were added for 30 min at 37°C, then samples were washed free of excess solution twice with PBS and three times with biotinylation buffer without biotin (215 mM NaCl, 4 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 5.5 mM glucose, 10 mM triethanolamine, and 2.5 mM Na3HPO4). Treatments were added back during the incubation with biotinylation buffer that contained 3 mg/ml bovine serum albumin and 2 mg/ml leupeptin in PBS with 1 mM dithiothreitol.

UT-A1-MDCK cells were homogenized in ice-cold isolation buffer and brought to 1% SDS for Western analysis of total cell lysate (25,29,32). Proteins were size-separated by SDS-PAGE on Laemmli gels, electrophobted to polyvinylidene difluoride membranes, and incubated with primary antibody overnight at 4°C. After washing, the blot was incubated with fluorescently labeled secondary antibody and visualized with the Odyssey infrared imaging scanner. Parallel gels were stained with Coomassie blue to verify uniformity of gel loading (LICOR, Lincoln, NE). Laser densitometry was used to quantify the intensity of the resulting bands on Western blot. Antibodies to anti–UT-A1 and anti-AQP2 were prepared by our laboratory; anti–calcium-sensing receptor (33,34) was a gift of Dr. Tyler Miller (Case Western Reserve University, Cleveland, OH).

Statistical Analyses

All data are presented as mean ± SEM. To test for statistical significance between two groups, we used a t test. To test for statistical significance among three or more groups, we used ANOVA followed by Fisher least significant difference. The criterion for statistical significance was P < 0.05.
Vasopressin Increases Plasma Membrane Expression of UT-A1 in Rat Inner Medulla

Vasopressin, administered to the rat, significantly increases UT-A1 protein abundance (densitometry in arbitrary units) in a membrane fraction that is enriched in plasma membranes (22,23,35) from basal rats (from 22 ± 1 to 26 ± 2; n = 4, P < 0.03; Figure 2). Vasopressin did not change UT-A1 abundance in a membrane fraction that is enriched in plasma membranes from rats that were made water diuretic for 2 wk (hydrated) to suppress endogenous vasopressin levels (from membranes from rats that were made water diuretic for 2 wk

Vasopressin significantly increases UT-A1 biotinylation (densitometry in arbitrary units) in inner medullas from basal rats (62 ± 3 to 71 ± 4; n = 5; P < 0.05; Figure 3). Figure 3 also shows that in the pool of biotinylated proteins from these cells, our antibody detects only bands that are consistent with the size of biotinylated UT-A1 (36).

Results

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Figure 1. Confocal micrographs of biotinylated proteins that were probed with Alexa 488–streptavidin. Isolated tubules (A and B) or UT-A1 MDCK cells that were grown on semipermeable supports (C and D) were biotinylated as described in the Materials and Methods section, then fixed with paraformaldehyde, permeabilized with NP40, labeled with fluorescence-tagged streptavidin (light), mounted on glass slides, and visualized with a Zeiss LSM 510 confocal microscope using a ×63 oil objective. Z stacks were collected. Shown are various cut planes from these stacks as follows: Isolated tubule xz sliced with luminal stain apparent (A), center slice 50% into the Z plane illustrating staining both apical and basolateral but with no staining inside the cell (B), UT-A1 MDCK cells at 33% Z cut from the top showing no evidence of interior biotin (C), and UT-A1-MDCK cells XZ cut plane view (D). Arrows mark the location of the supporting semipermeable membrane.

Figure 2. Acute vasopressin (AVP) administration increases UT-A1 abundance in the membrane fraction of rat inner medullas. Rats were given AVP (5 nmol subcutaneously) 45 minutes before being killed; inner medullary membranes were isolated and probed with anti–UT-A1. Left four lanes show UT-A1 in inner medullas from basal rats (duplicate samples shown). Right four lanes show UT-A1 in the inner medulla membranes from diuretic rats (water loaded for 2 wk). Arrows indicate the two prominent UT-A1 glycoprotein forms at 117 and 97 kD. Shown are two of four rats from each group.

Figure 3. Biotinylation of inner medullary (IM) proteins reveal that AVP treatment increases the abundance of biotinylated UT-A1. Rats were given AVP (5 nmol subcutaneously) 45 minutes before being killed, and an IM collecting duct (IMCD) suspension was prepared. The suspended IMCD were biotinylated, and the biotinylated protein pool was analyzed by Western blot for the presence of UT-A1. Left two lanes show UT-A1 abundance in the absence of vasopressin (duplicate samples shown). Right two lanes show UT-A1 in the membranes from rats that were treated with vasopressin. Arrows indicate the two prominent glycoprotein forms at 117 and 97 kD. Shown are two of five rats from each group.

Vasopressin and Forskolin Increase Plasma Membrane Expression of UT-A1 in Rat IMCD Suspensions

The preceding studies were performed by injecting vasopressin into rats and establish the physiologic response in the intact rat. In the following experiments, we isolated IMCD suspensions and treated them ex vivo with vasopressin or forskolin.

Vasopressin significantly increases biotinylated UT-A1 by 99 ± 29% (n = 7; P < 0.02; Figure 4A). Forskolin also significantly increases biotinylated UT-A1 by 49 ± 10% (n = 6; P < 0.01). As a positive control, we measured AQP2 and found that vasopressin significantly increases biotinylated AQP2 by 34 ± 7% (n = 7; P < 0.01; Figure 4B). As a control to ensure that our biotinylation protocol did not result in biotinylation of a non–plasma membrane protein, we probed the same samples using a mAb to the nuclear envelope proteins lamin A and C. We did not detect any biotinylated lamin in the biotinylated IMCD
suspensions (data not shown). As an additional control to ensure that the biotinylation reagent was not entering the cells and labeling the total cellular protein pool, we probed the total cell lysate (pre-streptavidin bead protein sample) for UT-A1. There was no difference in UT-A1 abundance among control, forskolin-, and vasopressin-treated IMCD suspensions (Figure 4C) or in AQP2 abundance between control and vasopressin-treated IMCD suspensions (Figure 4D). These controls indicate that our biotinylation protocol is technically correct and that the biotinylation reagent is not entering the cells.

Next, we tested whether direct activation of adenylyl cyclase with forskolin (18) would stimulate an increase in plasma membrane accumulation of UT-A1 in IMCD suspensions from hydrated rats. Forskolin significantly increases biotinylated UT-A1 in IMCD suspensions from hydrated rats (from 37 ± 9 to 62 ± 4; n = 3; P < 0.05; Figure 5A) and from additional basal rats (from 57 ± 8 to 90 ± 5; n = 3; P < 0.05) that were analyzed in parallel. Forskolin also increases biotinylated AQP2 in the same samples in both hydrated rats (from 55 ± 3 to 64 ± 2; n = 3; P < 0.05; Figure 5B) and from basal rats (from 78 ± 2 to 97 ± 9; n = 3; P < 0.05). As a control to ensure that the increases in UT-A1 and AQP2 were not part of a generalized and nonspecific response to forskolin, the same samples were probed for calcium-sensing receptor, an apical plasma membrane protein in the rat IMCD (37). The abundance of biotinylated calcium-sensing receptor did not change with forskolin in basal rats (Figure 5C).

**Forskolin and dDAVP Increase UT-A1 in the Apical Plasma Membrane of UT-A1-MDCK Cells**

Forskolin (10 μM, 55 min) significantly increases urea flux, consistent with our previous findings (14,28), and cAMP production in UT-A1-MDCK cells. Forskolin (10 μM, 20 min) significantly increases UT-A1 biotinylation (by 173 ± 15%; n = 6; P < 0.005) and hence UT-A1 protein abundance in the apical plasma membrane of the UT-A1-MDCK cells (Figure 6). dDAVP (10 nM, 15 min) also significantly increases UT-A1 biotinylation by 59% (from 35 ± 3 to 55 ± 12; n = 3; P < 0.05) in the apical plasma membrane of the UT-A1-MDCK cells (data not shown).

**Discussion**

The major findings in this study are that (1) vasopressin increases UT-A1 accumulation in the plasma membrane of the IMCD from basal rats; (2) forskolin increases UT-A1 accumulation in the plasma membrane of the IMCD, both in basal and in hydrated rats; and (3) forskolin and dDAVP increase UT-A1...
accumulation in the apical plasma membrane of UT-A1-MDCK cells. Two urea transporters, UT-A1 and UT-A3, are expressed in the IMCD (5,38,39). However, because we transfected only UT-A1 into the UT-A1-MDCK cells, we can conclude that the forskolin- and dDAVP-mediated increases in UT-A1 accumulation in the apical plasma membrane do not require the presence of UT-A3 in the UT-A1-MDCK cells; we cannot exclude the possibility that UT-A3 is involved in the rat IMCD. A vasopressin-stimulated increase in UT-A1 accumulation in the plasma membrane may be a mechanism that contributes to the vasopressin-stimulated increase in urea transport that occurs in the perfused rat terminal IMCD (9).

The only previous study to examine whether vasopressin-regulated trafficking of UT-A1 occurs concluded that it did not (16). However, this study examined Brattleboro rats, which lack endogenous vasopressin. Although that study was well performed, its findings have been generalized, resulting in the incorrect conclusion that vasopressin-regulated trafficking of UT-A1 to the plasma membrane does not occur (16). Our study suggests that vasopressin does regulate the accumulation of UT-A1 in the plasma membrane in basal Sprague-Dawley rats (which have endogenous vasopressin present). In addition, when forskolin is used to stimulate directly the catalytic subunit of adenyl cyclase (18), it significantly increases UT-A1 accumulation in the plasma membrane in IMCD suspensions from either basal or hydrated rats. Because collecting ducts in general are known to be conditioned in polyuric rats and their cAMP response to vasopressin is subdued (17,40,41), we speculate that the failure to detect an increase in UT-A1 accumulation after vasopressin administration in the Brattleboro rat (16) or our hydrated Sprague-Dawley rats may have resulted from an insufficient cAMP response. However, in Sprague-Dawley rats with a presumably normal cAMP response to vasopressin or when forskolin is used as the agonist, increases in both UT-A1 and AQP2 accumulation in the plasma membrane are clearly detected. These increases seem to be specific because biotinylated calcium-sensing receptor is unchanged.

Vasopressin also regulates the trafficking of AQP2 between subapical vesicles and the apical plasma membrane in rat collecting ducts, provided that at least one AQP2 molecule within a tetramer is phosphorylated at serine-256 (reviewed in reference [15]). Vasopressin-regulated trafficking of AQP2 occurs in both Sprague-Dawley and Brattleboro rats (reviewed in reference [15]). We speculate that this difference between vasopressin regulation of UT-A1 and AQP2 suggests that less cAMP stimulation may be needed to stimulate the plasma membrane accumulation of AQP2 than of UT-A1. Vasopressin also increases the apical plasma membrane accumulation of AQP2 in several cell culture systems (42–45).

In the whole-animal experiments (those in which the rat received a subcutaneous injection of vasopressin), the percentage changes are somewhat modest, although they are statistically significant (Figures 2 and 3). These data are important for establishing the physiologic response in the intact rat kidney. Because the magnitude of the change may have been limited by variable absorption of the injected vasopressin and/or delivery to the kidney collecting duct, we proceeded to study IMCD suspensions and treated them ex vivo with vasopressin or forskolin. A limitation to the IMCD suspensions is that the plasma membrane of an occasional IMCD cell may be damaged during the process of preparing them, thereby allowing the biotin reagent to enter the cytoplasm of the damaged cell (Figure 1). If this occurs, then it would be equally likely in control and in vasopressin- or forskolin-treated IMCD suspensions, thereby increasing the noise of the measurement and potentially underestimating the percentage increase in UT-A1 in the plasma membrane in the treated IMCD suspensions. UT-A1 accumulation in the plasma membrane (biotinylated UT-A1) consistently increases by 50 to 100% in the IMCD suspensions (Figures 4 and 5). However, even a 100% increase in UT-A1 in the plasma membrane may not account for the six- to eight-fold increase in urea permeability that was stimulated by vasopressin in the perfused tubule. We previously showed that vasopressin increases UT-A1 phosphorylation in rat inner medulla,

Figure 6. Forskolin (10 μM; ◼ and ◇, duplicate determinations) significantly increases urea flux (A) over control (▲) levels. As the final portion of the flux experiment, we add dimethyl urea (DMU) to inhibit carrier-mediated urea transport, and it reduced urea flux to the basal level. The cAMP production (B) in parallel wells (n = 4 duplicate determinations/group) of our UT-A1-MDCK cells was increased by a comparable 60-min treatment with forskolin. (C) Forskolin-induced increase in UT-A1. The cells that were grown on six-well cluster dishes were treated for 30 min with 10 μM forskolin, in parallel with control cells. This Western blot shows duplicate samples of both control (left) and forskolin-treated (right) cells that were probed with anti–UT-A1. Arrows indicate UT-A1. Shown are two of six wells of cells for each condition.
rat IMCD suspensions, and UT-A1-MDCK cells (13,14), with a time course that is similar to vasopressin-induced stimulation of urea transport in the perfused rat terminal IMCD (10,12,13,46). Thus, vasopressin may increase urea permeability through increases in both UT-A1 accumulation in the plasma membrane and UT-A1 phosphorylation.

Conclusion

Our study shows that vasopressin increases UT-A1 accumulation in the plasma membrane in basal Sprague-Dawley rats, and forskolin increases it in both basal and hydrated rats. In addition, our study further establishes our UT-A1-MDCK cells as a model system that reproduces the physiologic properties that we find in rat inner medulla and IMCD suspensions.

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