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

Janet D. Klein,* Otto Fröhlich,† Mitsi A. Blount,* Christopher F. Martin,* Tekla D. Smith,* and Jeff M. Sands*†

*Renal Division, Department of Medicine, and †Department of Physiology, Emory University School of Medicine, Atlanta, Georgia

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 \textit{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 \textit{V}_2 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 adenyl cyclase (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 midcapsular 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 decapitation. 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 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).

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**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 (400 μl of 100 mM HCl was added, and the cells were scraped off the filter with a rubber spatula. The cell suspension was centrifuged, and the supernatant cell extract was assayed for its cAMP concentration using a cAMP ELISA kit (Assay Designs, Ann Arbor, MI).

**Western Analysis**

Western analysis was performed as described previously (14,25,29–31). 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, electrophoblotted to polyvinylidene difluoride membranes, and incubated with primary antibody overnight at 4°C. After washing, the blot was incubated with fluorscently labeled secondary antibody and visualized with the Odyssey infrared imaging scanner. Parallel gels were stained with Coomassie blue to verify uniformity of gel loading (LI-COR, 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 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 Na₂HPO₄). Treatments were added back during the incubation with biotinylation buffer that contained 3 mg/ml biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester (cat. no. B1022, Sigma, St. Louis, MO) for 60 min at 4°C (16). This incubation procedure results in the biotinylation of both apical and basolateral plasma membrane proteins in rat IMCD suspensions but only apical plasma membrane proteins in UT-A1-MDCK cells (Figure 1). Cells then were washed free of unattached biotin by three washes with biotin quenching buffer (0.1 mM CaCl₂, 1 mM MgCl₂, and 260 mM glycine in PBS) with the last wash incubated for 20 min at 4°C. Next, samples were washed three times with lysis buffer without detergent, and the cells were solubilized for 1 h in lysis buffer that contained 1% NP-40 (150 mM NaCl, 5 mM EDTA, and 50 mM Tris). After centrifugation (14,000 × g, 10 min, 4°C) to remove insoluble particulates, streptavidin beads were added to the supernatant fractions and allowed to absorb biotinylated proteins overnight at 4°C. After washing with high-salt and no-salt buffers, Laemmli SDS-PAGE sample buffer was added directly to the pellets, samples were boiled for 1 min, and the pool of biotinylated proteins was analyzed by Western blot.

**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.
Results

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 20 ± 1 to 19 ± 2; n = 4; NS). The latter result is consistent with a previous study in which dDAVP (desmopressin, a V2-selective agonist) did not increase UT-A1 abundance in the membrane fraction that is enriched in plasma membranes from Brattleboro rats (which lack endogenous vasopressin) (16). There was no significant change in UT-A1 abundance in the vesicle fraction for either basal or hydrated rats (data not shown).

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).
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 ± 11 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 35 ± 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.
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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