Vasopressin rapidly increases phosphorylation of UT-A1 urea transporter in rat IMCDs through PKA

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Zhang, Chi, Jeff M. Sands, and Janet D. Klein. Vasopressin rapidly increases phosphorylation of UT-A1 urea transporter in rat IMCDs through PKA. Am J Physiol Renal Physiol 282: F85–F90, 2002. First published August 8, 2001; 10.1152/ajprenal.00054.2001.—The UT-A1 urea transporter plays an important role in maintaining the hyperosmolar milieu of the inner medulla. Vasopressin increases urea permeability in rat terminal inner medullary collecting ducts (IMCDs) within 5–10 min. To elucidate the mechanism, IMCD suspensions were radiolabeled with [32P]orthophosphate. UT-A1 was immunoprecipitated and analyzed by autoradiogram and Western blot. Both the 97- and 117-kDa UT-A1 proteins were phosphorylated. Vasopressin treatment increased the phosphorylation of both UT-A1 proteins at 2 min, which peaked at 5–10 min and remained elevated for up to 30 min. There was a discernable increase in UT-A1 phosphorylation with 10 pM and a 50% increase with 10–100 nM vasopressin. 1-Desamino-8-D-arginine vasopressin (dDAVP) or 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) also increased UT-A1 phosphorylation. The vasopressin-stimulated increase in UT-A1 phosphorylation was blocked by H-89 or a specific peptide inhibitor of protein kinase A. Phosphatase inhibitors (okadaic acid, calyculin) increased UT-A1 phosphorylation. We conclude that vasopressin increases UT-A1 phosphorylation via protein kinase A within 2–5 min in rat IMCDs. This suggests that phosphorylation of UT-A1 may be the mechanism by which vasopressin rapidly increases urea permeability in vivo.

V₉ receptor; adenosine 3’,5’-cyclic monophosphate; urine-concentrating mechanism; urea permeability; inner medullary collecting duct; protein kinase A

Vasopressin is a hormone that increases the permeability of the collecting ducts in the kidney, allowing the body to concentrate urine and conserve water. This study aimed to understand the mechanism by which vasopressin increases urea permeability in rat IMCDs (inner medullary collecting ducts).

The authors radiolabeled IMCD suspensions with [32P]orthophosphate to detect the phosphorylation of UT-A1. They found that vasopressin treatment increased the phosphorylation of both UT-A1 proteins within 5–10 min, with a peak at 5–10 min. This increase in phosphorylation was blocked by H-89 or a specific peptide inhibitor of protein kinase A. Phosphatase inhibitors also increased UT-A1 phosphorylation, suggesting that vasopressin increases UT-A1 phosphorylation via protein kinase A within 2–5 min in rat IMCDs.

The purpose of this study was to test an alternative mechanism by which vasopressin could increase urea permeability: that vasopressin, acting through cAMP, increases the phosphorylation of UT-A1 in the rat IMCD. The deduced amino acid sequence for UT-A1 contains several consensus sites for phosphorylation by protein kinase A (PKA), as well as protein kinase C or tyrosine kinase (11). Results indicate that UT-A1 is phosphorylated by vasopressin acting through PKA.

METHODS

IMCD suspensions. Male Sprague-Dawley rats (National Cancer Institute, Frederick, MD), weighing 200–250 g, were fed a standard rat chow, kept in filter-top cages with autoclaved bedding, and received free access to normal drinking water. Rats were injected with furosemide (5 mg ip) 20–30 min before they were killed. After death, both kidneys were rapidly removed, whole inner medullas were excised, and the tissue was transferred into microcentrifuge tubes with 1 ml of 10 mM Tris-HEPES buffer, pH 7.4.
MgSO$_4$, 5.5 glucose, and 5 sodium acetate, as well as 2 mg/ml 118 NaCl, 5 KCl, 4 Na$_2$HPO$_4$, 25 NaHCO$_3$, 2 CaCl$_2$, 1.2 MgSO$_4$, 2 CaCl$_2$, 1.2 MgSO$_4$, 5.5 glucose, and 5 sodium acetate, pH 7.4. Inner medullas were minced finely with a razor blade and put into digestion buffer containing (in mM) acetate, pH 7.4. Inner medullas were collected, and inner medullary collecting duct (IMCD) suspensions were prepared as described in METHODS. After the radiolabel was loaded, IMCDs were incubated for another 10 min with 10$^{-8}$ M vasopressin (arginine vasopressin; AVP)$_1$, then, washed IMCDs were solubilized and UT-A1 was immunoprecipitated with an anti-COOH-terminal UT-A1 antibody. Western blot showing equal proteins in control (AVP$^{-}$) and vasopressin-treated (AVP$^{+}$) samples. Left: autoradiograph showing phosphorylation of the 97- and 117-kDa UT-A1 proteins. Double-headed arrows highlight 97- and 117-kDa UT-A1 proteins.

of dissecting buffer containing (in mM) 118 NaCl, 2 K$_2$HPO$_4$, 25 NaHCO$_3$, 1.2 MgSO$_4$, 2 CaCl$_2$, 5.5 glucose, and 5 sodium acetate, pH 7.4. Inner medullas were minced finely with a razor blade and put into digestion buffer containing (in mM) 118 NaCl, 5 KCl, 4 Na$_2$HPO$_4$, 25 NaHCO$_3$, 2 CaCl$_2$, 1.2 MgSO$_4$, 5.5 glucose, and 5 sodium acetate, as well as 2 mg/ml collagenase B, 0.5 mg/ml BSA, and 540 U/ml hyaluronidase. After a 30-min incubation at 37°C, DNase I was added to a final concentration of 0.001%, and incubation was continued for another 20 min. The suspension was periodically agitated to break up large tissue fragments and to facilitate the digestion process. After the incubation, the resulting suspension was transiently (10 s) centrifuged at 50 g, the supernatant was discarded, and the pellet was resuspended in suspension buffer (digestion buffer without collagenase, BSA, or hyaluronidase). This process was repeated two additional times with suspension buffer and one time with phosphate-free DMEM (GIBCO, Grand Island, NY). The pelleted tubules were resuspended and pooled in phosphate-free DMEM, a small aliquot was removed and checked for the integrity of the tubule suspension using a dissecting microscope, and tubules were redistributed evenly into individual sample microcentrifuge tubes. In general, the two kidneys from a single rat yielded sufficient tubules for about one sample; i.e., five rats yielded sufficient tubules to compare five to seven experimental permutations.

Unincorporated $^{32}$P was removed by three washes with phosphate-free DMEM. Then, the IMCDs were lysed in 1 ml RIPA buffer (10 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 50 mM NaF, 1 mM Na$_3$P$_2$O$_7$·10H$_2$O, 1 mM phenylmethylsulfonyl fluoride; 1% Triton X-100, 10% glycerol, 1% deoxycholate, 1 μg/ml aprotinin, 0.18 mg/ml sodium orthovanadate) and sheared with a 26-gauge needle. After centrifugation at 14,000 g for 15 min to remove insoluble particulates, samples were incubated overnight with polyclonal anti-UT-A1 (12) at 4°C with gentle mixing; this antibody also detects UT-A2 and UT-A4. Immune complexes were precipitated with protein A-agarose (Pierce, Rockford, IL) for 2 h at 4°C; then, the pelleted beads were washed six times with RIPA and once with potassium-free phosphate-buffered saline. Washes were counted to ensure complete removal of unbound radiolabeled material. Laemmli-SDS-PAGE sample buffer was added directly to the pelleted beads and boiled; proteins were size-separated on two identical SDS-polyacrylamide gels. One gel was dried, and $^{32}$P incorporation into UT-A1 was analyzed by autoradiography. The proteins on the other gel were transferred to polyvinylidene difluoride membrane, and the amount of im-

Fig. 2. Time course of vasopressin stimulation of UT-A1 phosphorylation. After the 3-h radiolabel-loading period, 10$^{-8}$ M AVP was added at staggered times, and incubations were stopped with 3 rapid washes with phosphate-free DMEM, followed by solubilization in isolation buffer and RIPA as described in METHODS. The immunoprecipitated UT-A1 protein was analyzed for radiolabeled phosphate incorporation. A duplicate Western blot verified equal UT-A1 protein per lane (not shown). A: autoradiograph showing phosphorylation of the 97- and 117-kDa UT-A1 isoforms in response to incubation with AVP for 0, 2, 5, 10, 20, or 30 min. B: densitometry of 6 separate determinations. Increases in phosphorylation in response to vasopressin were statistically significant ($^{*}$P < 0.001) at all time points.
munoprecipitated UT-A1 protein was assayed by Western blot.

Statistics. All data are presented as means ± SE, and n is the number of rats. To test for the statistical significance between the results from two groups, Student’s t-test or the Mann-Whitney U-test was used. To test more than two groups, ANOVA was used, followed by Tukey’s protected t-test (20) to determine which groups’ results were significantly different. The criterion for statistical significance was P < 0.05.

RESULTS

Effect of vasopressin. There are two different glycosylated forms of UT-A1: 97 and 117 kDa (2). Both the 97- and 117-kDa UT-A1 proteins are phosphorylated in the absence of any added hormone (i.e., basal phosphorylation) and exhibit increased phosphorylation on treatment with 10^{-11} M vasopressin (Fig. 1). After the addition of 10^{-8} M vasopressin to the IMCD suspension, the phosphorylation of UT-A1 was significantly increased at 2 min, peaked at 5 min, and then remained increased for up to 30 min (Fig. 2). A significant increase in UT-A1 phosphorylation was evident with 10^{-11} M vasopressin, with a much larger effect with 10^{-8} and 10^{-7} M vasopressin (Fig. 3). A significant increase in the phosphorylation of UT-A1 was also observed with either 10^{-8} or 10^{-7} M dDAVP, a selective V2-receptor agonist (Fig. 4).

Role of PKA. Because vasopressin increases cAMP production in the rat terminal IMCD (21), we tested the effect of an exogenous cell-permeable cAMP analog, CPT-cAMP, and found that it significantly increased the phosphorylation of UT-A1 (Fig. 5). Next, IMCD suspensions were treated with the PKA inhibitor H-89. H-89 significantly blocked vasopressin’s stimulation of UT-A1 phosphorylation (Fig. 6). This result was confirmed by using the PKA inhibitor 14-22 amide, a specific, cell-permeable peptide inhibitor of PKA (Fig. 6, lanes 4 and 5, n = 1).

Next, IMCD suspensions were treated with the phosphatase inhibitors calyculin or okadaic acid. Incubation

Fig. 3. Dose-response of UT-A1 phosphorylation to varying concentrations of vasopressin. After the 3-h radiolabel-loading period, different amounts of vasopressin (from 10^{-11} to 10^{-7} M) were added to identical IMCD suspensions, and incubation was continued for another 10 min. IMCDs were processed and UT-A1 was immunoprecipitated as described in METHODS. A duplicate Western blot verified equal UT-A1 protein per lane (not shown). A: autoradiograph showing phosphorylation of UT-A1 in the presence of 10^{-11}, 10^{-10}, 10^{-8}, and 10^{-7} M AVP. The far right lane shows control (ctrl) IMCDs that received no exogenous vasopressin. B: densitometry of 5 separate determinations. Increases in phosphorylation in response to vasopressin were statistically significant (*P < 0.001) at all doses tested.

Fig. 4. 1-Desamino-8-[d-arginine] vasopressin (dDAVP) stimulates UT-A1 phosphorylation. After the radiolabel was loaded, IMCDs were incubated for another 10 min without (Ctrl) or with 10^{-8} M dDAVP; then, the washed IMCDs were solubilized and UT-A1 was immunoprecipitated with an anti-COOH-terminal UT-A1 antibody as described in METHODS. A duplicate Western blot verified equal UT-A1 protein per lane (not shown). A: representative autoradiogram showing phosphorylation of UT-A1 in IMCDs that received no dDAVP (Ctrl) or 10^{-8} M dDAVP. B: densitometry of 7 separate determinations of the effect of 10^{-8} M dDAVP, showing that the increase in UT-A1 phosphorylation is significant (*P < 0.002).
with either phosphatase inhibitor significantly increased the phosphorylation of UT-A1 (Fig. 7). Finally, we immunoprecipitated UT-A1 from IMCD suspensions and then probed the immunoprecipitate with an anti-phosphotyrosine antibody. Although UT-A1 protein was present in the IMCD samples (Fig. 8, center lanes, preimmunoprecipitation samples), no protein was detected when the same sample was probed with the anti-phosphotyrosine antibody (PY-20; Pharmingen/Transduction Laboratories, San Diego, CA). In addition, anti-UT-A1 did not recognize any proteins in the immunoprecipitated phosphotyrosine protein pool, and PY-20 did not recognize immunoprecipitated UT-A1. This is consistent with phosphorylation occurring in serine or threonine residues, which are the likely targets for PKA.

DISCUSSION

The major result of this study is that vasopressin, acting through PKA, increases the phosphorylation of UT-A1 in freshly isolated suspensions of rat IMCDs. The time course for vasopressin-mediated increases in UT-A1 phosphorylation matches that for vasopressin-mediated increases in facilitated urea permeability in perfused rat terminal IMCDs (14, 21, 23). These findings strongly suggest that phosphorylation of UT-A1 is a mechanism by which vasopressin increases facilitated urea permeability in rat terminal IMCDs in vivo.

The terminal IMCD is generally thought to express only V2 receptors (5, 6), although one study did find evidence for V1a receptors by RT-PCR (22). Previous studies showed that vasopressin stimulates urea permeability in perfused terminal IMCDs via the V2 receptor by showing that dDAVP, a selective V2 agonist, mimics the effect of vasopressin (21). The present study shows that dDAVP also mimics the effect of vasopressin to increase UT-A1 phosphorylation.

Plasma vasopressin levels generally range between $10^{-11}$ and $10^{-10}$ M (4). These levels of vasopressin stimulate cAMP production in microdissected rat IMCDs (21). However, higher vasopressin levels ($10^{-8}$–$10^{-7}$ M) stimulate substantially higher levels of cAMP production (21) and UT-A1 phosphorylation (present study). We chose to use $10^{-8}$ M vasopressin in the present experiments because this concentration resulted in a maximal rate of cAMP accumulation (21); 2) has been used in a large number of the perfused tubule measurements of urea permeability (reviewed in Ref. 18); and 3) provided an optimal signal-to-noise for assessing inhibition of the PKA pathway. Although this vasopressin concentration is higher than that

![Image of Fig. 6: Inhibitors of protein kinase A (PKA) inhibit vasopressin-stimulated phosphorylation of UT-A1. After 3-h radiolabel loading, either H-89 (5 μM, n = 3) or the more specific peptide inhibitor PKA inhibitor 14–22 amide (5 μM, n = 1) was added to the IMCD suspension. After a 15-min preincubation, AVP was added and incubation was continued for another 20 min. IMCDs were processed and UT-A1 was immunoprecipitated as described in METHODS. A: autoradiograph of the UT-A1 proteins from control (Ctrl), H-89 alone, H-89 then AVP, AVP alone, and peptide inhibitor (Inhib.) then AVP. B: densitometry of 3 separate determinations of the effect of H-89, showing that H-89 blocks AVP-stimulated UT-A1 phosphorylation (*P < 0.05).]
found in systemic plasma, vasa recta vasopressin levels could be higher than plasma levels due to counter-current multiplication. Because terminal IMCDs are exposed to vasa recta rather than systemic plasma, it is possible that these higher vasopressin concentrations may be physiological in the deepest portions of the inner medulla.

We also found that inhibitors of PKA reduce both the vasopressin-stimulated and basal levels of phosphorylation of UT-A1. This result strongly suggests that PKA is phosphorylating UT-A1, both basally and in response to vasopressin stimulation. The reduction in basal phosphorylation by H-89 suggests that adenylyl cyclase may be constitutively phosphorylating UT-A1 in the IMCD. However, we cannot determine from the present studies whether PKA is directly phosphorylating UT-A1 or whether it phosphorylates UT-A1 indirectly by activating another kinase (or kinases), which then phosphorylates UT-A1.

PKA typically results in the phosphorylation of serine or threonine residues. The increase in UT-A1 phosphorylation by okadaic acid or calyculin is consistent with a serine or threonine phosphorylation site (1, 3, 9), and we showed that vasopressin does not phosphorylate a tyrosine residue. Future studies will be needed to determine the residues in UT-A1 that are phosphorylated by vasopressin.

Finally, the present study suggests a mechanism by which two vasopressin-regulated transport processes, water and urea transport, can be independently regulated: 1) urea permeability is regulated primarily by phosphorylation of UT-A1; whereas 2) osmotic water permeability is regulated primarily by the regulated trafficking of AQP2, although phosphorylation of AQP2 is important for its insertion into the apical membrane and the formation of tetramers (10). Atrial natriuretic factor inhibits vasopressin-stimulated osmotic water permeability, but not vasopressin-stimulated urea per-
meability, in perfused rat terminal IMCDs (17). This result could be explained if atrial natriuretic factor were to affect the trafficking of AQP2, but had no effect on phosphorylation, in response to vasopressin. Future studies will be needed to test this hypothesis.

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