Urea transport in MDCK cells that are stably transfected with UT-A1

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The primary method for investigating the rapid regulation of urea transport has been perfusion of rat IMCDs. This method provides physiologically relevant functional data, although it cannot determine which urea transporter isoform is responsible for a specific functional effect, because both UT-A1 and UT-A3 are expressed in this nephron segment. Progress in understanding the cell biology of urea transporters and the functions of the separate isoforms has been hampered by the lack of an appropriate cell culture system. The goal of the present study was to create a polarized epithelial cell line that stably expresses the UT-A1 urea transporter and reproduces many of the functional properties of urea transport in the IMCD.

In early studies of type I, high-resistance Madin-Darby canine kidney (MDCK) cells, it was shown that they responded to arginine vasopressin (AVP, antidiuretic hormone) by increasing the adenylyl cyclase activity, cAMP levels, and the synthesis of prostaglandins (reviewed in Ref. 8). Addition of apical AVP (110 nM = 50 mM/µl) also increased the rate of tracer Na⁺ efflux from the cells, but the effect was smaller and delayed compared with the increased rate of efflux seen with 1 mM cAMP (8).

METHODS

MDCK cells, the kind gift of Dr. James Schafer (University of Alabama at Birmingham), were originally selected in Rossier’s laboratory (University of Lausanne) for low epithelial sodium channel expression. They were then used and passaged in Rotin’s laboratory (University of Toronto) and then in Schafer’s laboratory (19) and are a type I, high-resistance cell line (2, 25). These cells were cultured in T-75 Corning Costar flasks (Corning, Acton, MA) in a humidified atmosphere (5% CO₂ in CO₂ buffer, pH 7.4, with 5 mM urea. The baseline fluxes were not different between unstimulated UT-A1-transfected MDCK cells and nontransfected or sham-transfected MDCK cells. However, only in the UT-A1-transfected cells was UT-A1 protein expressed (as measured by Western blot analysis) and urea transport stimulated by forskolin or arginine vasopressin. Forskolin and arginine vasopressin also increased the phosphorylation of UT-A1. Thionicotinamide, dimethyurea, and phloretin inhibited the forskolin-stimulated [¹⁴C]-urea fluxes in the UT-A1-transfected MDCK cells. These characteristics mimic those seen in rat terminal inner medullary collecting ducts. This new polarized epithelial cell line stably expresses UT-A1 and reproduces several of the physiological responses observed in rat terminal inner medullary collecting ducts.

urea transporter-A1; arginine vasopressin; collecting duct; Madin-Darby canine kidney cells

DURING THE PAST DECADE, substantial progress has been made in understanding the regulation of urea transport proteins in kidney. Major advances include the cloning of two urea transporter genes and several cDNA isoforms as well as the creation of polyclonal antibodies to different protein isoforms (reviewed in Ref. 27). The UT-A family of urea transporters currently consists of five isoforms plus three cDNA variants that differ in the 3'-untranslated region. UT-A1, UT-A2, UT-A3, and UT-A4 are expressed in kidney, but UT-A5 is found only in the testes (6). UT-A1 is the largest UT-A protein, with its 97- and 117-kDa glycosylated forms. It is expressed in the apical membrane of the inner medullary collecting duct (IMCD) in humans (17) and rodents (23, 30). Stimulation of protein kinase A increases urea transport in the rat terminal IMCD (28, 31), increases the phosphorylation of UT-A1 in IMCD suspensions (32), and increases urea flux in Xenopus oocytes that heterologously express human or rodent UT-A1 (7, 24, 30).

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and with pcDNAs/FRT/UT-A1, an expression vector that contains the coding region for UT-A1 and possesses an FRT site for homologous recombination. After insertion of the UT-A1 gene into the FRT site, UT-A1 transcription was driven by the human cytomegalovirus immediate-early enhancer/promoter. The latter vector also contains the hygromycin resistance gene under the control of the SV40 promoter to permit selection for recombinant clones in 800 μg/ml hygromycin. The homologous recombination inactivates the lacZ-Zeoicin fusion gene. These cells were then grown and passaged in T-75 flasks by using 500 μg/ml hygromycin in DMEM to maintain selection. The final MDCK-UT-A1 cells, which express UT-A1 by functional assay and Western blot analysis, are sensitive to Zeocin, resistant to hygromycin, and lack β-galactosidase activity. The cells were grown and passaged in DMEM containing HEPES and bicarbonate buffers by using standard techniques.

Collagen-coated Costar Transwell inserts (1 cm² growth surface area; Corning) were used to grow an epithelial layer of MDCK cells. After 1-h incubation of the Transwells in DMEM at 37°C, a suspension of selected, trypsinized cells was prepared, and 7.5 × 10⁶ cells were loaded onto each Transwell and fed after 1 h without hygromycin. These cells grew to confluence 5–7 days later. The transmembrane resistance was measured daily by using an epithelial resistance meter (EVOMX-G; World Precision Instruments, Sarasota, FL). Insertions in which there were not orderly increases in the transepithelial electrical resistance from <0.1 kΩ to >1 kΩ were discarded. We used only membranes with 1.5 kΩ or higher resistance for flux measurements. We transferred the membranes from the CO₂ incubator to the flow plate in the following manner to minimize changes in ionic conditions. The urea flux medium contained Hanks’ balanced salt solution with bicarbonate (HBBS; GIBCO/Invitrogen) supplemented with 20 mM HEPES from a 1-M stock (GIBCO/Invitrogen). Flux medium made of HBBS-HEPES containing 5 mM urea was aliquoted (1.5 ml) into the wells of a 12-well plate and placed with the lid open for ~1 h in the humidified tissue culture incubator (PCO₂ = 40 mmHg, 37°C). We replaced the lid before we moved the covered plates to the experimental bench, where we temporarily transferred them to a 37°C water bath and then placed them on top of a thermostated aluminum block.

Urea flux measurements. Immediately before initiating the flux measurements, we removed four epithelial membrane inserts (Costar) from their culture plates and placed them into empty wells of a 12-well plate. We carefully removed the 500–600 μl of culture medium over each epithelial layer and added 400 μl of prewarmed flux medium containing 0.4 μCi of [¹⁴C]urea. The four inserts were then transferred to the first row of wells, each containing the 1.5 ml of flux medium to initiate the transepithelial flux. We removed the lid only to add the inserts and then three more times during the flux as the four inserts were moved at designated times from row to row, ending one and initiating the next flux period (Fig. 1). We always measured the [¹⁴C]urea flux from the cis solution (insert or apical membrane bathing solution or upper solution) to the trans solution (well or basolateral membrane bathing solution or the lower solution) because it was difficult to rapidly and carefully remove the upper solution without damaging the cells growing on the filter that forms the base of the insert. Forskolin (10 μM stock in DMSO), AVP (10⁻⁴ M stock in H₂O), phloretin (100 mM stock in ethanol), dimethylurea, and thionicotinamide (Sigma) were certified grade and were added to the trans solution.

Western blot and phosphorylation methods. We radiolabeled cells using our previously published methods (13, 14). Briefly, we washed confluent cell layers with phosphate-free DMEM and then incubated them with 1 μCi of phosphate-free DMEM containing 0.1 mCi/ml of [³²P]orthophosphate for 3 h, 37°C, 5% CO₂, and 100% humidity. At
the end of the 3-h loading period, inhibitors or activators were added for further incubation. We then washed and solubilized the cells in RIPA buffer. All cellular material was collected. Each phosphorylation immunoprecipitation sample contained the contents of two wells from a six-well plate. We sheared the cells in the samples with a 26-gauge needle, centrifuged the samples for 15 min at 14,000 g, and removed the top 80% of each supernatant fraction to a fresh tube containing 10 μl of antibody. We removed an additional 50 μl, which we added to an equal volume of Laemmli sample buffer and boiled before using it as a preimmunoprecipitation control sample in Western blots.

For immunoprecipitation, we incubated samples with UT-A1 antibody at 4°C overnight with gentle agitation. Protein A-agarose beads (25 μl) were added, and cold incubation continued for a further 2 h. We pelleted the beads in a microcentrifuge and then washed the pellet seven times with RIPA buffer. We verified the completeness of the washing protocol by counting all of the washes as well as the supernatant and sample. On the basis of the counts present on the protein A-agarose beads, we added an amount of Laemmli buffer to the beads and boiled the samples for 1–3 min.

For electrophoretic analysis, proteins were size separated by SDS-PAGE on Laemmli gels and then either stained with Coomassie blue and dried for autoradiography or electrobotted to polyvinylidene difluoride membranes for Western blot analysis as described previously (12–15, 32). Western blots were incubated with our antibody to the COOH terminus of UT-A1 (5% milk, Tris-buffered saline, 0.05% Tween-20) overnight at 4°C (20). The secondary antibody we used depended on the method of analysis. For enhanced chemiluminescence detection, we further incubated the blot with horseradish peroxidase-linked goat anti-rabbit IgG at a dilution of 1:5,000, 2 h, room temperature, and then we washed and visualized the blot by using the LI-COR Odyssey gel scanning system (LI-COR Biotechnology, Lincoln, NE). We stained gels in parallel with Coomassie blue to verify the uniformity of gel loading.

UT-A1 protein was immunoprecipitated from equal amounts of the whole cell lysates by using our previously described method (13, 14, 32). Proteins were size separated on two identical SDS-polyacrylamide gels containing an equal portion of the total immunoprecipitated protein per lane. The proteins on one gel were transferred to a polyvinylidene difluoride membrane, and the amount of immunoprecipitated UT-A1 was assayed by Western blot. The other gel was dried, and 32P incorporation into UT-A1 was analyzed by autorigraphy.

Expression of UT-A1 protein in MDCK-UT-A1 cells. We tested whether the newly derived cell line expresses the desired urea transporter protein by Western blot analysis. Figure 2 demonstrates that these cells express significant amounts of newly expressed UT-A1 protein. The protein is predominantly present as the lower-glycosylation form of 97 kDa.

Stimulation of UT-A1-mediated urea fluxes by AVP. We tested the stimulation of trans-epithelial urea flux by six concentrations (0–10−8 M) of AVP on six separate epithelial membranes (Fig. 3). After six control flux periods, AVP was added to the basolateral side (well) at the nominal concentrations stated in Fig. 3. At stimulation for 33 min, 5 mM thionicotinamide was present during the three final time peri-
The increase in urea flux in response to AVP is mimicked by the increase in UT-A1 phosphorylation. Figure 4 shows both uniform protein and increased phosphorylation of UT-A1 (arrows) in cells treated with AVP stimulation is apparent at $10^{-10}$ M and increases to a maximum at $3 \times 10^{-9}$ and $10^{-8}$ M. The membrane stimulated by $10^{-8}$ M AVP had a higher control flux and an equally higher flux in the presence of thionicotinamide. Thus the absolute increase in the flux was not different from that in $3 \times 10^{-9}$ M AVP. The average flux without AVP and with 5 mM thionicotinamide was 1.6 ± 0.1 nmol-min$^{-1}$ cm$^{-2}$ (all values are means ± SD). The average flux without AVP and with 5 mM thionicotinamide was 1.6 ± 0.1 nmol-min$^{-1}$ cm$^{-2}$.

Phosphorylation of UT-A1 by AVP. The increase in urea flux in response to AVP is mimicked by the increase in UT-A1 phosphorylation. Figure 4 shows both uniform protein and increased phosphorylation of UT-A1 (arrows) in cells treated with $10^{-8}$ M AVP for 15 min.

Persistence and reversal of forskolin activation. To determine how rapidly the activation by forskolin was reversed by its withdrawal and how long it would persist in the presence of forskolin, we measured the time course of the activation of the $[^{14}\text{C}]$urea flux in five inserts (Fig. 7). In the four inserts where forskolin-stimulated flux remained elevated for up to 30 min after forskolin removal. The membranes shown by the squares and circles demonstrate the range of differences in stimulation by forskolin.

In the control cells, forskolin did not stimulate the $[^{14}\text{C}]$urea flux significantly and thionicotinamide essentially did not inhibit the flux. This suggests that the native MDCK cells have little, if any, intrinsic thionicotinamide-inhibitable urea transport and that it is not stimulated by forskolin. Without forskolin treatment, UT-A1-transfected cells also exhibited a low urea flux and no significant inhibition by thionicotinamide. In contrast, when the UT-A1-transfected cells were exposed to 10 μM forskolin, transepithelial urea fluxes were stimulated two- to threefold (and sometimes as much as fourfold) over the course of 30–60 min. This stimulated urea flux is nearly completely inhibited by thionicotinamide, indicating that it is mediated by UT-A1.

Phosphorylation of UT-A1 by forskolin. A 97-kDa UT-A1 protein is expressed in the stably transfected MDCK-UT-A1 cells (Fig. 2). This 97-kDa UT-A1 protein is basally phosphorylated in unstimulated MDCK-UT-A1 cells, but phosphorylation is increased 2- to 10-fold within 2 min of stimulation with 10 μM forskolin. This is shown in Fig. 6, where, despite equal amounts of immunoprecipitated UT-A1 protein, the phosphorylation level was substantially increased within 2 min of forskolin treatment.

Persistence and reversal of forskolin activation. To determine how rapidly the activation by forskolin was reversed by its withdrawal and how long it would persist in the presence of forskolin, we measured the time course of the activation of the $[^{14}\text{C}]$urea flux in five inserts (Fig. 7). In the four inserts where forskolin-stimulated flux remained elevated for up to 30 min after forskolin removal. The membranes shown by the squares and circles demonstrate the range of differences in stimulation by forskolin.
10 μM forskolin was applied, the flux was activated over ~20–30 min and then appeared to reach a plateau value. When after 42 min of stimulation the forskolin was removed from two of the inserts, the flux in the absence of forskolin remained stimulated for ~20 min and then declined ~40% over the next 35 min before 5 mM thionicotinamide was included on the basolateral side to inhibit the flux. In the continued presence of forskolin, the urea flux remained stimulated for a significantly longer period and then decayed more slowly. The control fluxes in the absence of forskolin were nearly constant (average 2.6 ± 0.3 nmol·min⁻¹·cm⁻²) over the same time period.

Concentration dependence of thionicotinamide inhibition on the basolateral surface. We tested the concentration dependence and reversibility of thionicotinamide inhibition by measuring the forskolin-simulated [¹⁴C]urea flux on four membranes with either increasing or decreasing concentrations of thionicotinamide in the bottom well solution (Fig. 8). There were no significant differences in the fluxes with raising or lowering the thionicotinamide concentration. The data were fit to the equation: 

\[ V_{TN} = V_{inf} + (V_0 - V_{inf})(1 + [TN]/K_i) \]

where \( V_{TN} \) is the extrapolated flux at infinite thionicotinamide concentration ([TN]), \( V_0 \) is the flux at zero thionicotinamide concentration, and \( V_{inf} \) is the flux at a given thionicotinamide concentration. \( K_i \), the apparent inhibitor constant of thionicotinamide on the basolateral membrane, was 1.2 mM, \( V_0 \) was 10.8 nmol·min⁻¹·cm⁻², and \( V_{inf} \) was 2.4 nmol·min⁻¹·cm⁻².

**Inhibition of UT-A1-mediated [¹⁴C]urea fluxes by basolateral dimethylurea.** Dimethylurea inhibited urea fluxes to the same extent as thionicotinamide (Fig. 9). In preliminary experiments, we found that 300 mM dimethylurea rapidly inhibited the flux to the maximum extent but that the flux needed >10 min to be reversed after the dimethylurea solution was removed from the bottom well. However, after treatment with <20 mM dimethylurea the flux was rapidly and fully reversible. Figure 9 shows a fit to the data that yielded an apparent inhibitor constant for dimethylurea of 4.9 mM. Dimethylurea appears to be a relatively potent, complete, and reversible inhibitor of the UT-A1-mediated flux.

**Inhibition of UT-A1-mediated [¹⁴C]urea fluxes by basolateral phloretin.** Phloretin also inhibits the urea flux to the same extent as thionicotinamide and dimethylurea (Fig. 10). About 300 μM phloretin is sufficient to achieve near-complete inhibition.

**DISCUSSION**

The major result of the present study is that we have created a stably transfected MDCK-UT-A1 cell line. We chose MDCK cells as parent cells because they are a model for distal nephron epithelium and are most likely to possess the same sets of signaling pathways as the distal nephron in vivo (21). MDCK cells can form a high-resistance epithelium (25) with low permeabilities for nonelectrolytes and water (18, 26). The value reported by Lavelle et al. (18) for the urea permeability of type I MDCK cells (7.3 × 10⁻⁶ cm/s) is the same as what we measured: our control fluxes in the range of 1.6–2.4 nmol·min⁻¹·cm⁻² (Figs. 3, 5, and 7) correspond to 5–8 × 10⁻⁶ cm/s.

Our Western blot analysis of urea transporter protein showed no evidence for intrinsic urea transporters in the parental
untransfected MDCK cells (Fig. 2). A negative Western blot could be obtained if the antibody, which was raised against a COOH-terminal peptide from rat UT-A1, does not recognize the corresponding canine peptide sequence. However, our flux experiments in untransfected cells also showed no change in basal transport after treatment with AVP or forskolin, both of which produced a strong stimulation in transfected cells. Since expression of UT-A1 without hormonal activation did not increase the $[^{14}C]$urea flux above the native flux rate, all of the hormonal activation can be ascribed to the expressed rat UT-A1. It therefore appears that the MDCK cells did not express a native canine urea transporter or, if they did, that it did not react with the antibody and was not activated by forskolin or AVP.

The MDCK-UT-A1 cells expressed significant amounts of UT-A1 protein as shown by Western blot analysis (Fig. 2), and our transport experiments showed that it was functionally active. In the renal tubule in vivo, the UT-A1 protein was present in two different glycosylation states, with apparent molecular weights of 97 kDa and 117 kDa (1), which differ in their degree of glycosylation and whose relative abundance depended on physiological and pathological conditions (1, 3, 11, 16). In comparison, in the MDCK-UT-A1 cells, the UT-A1 protein was primarily present in the 97-kDa form. However, this did not appear to impair the functional status of the protein, because the MDCK-UT-A1 cells exhibited a robust urea permeability after stimulation with forskolin.

In MDCK-UT-A1 cells, AVP and forskolin increased UT-A1 phosphorylation (Figs. 4 and 6) and both agonists activated $[^{14}C]$urea fluxes (Figs. 3 and 5). The activated urea fluxes were inhibited by three urea transport inhibitors: thionicotinamide, dimethylurea, and phloretin (Figs. 8–10). Since the MDCK-UT-A1 cells increased their phosphorylation of UT-A1 and increased their flux in response to low concentrations of AVP or forskolin, which activates protein kinase A, these cells appeared to have functional V$_2$ receptors for AVP. To our knowledge, this is the first epithelial cell model to stably express UT-A1, the urea transporter that is expressed in the IMCD.

The time courses of phosphorylation and flux activation of UT-A1 by forskolin were different. The phosphorylation was rapid (2–5 min; Fig. 6) compared with the flux activation (10–30 min; Fig. 5). There are many possible explanations for the delay in flux activation. This may be due to phosphorylation of UT-A1 at multiple sites, many of which are unrelated to activation or many of which must be phosphorylated before the one critical activating site is phosphorylated. Alternatively, phosphorylation of UT-A1 may be a parallel phenomenon unrelated directly to its activation that may result from the phosphorylation of another protein, or linked chain of proteins, which then activate UT-A1 already in the plasma membrane.

The delay in MDCK cells may be due to the slow insertion of UT-A1-containing vesicles into the plasma membrane, although this mechanism has been disproved for AVP activation of urea transport in the renal IMCD of Brattleboro rats (which lack AVP) (10, 22).

AVP increased urea permeability in perfused rat terminal IMCDs (28) and increased UT-A1 phosphorylation in IMCD suspensions (32). Forskolin also increased urea permeability in perfused rat terminal IMCDs (9). Phloretin inhibited AVP-stimulated urea transport in perfused rat terminal IMCDs (4).

Although neither dimethylurea nor thionicotinamide has been tested in the perfused IMCD, methylurea and acetamide did inhibit urea transport in the rat terminal IMCD (4). Thus the properties of our stably transfected MDCK-UT-A1 cells reproduced the properties of urea transport in the rat terminal IMCD. This suggests that our transfected cells will be a useful model system for studying the cell biology and signaling pathways that regulate urea transport by each of the urea transporter isoforms.

A potential advantage of the approach that we used to create this cell line is that we initially cloned an FRT site into the genome of the parental MDCK cells and created a stable line of MDCK-FRT cells. By cloning UT-A1 into the MDCK-FRT cells, the UT-A1 cDNAs can only incorporate into the FRT site that we introduced into the genome of the parental MDCK cells. In future studies, we will be able to clone other urea transporter isoforms into the MDCK-FRT cells, and these UT-A cDNAs should only be incorporated into the same FRT sites. Thus any difference in function between MDCK cell lines that are stably transfected with different UT-A cDNAs should result from differences in the UT-A protein that is expressed and not from where the transgene is incorporated into the MDCK cell genome.

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