Regulation of UT-A1-mediated transepithelial urea flux in MDCK cells

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The urea transport proteins produced by the UT-A gene (Slc14A2) play an important role in concentrating the urine. Four different protein isoforms, which are the products of alternative promoters and alternative splicing of UT-A transcripts, are expressed in specific segments of the nephron, where they provide a selective pathway for urea permeation across the tubular wall. The UT-A1 isoform, which is the best-characterized form of UT-A, is expressed only in the inner medullary collecting duct (IMCD) and is the focus of this report. UT-A2 is expressed in apical and basolateral membranes of the thin descending limb of the loop of Henle (6). UT-A3 is expressed in the apical membrane of the IMCD in the rat (23) and in the basolateral membrane of the IMCD in the mouse (22). The membrane location of UT-A4 protein is not known, but its message is expressed in the renal inner and outer medulla (10).

UT-A1 is located in the apical membrane in the human (2) and rat (14) IMCD and in the apical and basolateral membranes in the mouse IMCD (6). The interstitial medullary urea concentration and, secondarily, the maximum urine concentrating ability are regulated by UT-A1-mediated transport across the apical membrane (16). The active transport of NaCl in the thick ascending limb provides the power for concentrating urea in the IMCD luminal fluid by enabling movement of water down its thermodynamic gradient in the collecting duct. The physiological importance of UT-A1 for the urinary concentration mechanism has been demonstrated in knockout mice not expressing the UT-A gene (4, 5).

Along with increasing water permeability, vasopressin (arginine vasopressin, AVP) increases urea permeability in the terminal segment of the IMCD (17, 21) and allows the passive movement of concentrated urea from the tubular lumen into the papillary interstitium. The generally accepted scheme for this regulation includes steps in which AVP binds to the high-affinity V2 receptor. This receptor is coupled to the heterotrigemeric G protein, Gs, through which it activates adenylyl cyclase, which in turn raises cAMP levels and activates protein kinase A (PKA) (9). The activation of PKA causes phosphorylation of UT-A1 (26) and coincidentally increases the urea flux, presumably mediated by UT-A1 (17).

To study the pathway or pathways by which urea permeability may be increased in the IMCD, we have developed (7) a Madin-Darby canine kidney (MDCK) cell culture model that heterologously expresses UT-A1. This model has the experimental advantage that it expresses only one UT-A isoform, eliminating the contributions of any of the other UT-A isoforms (UT-A3, UT-A4) that might be functional in vivo. It also lends itself to well-defined studies of transepithelial tracer urea fluxes. Using this cell system, we demonstrated (7) that UT-A1-mediated urea permeability is induced by AVP and forskolin and that UT-A1 protein is phosphorylated in response to these agonists.

In the present study we examined the stimulation of UT-A1-mediated transepithelial urea fluxes in more detail. We found that the time course of urea flux stimulation by AVP or forskolin consisted of two components, a low-amplitude and possibly transient component during the first 10–15 min and a large delayed component that took 40 min to reach maximum. The rapid component is not blocked by the PKA inhibitor H-89. We conclude that the activation of UT-A1-mediated transport in MDCK cells is at least in part mediated by cAMP as second messenger. However, the cAMP responsible for flux activation may be compartmentalized by being generated and

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degraded in a restricted region or regions of the cell, and it may activate urea transport at least in part through non-PKA signaling pathways.

METHODS

Reagents. All chemicals were reagent grade. Forskolin and the protein kinase inhibitor H-89 (Sigma, St. Louis, MO) were dissolved in DMSO as a 1,000× stock solution. AVP (Sigma) was dissolved in water as a 10−4 M stock solution. The AVP stock solution was stored frozen at −70°C, thawed only once, and further diluted for final use, and the unused portion was discarded after the experiment. The V2-selective vasopressin receptor antagonist OPC-31260 (Otsuka Pharmaceutical, Tokushima, Japan) was dissolved in DMSO.

UT-A1-expressing MDCK cells. Construction and selection of the MDCK-FLP recombinase target (FRT) and the MDCK-FRT-UT-A1 cells were performed as described previously (7). We have found that plating 2 × 10⁶ cells per square centimeter of growth area and measuring the urea flux on the confluent high-resistance (>800 Ω-cm²) polarized epithelial membranes 4 days later give the most consistent results.

Flux measurements. The measurement of [¹⁴C]urea flux with collagen-coated Costar Transwell inserts with 1-cm² growth surface area was described previously, along with a number of controls to demonstrate that the methods are reliable (7). All fluxes were determined at 37°C in the apical-to-basolateral direction by adding the [¹⁴C]urea to the apical medium and collecting the radioactivity in the basolateral medium in 3-min intervals. Each experiment had internal controls: we initially measured the baseline flux in the absence of an activator, and, at the end of the experiment, we added the inhibitor dimethylurea (DMU) at 100 nM to the basolateral medium, which reduces the flux to close to the baseline level. The initial unstimulated baseline urea flux was 2.0 nmol·cm⁻²·min⁻¹ (SD 1.7, n = 1,746), and the final flux after addition of the urea transport inhibitor DMU at 100 nM was 1.6 nmol·cm⁻²·min⁻¹ (SD 0.8, n = 1,076), where three to six independent flux measurements from 405 different epithelial membranes were averaged. We used the DMU-inhibitable flux as a measure of the UT-A1-mediated urea flux. In the 5 mM urea solutions used in all experiments, a flux of 2.1 nmol·cm⁻²·min⁻¹ corresponds to a permeability of 7 × 10⁻⁶ cm/s. All measurements were performed at pH 7.4 at 37°C in a medium containing (mM) 140 NaCl, 1.6 K₂HPO₄, 5.5 t-glucose, 1 CaCl₂, 0.81 MgCl₂, and 24 HEPES titrated with NaOH.

Immunofluorescence experiments. Cells were grown on Transwell filters to confluence as if they were to be used for flux experiments. Cells were washed three times with phosphate-buffered saline (PBS) and then exposed to 3.7% paraformaldehyde (PFA) for 15 min at room temperature. The PFA was replaced with 0.3% Triton X-100 in PBS for 5 min. The detergent was removed by two washes with PBS followed by three washes with PBS containing 1.5% bovine serum albumin (PBS-BSA). Cells were then incubated with primary antibody [anti-COOH-terminal UT-A (11) diluted 1:5,000 in PBS-BSA] for 1 h at room temperature. Unbound antibody was removed with five washes of PBS-BSA, and secondary antibody (anti-rabbit Alexa 546 diluted 1:250 in PBS-BSA) was added for an additional 1 h. If included, FITC-phalloidin diluted 1:250 in PBS was added with the secondary antibody. To remove secondary antibody, cells were washed three more times with PBS-BSA and one time with deionized water. The cells were mounted between slide and coverslip with Vectashield mounting medium and sealed with nail polish.

Data presentation and statistics. A typical experimental series consisted of parallel flux experiments with eight filter inserts. When data are presented in bar graphs, each bar represents one flux experiment and the error bar is the SD of the last three or four time point measurements of that flux. As one might expect, we found significantly less variability within an experimental series than among different series performed on different days, where maximally stimulated flux values could vary by 10–20%.

RESULTS

We previously showed (7) that forskolin stimulated transepithelial tracer urea fluxes in our UT-A1-expressing MDCK cell line by four- to fivefold. For the present experiments, we reisolated a subclone of this cell line that exhibits uniform expression of UT-A1 (Fig. 1) and significantly higher rates of stimulated urea transport (Fig. 2A), with typical flux values of ~25 nmol·cm⁻²·min⁻¹ at saturating concentrations of forskolin (10 μM). The time course of activation appears to occur in two phases, an initial phase of 10–15 min that is characterized by a slow ramp of activation and a relatively small amplitude, and a second phase during which urea flux activation is accelerated before leveling off. Overall, it took 40–50 min for urea transport to reach this plateau level that we interpret as maximal stimulation under these conditions. At first glance, the initial phase of activation may appear like a lag period after which activation accelerates. However, close inspection (Fig. 2B) reveals that there is no lag. Instead, urea flux activation begins within the first time point (3 min) after exposure to forskolin, and the flux increases at a steady slope over the first 12 min, until it is accelerated during the second phase.

We determined the concentration dependence of flux activation by forskolin. Forskolin activated the transepithelial urea fluxes with a high apparent affinity, with half-maximal activation (0.7 μM (Fig. 3). This value is typical for processes that are stimulated by forskolin through its action as an activator of adenylyl cyclase (18). Forskolin’s action on urea transport in our MDCK cells is consistent with the activation of transepithelial urea transport in the IMCD by agents that raise intracellular cAMP levels (8, 21).

Figure 4 is a summary of the plateau urea fluxes after 51–63 min of treatment with the indicated compounds. All these agents were added to the basolateral side of the epithelial membrane. There was no activation by dideoxyforskolin but a large activation by forskolin, indicating that all of the diterpene activation was due to the activation of adenylyl cyclase and synthesis of cAMP in some region of the cells. Both AVP and the V₂-specific agonist 1-desamino-8-d-arginine vasopressin (DDAVP) were approximately equipotent, indicating that AVP also probably acted through V₂ receptors. In the absence of the phosphodiesterase inhibitor IBMX, both compounds activated urea transport to ~10–15 nmol·cm⁻²·min⁻¹, which is less than the activation achieved by forskolin. The modest activation (a slow continuous rise typically to 5 nmol·cm⁻²·min⁻¹ after 50 min) by IBMX indicates that endogenous activities can accumulate cAMP to concentrations sufficient to activate urea flux if cAMP breakdown is inhibited. This suggests that even in the absence of an externally applied stimulus there was a low constitutive rate of cAMP production in these cells. Surprisingly, the cAMP analogs 8-bromoadenosine 3',5'-cyclic monophosphate 8-BrCAMP and 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) were poor activators even at the moderately high concentrations used (300 μM).

Although IBMX alone caused only moderate activation of urea transport, it augmented, as expected for an inhibitor of phosphodiesterase, the effect of agonists that stimulated the
formation of intracellular cAMP. In the presence of 200 μM (basolateral) IBMX, AVP activated urea transport to the same maximal level as forskolin. Under this condition, AVP activated urea transport with an apparent affinity of $3 \times 10^{-11}$ M (Fig. 5). This high affinity indicates that the effect of AVP is mediated by binding $V_2$ receptors (19). We confirmed this receptor specificity by demonstrating that the $V_2$-selective nonpeptide antagonist OPC-31260 nearly completely blocked the activation of urea transport by AVP (Fig. 6), similar to its ability to block the recruitment of aquaporin-2 protein into the apical membrane of the renal inner medulla (3).

$V_2$ receptors are believed to be present primarily at the basolateral side of MDCK cells. We confirmed this by demonstrating that the action of AVP is asymmetric in that AVP activated urea transport only when it was present on the basolateral side; AVP had no effect when present only on the apical side (data not shown). This demonstrates that in the MDCK-UT-A1 cells the $V_2$ receptors are functionally present almost exclusively on the basolateral membrane.

In general, the mode of activation of a physiological process in the kidney by AVP is held to be mediated by activation of adenyl cyclase, which produces the cAMP that activates PKA (9). To test for the direct activation of urea transport by cAMP in our MDCK-UT-A1 cells, we treated them with the membrane-permeant cAMP analog 8-Br-cAMP. We found no effect on urea transport at 100 μM, but we did observe significant activation when 300 μM 8-Br-cAMP was added to the basolateral side (Fig. 7). However, when added to the apical side, 300 μM 8-Br-cAMP had no effect, and even in the presence of IBMX it did not activate urea transport beyond that observed for IBMX alone.

To test whether the elevated cAMP levels led to urea transport stimulation through activation of PKA, we tested the effect of the PKA inhibitor H-89. Figure 8 shows that H-89 only partially blocks the AVP-induced cAMP permeability. Similarly, the activation of urea fluxes by forskolin was also only partially inhibited by H-89 (Fig. 9). Even at concentrations as high as 30 μM, H-89 inhibited the forskolin activation only by
This suggests that other signaling pathways besides that involving PKA play a role in the activation of urea transport by AVP or forskolin. Furthermore, the shape of the time course curve during the early phase of urea transport activation differed with different activators. As seen in Fig. 2, forskolin caused a slow initial increase, followed by the steeper activation of the second phase. When stimulated by AVP, the two phases were more clearly separable in that the initial phase manifested itself as a pronounced shoulder. In many cases, the initial phase took the shape of a transient maximum. Such an initial peak is discernible in Fig. 8, although a clear transient has not been observed in all experiments. The magnitude of the transient peak was not influenced by the length of the preincubation time in flux medium (for 9–36 min) before the exposure to AVP (data not shown).

**DISCUSSION**

We report here more detailed flux experiments with MDCK cells permanently transfected with the urea transporter isoform UT-A1 (7). This is a clonal cell line in which all cells express the UT-A1 protein, as demonstrated by the fluorescence images of Fig. 1. As observed previously, in 5 mM urea solutions and in the absence of a stimulus the expressing cells exhibited a low flux of 2.0 nmol·cm⁻²·min⁻¹. When averaged over several hundred flux experiments, the flux was slightly, but not significantly, decreased to 1.6 nmol·cm⁻²·min⁻¹ in the presence of 100 mM DMU. This suggests that most, if not all, of the urea movements in the unstimulated MDCK-UT-A1 cell are by nonspecific means, enabled by the diffusional permeability of the lipid bilayer. Indeed, the measured flux corresponds to a permeability of 7 × 10⁻⁶ cm/s, which is the same as measured for nontransfected MDCK cells (7, 13). If there is a small component of DMU-inhibitable urea flux, this could be due to a very small fraction of endogenously activated transporters in the absence of exogenous stimulants and reflect the low resting steady-state cellular content of cAMP.

Urea permeability was strongly increased in the presence of forskolin. Although subject to some fluctuations between experi-
ments, the maximal urea flux at saturating concentrations of forskolin (10 μM) was typically around 25 nmol·cm⁻²·min⁻¹. This corresponded to a 12-fold stimulation over the fluxes observed in the absence of an externally applied stimulus. AVP activated urea fluxes to a lesser extent than forskolin, reaching typical values of 10–15 nmol·cm⁻²·min⁻¹ in the presence of 10⁻¹⁰–10⁻⁸ M AVP. However, in the presence of the phosphodiesterase inhibitor IBMX the activation by AVP was as strong as that by forskolin alone, whereas the activation by forskolin was not further enhanced by IBMX (data not shown). The fact that IBMX by itself caused a slow upward drift in the urea flux that reached 5–10 nmol·cm⁻²·min⁻¹ after 50 min indicates that even in the absence of an externally applied stimulus there is a constitutively active rate of cAMP formation. It also indicates that this continually formed cAMP is continually degraded and kept to a basal level by a strong phosphodiesterase activity. In fact, the phosphodiesterase activity in the absence of IBMX appears to be so strong that a 300 μM basolateral concentration of 8-BrcAMP was required to achieve significant activation of urea transport. In comparison, in the presence of IBMX the same concentration of 8-BrcAMP caused a robust activation of urea transport to as high as 15–20 nmol·cm⁻²·min⁻¹ after 50 min. It is interesting that when added to the apical side, 300 μM 8-BrcAMP did not activate urea transport even in the presence of (basolaterally added) IBMX. Virtually identical observations were made with CPT-cAMP (data not shown). This asymmetric response to externally added cAMP analogs could be due to different permeabilities or surface areas of the apical and basolateral membranes, or it could be due to an asymmetric distribution of cAMP-degrading enzymes within the cell or of other components of the cAMP signaling pathway. In control experiments, we observed that in the range of submaximal activation it took about twice as much apical forskolin to achieve the same activation of urea transport as achieved by basolateral forskolin (data not shown).

The response of urea transport to added AVP was also asymmetric. Basolateral AVP elicited a strong activation, whereas apical AVP had no noticeable effect (data not shown). This is most readily explained by a nearly exclusive presence of V₂ receptors on the basolateral side. The functional asymmetry is certainly much stronger than could be caused by the differences in membrane area (at constant receptor densities) on the two sides. A strong asymmetry of V₂ receptor density in MDCK cells was demonstrated previously by immunologic techniques (1). However, the present flux data suggest that the observed asymmetric distribution of overexpressed, epitope-labeled V₂ receptors in receptor-transfected cells is still less than the asymmetric distribution of natively expressed receptor proteins in MDCK cells, which is similar to findings in isolated renal tubules (15).

Our data suggest that the activation of UT-A1-mediated urea transport in our MDCK cells by forskolin and AVP occurs in...
at least two different phases. We observed a rapid response of low amplitude whose onset occurred within the first time point taken after addition of the stimulant (3 min), followed by a slower component that exhibited a lag of up to 15 min. When urea transport was activated by forskolin, the rapid response component was visible only as a weak shoulder riding on the onset of the slower component with its much larger amplitude. In contrast, when urea transport was activated by AVP, the early component was much more distinct, probably because of the smaller amplitude of the slower component in the presence of AVP. Under these conditions, urea flux briefly decreased, suggesting that the early component may be a transient response to the stimulus.

This biphasic response is reminiscent of the response of urea and water permeability to AVP in isolated rat terminal IMCD tubules (24). In these experiments, urea and water permeabilities exhibited a rapid phase that reached completion around 10 min, followed by a slower component over the next 30 min. The difference between the two cell systems, however, is that in the IMCD the early component had the larger amplitude, contributing 80–90% to the maximal response, whereas in the MDCK cells it contributes 10% to the response to forskolin and up to 30% to the response to AVP. The nature of this quantitative difference is not clear at this time. There are several differences between the rodent tubule and the canine cell line beyond the difference in species that could account for possible differences in the regulation of renal urea transport. One possibility would be that through the long history of culture of the MDCK cells the relative strength of signaling or regulatory pathways may have shifted; a component could even have been lost. Because it is likely that the MDCK-UT-A1 cells overexpress the UT-A1 protein, the elevated level of heterologous protein could be recruited into a membrane pool that is differently regulated. Nonetheless, at this time the MDCK-UT-A1 cells represent the best available cell culture model for studying the regulation of inner medullary urea transport.

The mechanisms underlying urea flux activation in the presence of forskolin and AVP in the MDCK cells are also not clear. The generally held notion is that the activation of urea transport in the IMCD by AVP occurs through activation of adenyl cyclase, cAMP formation, and activation of PKA (9), followed by phosphorylation of the UT-A1 urea transporter (26). If phosphorylation of UT-A1 is mediated solely through activation of PKA, one would expect the activation of urea flux to be nearly completely inhibited in the presence of 1–5 μM of the PKA inhibitor H-89. However, the activated urea flux was only partially suppressed even in the presence of 30 μM H-89. This strongly suggests that in the MDCK cells the slow activation of urea transport is mediated by at least one additional signaling pathway that does not involve PKA.

The observed incomplete block of urea transport activation by H-89 is consistent with previous work on the effect of PKA-specific inhibitors on the phosphorylation of UT-A1 in IMCD suspensions. Zhang et al. (26) found that H-89 and the specific peptide PKA inhibitor 14-22 amide only partially blocked the phosphorylation of UT-A1 protein induced by AVP. This parallel behavior of UT-A1 protein phosphorylation and urea transport activity suggests that UT-A1 protein phosphorylation in the isolated IMCD and urea transport activation in cultured MDCK cells are subject to the same set of signaling pathways.

Not only was H-89 an incomplete inhibitor of the activated urea transport at the (maximum) plateau phase of urea flux activation, we observed no inhibitory effect at all during the early phase (Fig. 8). During this early phase, which comprises the first 10–15 min of stimulation, there was no difference whether the cells had been preincubated with H-89 for 27 min or not. This suggests that during this early phase of activation PKA is not involved at all. That PKA might not be the sole mediator of urea transport activation is more readily understood for AVP because in addition to stimulating cAMP production AVP also raises intracellular Ca\(^{2+}\) levels (21, 25) and thus activates additional pathways that can lead to UT-A1 phosphorylation and activation. However, no such behavior is known for forskolin, at least not stimulation that is specific to forskolin over dideoxyforskolin.

In recent years a new family of related proteins that can function as nonkinase effectors of several second messengers has been identified (20). The subfamily of Epac/cAMP-GEF proteins can bind cAMP and in response activate a small G protein, Rap1, which in turn can trigger downstream kinase cascades such as MEK/Erk. Thus Epacs can transduce signals...
to the same effector molecules as PKA. Epac-1 is ubiquitously expressed, including in the kidney, where it has been shown to mediate the activation of H-K-ATPase by cAMP (12). It will be of interest to explore whether Epac-1 also participates in the activation of urea transport in response to AVP.

In summary, we have demonstrated that constitutively expressed UT-A1 protein in transfected MDCK-UT-A1 cells mediates urea transport only after activation by forskolin and AVP. Much of this activation is expected, based on the action of these agents in other systems including isolated IMCD tubules. However, there are also significant quantitative differences in the time course of activation. It is possible that the MDCK-UT-A1 cells provide a model to dissect out signaling pathway components that are not as readily observed in intact tubules.

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