Stimulation of UT-A1-mediated transepithelial urea flux in MDCK cells by lithium

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Submitted 26 July 2007; accepted in final form 30 December 2007

Lithium treatment has proved to be an effective pharmacological agent to control, among others, bipolar affective disorder (19), through mechanisms that are not yet well understood. Unfortunately, lithium (Li) treatment has toxic side effects on several organ systems including the kidney (31). Li treatment can result in an impaired ability to reabsorb water and therefore to concentrate the urine, leading in some cases to polyuria and polydipsia (1). Prolonged treatment can render this impairment progressively irreversible and nonresponsive to the action of vasopressin (AVP) (21, 25, 32).

Studies with animal models of Li treatment confirmed the polyuric effect of Li treatment (3, 35) and work with isolated renal tubular segments demonstrated that Li inhibits the AVP-induced water permeability, with the inhibition occurring at the level of cAMP generation (3–5). In rat medullary collecting duct cells, it has been suggested that this reduced cAMP generation results from activation of the inhibitory GTP binding protein, Gi (35), whereas in cultured LLC-PK1 cells, Li appears to inhibit adenylyl cyclase activity by disrupting the interaction between adenylyl cyclase and the stimulatory GTP binding protein, Gs (10).

More recent experiments with animals demonstrated that chronic Li treatment also leads to altered expression of transport proteins in the collecting duct. Of particular importance with respect to water handling is the observation that the AVP-regulated water channel aquaporin-2 (AQP-2) is dramatically downregulated in rats after 25 days of Li treatment (2, 15, 21). The same effect has been noted in a cultured cortical collecting duct cell line (20). Among the other aquaporins found in the kidney, AQP-3 is also downregulated, whereas AQP-1 and AQP-4 and several cation transporters are unaffected (17), but evidence is accumulating that chronic Li might have a fairly broad effect on gene expression (2, 28).

Much less attention has been given to the renal urea transporter whose physiological role is to maintain a high interstitial urea concentration and to support the formation of the cortico-medullary osmotic gradient (29). Klein et al. (15) showed that both urea transporter isogenes, UT-A1 in the renal tubule and UT-B in the vasa recta, are downregulated after 25 days of Li feeding. Furthermore, Li treatment reduced the phosphorylation of UT-A1 in response to AVP. However, there is no report in the literature on a possible effect of acute Li exposure on urea transport activity in the renal collecting duct.

The purpose of the present experiments was to explore the effect of acute Li treatment on short-term regulation of urea transport in a cellular model. We developed a permanently transfected Madin-Darby canine kidney (MDCK) cell line that constitutively expresses the UT-A1 protein. In the absence of agents known to stimulate the cellular cAMP levels, these cells exhibit a low urea permeability, comparable to that expected for the permeability of the lipid bilayer, but in the presence of AVP or forskolin, the transepithelial urea permeability is greatly enhanced through activation of UT-A1 (7, 8). The goal of the experiments was to test whether Li interfered with the activation of urea transport by AVP or forskolin and to compare this with what had been observed for osmotic water permeability.
MATERIALS AND METHODS

Reagents. All chemicals were reagent grade. Forskolin and the protein kinase inhibitor H-89 (Sigma Chemicals, 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 and thawed only once, further diluted for final use and the unused portion was discarded after the experiment.

UT-A1-expressing MDCK cells. The cell line used in these experiments, MDCK-UT-A1, is permanently transfected with the gene encoding the rat UT-A1 form of the urea transporter. The UT-A1 protein is constitutively expressed, but unless an activator such as AVP or forskolin is added, there is no significant UT-A1-mediated urea transport across the epithelium (8). The method of construction of this cell line and of characterizing the activation of urea transport by activators of cAMP levels has been described earlier (8). Cells were maintained in DMEM (GIBCO). To prepare them for a flux experiment, the cells were plated at a density of 2 × 10⁵ cells/cm² on collagen-coated Costar Transwell inserts (Corning, Acton, MA). Confluence was generally obtained after 4–5 days, at which point the filters reached a transepithelial resistance of typically >800 Ω·cm⁻².

Media. The flux media contained (in mM) 140 NaCl (“Na medium”) or 140 LiCl (“Li medium”), 5 urea, 1.5 K₂HPO₄, 5.5 glucose, 0.8 MgCl₂, 1.0 CaCl₂, and 24 HEPES, titrated to pH 7.64 at room temperature. To vary the Li concentration, the desired proportions of Na medium and Li medium were mixed. In most experiments, the flux medium was the same on both sides of the epithelium.

Urea flux measurements. Details of the method have been described previously (7, 8). Apical-to-basolateral tracer urea flux at 37°C was measured with cells grown to confluence in Transwell inserts. To start an experiment, an insert was placed into a well of a 12-well culture dish that resided on a thermostatted block, the (apical) medium was removed and from the insert replaced with ¹⁴C-urea-containing medium. At 3-min intervals, the insert was moved into a new well containing fresh flux medium. The flux was calculated from the radioactivity that had accumulated in the well (basolateral solution) during this period. The experimental setup permitted eight inserts to be fluxed in parallel in one experimental series. Typically, a flux experiment consisted of determining the unstimulated flux over three time points (9 min), followed by an experimental period of 54 min and ending with three time points during which the inserts were placed in medium containing 100 mM dimethyurea (DMU). The urea transport inhibitor DMU served to demonstrate that the activated flux was mediated by the urea transporter and not due to a nonspecific leak pathway. The summary bar graphs in the figures are the averages and standard deviations were calculated from the last four time points of the experimental treatment period (before the urea activation period) during this period. The experimental setup permitted eight inserts to be fluxed in parallel in one experimental series. Typically, a flux experiment consisted of determining the unstimulated flux over three time points (9 min), followed by an experimental period of 54 min and ending with three time points during which the inserts were placed in medium containing 100 mM dimethyurea (DMU). The urea transport inhibitor DMU served to demonstrate that the activated flux was mediated by the urea transporter and not due to a nonspecific leak pathway. The summary bar graphs in the figures are the averages and standard deviations of the last three to four time points of the activation period (typically 45–54 min after the start of flux activation).

Cellular cAMP determinations. Cells grown on Transwell filter inserts and exposed to the desired experimental conditions were harvested by transferring the filter inserts into an empty well of a 96-well plate precoated with collagen. Cells were scraped off the filter, collected together with the HCl supernatant in a microcentrifuge tube, and kept at room temperature for 10–15 min. They were stored in a −80°C freezer until the time they were analyzed. At that time, the samples were thawed at room temperature and centrifuged to pellet all insoluble cellular material. The cAMP assay was performed on typically 50 μl of the supernatant using the Direct Cyclic AMP immunoassay kit from Assay Designs (Ann Arbor, MI) and following the manual’s instructions. Each filter sample was assayed in duplicate. We added no phosphodiesterase inhibitor such as IBMX to the cells, unless specifically indicated for a given experiment.

Statistical analysis. Most figures report the results of a set of flux experiments on eight Transwell inserts. For the summary histograms, averages and standard deviations were calculated from the last four time points of the experimental treatment period (before the urea transport inhibitor DMU was added to test for UT-specific fluxes). Statistical significance is indicated by an asterisk above the bar. The summary graphs of Fig. 8 are the result of five separate series of experiments in which cells were both subjected to flux measurements and then harvested to determine cAMP levels. Statistical significance was determined by paired t-test.

RESULTS

Acute exposure to Li enhances AVP-induced urea transport. In experiments reported in the literature on isolated renal tubules, acute exposure to Li-containing medium attenuated the stimulatory effect of AVP on osmotic water permeability (4, 5). We therefore tested the effect of Li exposure on the activation of urea transport by AVP in our MDCK-UT-A1 cells (Fig. 1). In agreement with previous experiments, in the absence of an activator the trans-epithelial urea flux in the presence of 5 mM urea was low (1–3 nmol·cm⁻²·min⁻¹) and corresponded to the urea permeability of the plasma membrane (8). When the cells were exposed to the vasopressin analog
AVP, urea flux rose over the next 30 min to typically 10–15 nmol·cm⁻²·min⁻¹. In contrast, when the cells were transferred into a medium in which LiCl replaced NaCl as major salt, we observed two effects of Li. First, in the presence of Li alone, urea flux gradually increased to typically 5 nmol·cm⁻²·min⁻¹ over 60 min. Second, the activation of urea flux by AVP was greatly enhanced, with fluxes rising to typically 40 nmol·cm⁻²·min⁻¹.

Acute exposure to Li enhances the effect of other activators of urea flux. To test whether this effect was specific to AVP or could also be observed with other agents known to increase intracellular cAMP levels, we measured urea fluxes in cells in the presence of 10 μM forskolin, 200 μM phosphodiesterase inhibitor IBMX, and a combination of 200 mM IBMX and 10⁻⁸ M AVP (Fig. 2). As found with AVP, the effect of all three treatments was also significantly augmented by the presence of Li in the medium. Even the effect of forskolin, the strongest single activator in our previous experiments (7, 8), was further enhanced in Li medium.

Li enters the cell from the basolateral side on a Na-H exchanger. To test the concentration dependence of the Li effect, we mixed NaCl and LiCl media at several different ratios. Furthermore, to enhance the effect of Li, which by itself exhibited only moderate activation, we added IBMX (200 μM) to the basolateral medium. Figure 3 shows that the Li concentration dependence was essentially linear in the range up to at least 150 mM.

Figure 4 shows that the Li effect was asymmetric. When Li was present only on the apical side, it had no activating effect on urea transport. On the other hand, when added to the basolateral side only, its effect was comparable to that seen with Li present on both sides. However, we were able to elicit an effect by apical Li when we added the ionophore monensin to the apical side in the presence of Li (Fig. 4). This suggests that the Li effect is caused by Li entering the cell and not simply by binding to an extracellular basolateral site.

Furthermore, the Li effect appears to be specific to Li over other cations. In media in which NaCl was replaced isotonically by choline-Cl or MgCl₂, urea transport remained constant and at the same level as found for NaCl medium (Fig. 5).

To determine how Li might enter the cell, we employed several inhibitors of transport systems that are known to carry Li as a possible substrate. Figure 6 shows that neither chlorothiazide, an inhibitor of Na-Cl cotransport, nor furosemide, an inhibitor of Na-K-Cl cotransport, had any noticeable effect on the way Li activated urea transport. Neither was there an effect of the Na pump inhibitor ouabain (Fig. 6B). However, amiloride and its derivatives, especially those with a higher affinity for the Na-H exchanger, significantly suppressed the effect of Li. It therefore appears that Li entered the cells by means of a basolateral Na-H exchanger.

Li effect does not involve protein kinase A. The generally held notion of how AVP activates urea transport is by binding to the V₂ receptor which through the Gₛ protein activates adenylate cyclase and raises cAMP levels (13), in turn activating protein kinase A (PKA). In a previous study, we suggested that phosphorylation of the urea transporter and/or another mediator protein leads to an increased trans-epithelial urea permeability (7, 37). We also showed that the PKA inhibitor, H-89, blocked approximately one-half of the urea transport activation by AVP or forskolin in MDCK-UT-A1 cells, which suggests that at least some of the activation of was mediated by PKA (7). However, when we tested H-89 for its effect on the activation of urea transport by Li, we found that it had no inhibitory effect (Fig. 7).
Fig. 5. Urea fluxes in the presence of other cations substituted isosmotically for Na. Only Li exerted a stimulator effect.

Fig. 6. Effect of cation transport inhibitors on Li-stimulated urea fluxes. A: chlorothiazide: Na-Cl − cotransport; furosemide: Na-K-Cl − cotransport; amiloride: Na-H exchanger (low affinity) and epithelial Na channel (high affinity). B: ouabain: Na-K-ATPase; benzamil and hexamethylene amiloride (HMA) are higher-affinity inhibitors of Na-H exchange than amiloride.

Fig. 7. Effect of the protein kinase A inhibitor, H-89, on Li-stimulated urea fluxes. Basolateral H-89 was present throughout the entire experimental time course, including the first 3 time points before the switch to Li medium.

Fig. 8. Combined measurements of urea fluxes and cellular cAMP contents. Data are averages and standard deviations of 5 separate experimental series. At the end of the flux experiment (A), the cells from each filter were collected for determination of cAMP contents (B). Paired comparison of the measurements in Na vs. Li medium were performed to determine statistically significant differences (indicated by *) between the 2 groups.

Li can activate urea transport without stimulating cellular cAMP formation. Since activation of urea transport has, in general, been associated with increased cellular cAMP levels, we determined to which extent Li affected the cAMP levels in the MDCK-UT-A1 cells. In five series of experiments we combined measurements of urea flux after exposure to AVP, forskolin, and IBMX, in control (NaCl) media and in LiCl media, with measurements of the cellular cAMP content at the end of the flux experiment. In agreement with earlier experiments, Li enhanced the activation of urea flux in the absence and presence of flux activators AVP, forskolin, and IBMX (Fig. 8A). In particular, the combined stimulatory effect of Li and IBMX was significantly stronger than expected from the individual effects of Li and IBMX on the urea permeability. However, cAMP levels did not vary in accordance with the urea fluxes. Li by itself slightly stimulated cAMP levels but had no significant effect on cAMP levels in the presence of AVP and IBMX. However, it significantly suppressed forskolin-dependent cAMP formation (Fig. 8B).
To test whether the observed Li-dependent urea flux activation was due to increased movement of UT-A1 protein into the apical membrane, we also performed biotinylation experiments. Previously, we demonstrated that exposing UT-A1-MDCK cells to dDAVP or forskolin increased the amount UT-A1 protein in the apical membrane (14). However, exposing UT-A1-MDCK cells to Li under the same conditions as the flux experiments had no influence on the amount of apical biotinylation of UT-A1 protein (data not shown).

**DISCUSSION**

In this study, we tested our MDCK-UT-A1 cell line for its usefulness as a cellular model to investigate the acute effects of Li on the activation of inner medullary urea transport. The initial working hypothesis was that, since regulation of inner medullary urea permeability by AVP roughly parallels that of water permeability (33), Li would have similar effects on urea and water transport.

However, in apparent contrast to the generally found inhibitory effect of Li on osmotic water permeability, we found that, in the MDCK-UT-A1 cells, exposure to LiCl medium stimulated rather than inhibited the trans-epithelial permeability to tracer urea. This increased permeability was mediated specifically by the urea transporter protein, UT-A1, as evident from the return of the stimulated flux to baseline levels when the transport inhibitor DMU was added to the medium.

The Li effect was not duplicated by replacing NaCl with MgCl₂ or choline-Cl instead of LiCl. Thus, the activation of urea transport was not caused by the absence of Na but by the presence of Li in the medium. Even though Li by itself was active only when present on the basolateral side, it did activate urea fluxes from the apical side when the ionophore monensin was present on the apical side at the same time. In addition, we observed that amiloride and its analogs were able to inhibit the effect of basolateral Li. Taken together, these results suggest that Li had to enter the cell to exert its activating effect and that it did so by being transported on a basolateral Na-H exchanger.

The apical/basolateral asymmetry of the Li effect observed is opposite to the asymmetry found in rabbit cortical collecting tubules where only luminal Li inhibited cAMP formation (4). The reason for this apparent discrepancy is not known but could lie in the different segmental origins of the two cell types, as MDCK cells exhibit properties reminiscent of medullary collecting duct cells. It is possible that cortical and medullary collecting ducts possess different Li entry pathways. For example, the rat cortical collecting duct is known to contain apical ENaC channels that are permeable to Li (9). Since amiloride has an inhibitory potency for ENaC channels several orders of magnitude higher than that apparent in our studies (near 1 mM; Fig. 6B), it is most likely that in the case of the MDCK cells Li entered through a basolateral Na-H exchanger. In support of this notion, MDCK cells have been demonstrated to possess a Na-H exchanger (27). It is interesting, though, that in the isolated rabbit cortical collecting tubule Li exerted its (inhibitory) effect on osmotic water permeability from the apical but not from the basolateral side (4). Perhaps in this case Li entered through apical Na channels. It is not clear from which side Li entered in the more recent study on mouse cortical collecting duct-derived MpkCCD cells (27) as Li was added to both sides of the cultured cells.

The Li concentrations used in most of our experiments were higher than those used in previous experiments studying the effect of acute Li exposure (150 vs. 10–20 mM) and significantly higher than the estimated plasma Li concentrations in chronically exposed animals (as low as 1 mM). Using such a high concentration was a useful tool to increase the experimental signal, but the apparently linear dependence of the stimulatory effect on the Li concentration (Fig. 3) suggests that activation of urea transport already occurs at less than 50 mM Li without an additional activator. It should be noted that in our experiments activation of urea transport could be observed as early as after 15 min of exposure, whereas in the published studies the tissues were exposed for up to several hours before being assayed.

A major difference between the present experiments and most studies reported in the literature is the mode of exposure to Li. As expected for a drug that is used as chronic treatment, most studies have explored the effect of long-term (many days to weeks) exposure on renal function. It is clear from these reports that Li exposure for several days or weeks not only affects the gene expression of AQP-2 (17, 21) and UT-A (15) but also that of other transporters (17, 18, 28) and enzymes (16, 26) and eventually even alters the cellular composition of the collecting duct (2). In the case of AQP-2 it had appeared likely that cAMP influenced gene expression through cAMP-responsive transcription elements (22, 36), but more recent experiments point toward a cAMP-independent effect of chronic Li treatment (20). Considering the broad effect of Li on a long list of enzymes (11), other signaling pathways could also be involved in this pleiotropic response to chronic Li exposure (7, 20). With respect to the present study, it would appear reasonable to presume that only a small number of signaling pathways, excluding transcriptional processes, is responsible for the observed acute upregulation of the urea permeability in MDCK-UT-A1 cells.

Li alone stimulated urea transport, and it also enhanced the stimulation by AVP, forskolin, and IBMX, activators known to raise intracellular cAMP levels. Even at a saturating concentration of forskolin urea flux was further stimulated by Li. The Li effect thus appears to be additive to that of the other agents, as if Li raised the cAMP levels even further. However, this would contradict previous suggestions that Li inhibits adenylate cyclase activity (3–5, 10, 35). Our cAMP results are consistent with Li inhibiting adenyl cyclase because Li exposure greatly suppressed cAMP formation in the presence of forskolin. Alternatively, Li may also activate, directly or indirectly, a phosphodiesterase activity that is responsible for degrading cAMP. However, by itself Li did appear to raise the cAMP levels slightly. The mechanism for this weak stimulation is not clear. We can eliminate the involvement of PKA as a major participant in the activation process because H-89 had no effect on this activation. However, this observation by itself would not eliminate a more indirect activation pathway involving cAMP since we found that AVP- and forskolin-activated urea fluxes are only partially inhibited by H-89 (7). It is known that cAMP can activate the MAP kinase Erk1/2 cascade via Ephc/CAMP-GEF proteins, guanine nucleotide exchange factors for the small GTPase Rap1 (6) which feeds into this signaling cascade. It will be important to examine, in the future, whether in the UT-A1-MDCK cells, this pathway is...
activated in parallel to urea flux activation by AVP or forskolin.

From the comparison of the Li effects on urea flux activation and on cAMP generation, it would appear that urea transport can be activated without a concurrent stimulation of cAMP formation. It is unknown through which alternative signaling pathway Li may affect urea permeability. The hydrated Li ion is similar in size to magnesium and is known to interfere with Mg-requiring enzymes, including several different phosphatases and kinases (11). However, the cAMP values measured in cell extracts do not necessarily reflect the cAMP concentrations relevant for activating urea fluxes. Recent studies on several cellular systems demonstrated that signaling pathways involving cAMP can be physically isolated from the bulk cytoplasmic phase. For example, pathway components (adenylyl cyclase, PKA, and/or phosphodiesterase) can be anchored to compartments that are diffusively separated from the rest of the cell (12, 23, 30, 34).

Since one of the physiological end results of AVP binding to inner medullary collecting duct cells is activation of trans-epithelial urea transport, any signaling pathways to that effect necessarily converge at this point. Current evidence suggests that the point of convergence involves protein phosphorylation and trafficking of the UT-A1 urea transporter protein to the plasma membrane, similar to what has been found for the AQP-2 protein (14, 24). Therefore, one could surmise that the Li-mediated rise in trans-epithelial urea flux might also be accompanied by a UT-A1 protein shift into the apical membrane. However, we saw no such increase in the extent of biotinylation of UT-A1 protein upon exposure to Li (data not shown). This would suggest that activation of urea transport across UT-A1-MDCK cells involves not only trafficking to the membrane, but also modulation of individual channel activity after incorporation into the apical membrane.

In summary, the question about the detailed mechanism underlying the acute regulation of urea transport by Li is not clear. Cytoplasmic Li clearly has a suppressing effect on bulk cytoplasmic cAMP levels when these were raised by one of the traditional agonists, but it is possible that the cAMP that determines urea flux activity differs from the bulk cAMP because of diffusional isolation in a domain near the membrane. In addition, it will be of interest to examine the Li sensitivity of the components of the signaling cascade that governs the activation of urea transporter.

GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant PO1-DK-6152.

REFERENCES


