Amiloride restores renal medullary osmolytes in lithium-induced nephrogenic diabetes insipidus

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Lithium is a cheap and effective drug which is widely used in the treatment of bipolar disorder (28). However, a confounding problem is the induction, in some 70% of patients, of overt polyuria and polydipsia (9, 19, 41), which in ~20% of sufferers becomes irreversible and lead ultimately to nephrogenic diabetes insipidus (NDI). A small percentage progresses to chronic renal failure (3, 11, 29). A large number of papers in recent years have demonstrated that the prime cause of the lithium-induced polyuria is the failure of vasopressin-mediated insertion of the water channel protein aquaporin-2 (AQP2) into the apical membranes of the principal cells of the renal collecting ducts (17, 18, 22, 30). Lithium, entering the principal cells through the epithelial sodium channel (ENaC), inhibits the formation of cAMP (41), thus interrupting the chain of events in which the phosphorylation of PKA by cAMP leads, in turn, to the phosphorylation of one or more sites on AQP2, allowing translocation and insertion of AQP2 into the apical membrane (12). In the absence of apical AQP2, the normally increased water permeability of the membrane, induced by vasopressin, which would allow diffusion of water back into the renal interstitium down the established medullary osmotic gradient, does not occur. Additionally, reduction of cAMP levels, which normally act via the cAMP-response element to stimulate AQP2 production by binding to the promoter region of the AQP2 gene (23), leads to a decrease in AQP2 production. That lithium affects other routes of AQP2 insertion, however, is evidenced by the demonstration that lithium-induced downregulation of AQP2 and the subsequent development of NDI can occur independently of adenyl cyclase activity (20).

In addition to its effects on AQP2, therapeutic doses of lithium reduce the abundance of the urea transporters UT-A1 and UT-B in the renal medulla of rats and also inhibit the vasopressin-mediated phosphorylation of UT-A1 (14), thus potentially contributing to the loss or reduction of the medullary osmotic gradient. However, there have been no studies to date which have documented the lithium-induced changes in renal medullary osmolyte concentrations.

Lithium also has more widespread chronic renal effects. Microarray screening of gene expression in the renal medulla of lithium-treated compared with control rats demonstrated altered transcription and mRNA expression of a number of genes, including those involved in cellular proliferation and regulation of the actin cytoskeleton (6, 7, 13, 16, 31).

Since lithium enters the cells of the collecting duct through the ENaC, which has a higher permeability for lithium than sodium (10), early treatments to ameliorate the polyuria resulting from lithium administration utilized the ENaC channel blocker amiloride (1, 34). While this met with some empirical success, no studies have investigated in established lithium-induced NDI the effects of amiloride on renal medullary osmolyte concentrations and the interrelationship with aquaporin and urea transporter status. Here, we demonstrate that lithium treatment diminishes the osmotic gradient in the renal medulla, reflected in a marked reduction in both organic osmolyte and urea content. Following administration of amiloride to rats with established, lithium-induced NDI and continued lithium treatment, there is restoration of the urine concentrating mechanism, upregulation of AQP2, AQP3, and UT-A1 expression and an associated increase in medullary osmolytes.

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MATERIALS AND METHODS

Animal protocols. Ethical approval for the protocols described below was given by the University of Otago Animal Ethics Committee (ethics no. 15/04) under New Zealand national guidelines. Twenty-four Wistar male rats (200 g), from the HerCs-Taiier Resource Unit, University of Otago, were separated into three groups. To induce NDI, the protocol of Kwon and colleagues (17) was followed. Briefly, the control group (8 rats) was kept for 7 wk on a standard rodent diet (F49, Alliance Foods) and given tap water ad libitum. Two experimental groups received tap water and the same standard rodent food containing 40 mmol lithium carbonate/kg dry food for the first 7 days, followed by 60 mmol lithium carbonate/kg dry food (lithium diet) for the following 6 wk. It was found (16) that this protocol resulted in plasma lithium levels comparable to therapeutic levels in human serum (0.8–1.3 mmol/l) and minimized the weight loss caused by lithium. All rats administered lithium were given access to a salt block to maintain sodium balance and prevent lithium intoxication. Body weight and water intake were measured daily. At the end of week 4 (once NDI had been established), one group of rats on the lithium diet (lithium plus amiloride) was given drinking water containing amiloride (0.2 mmol/l), while the other group (lithium) continued on their lithium diet. A preliminary study to determine an effective dose-response curve for amiloride (data not shown) established that a concentration of 0.2–0.5 mmol/l amiloride in the drinking water was adequate to achieve a concentration in the urinary collecting ducts sufficient to completely block ENaC, with little difference between 0.2 and 0.5 mmol/l amiloride. Therefore, in the experimental studies 0.2 mmol/l amiloride was chosen as an appropriate dose for this study. Rats were housed individually in metabolic cages for 24 h on days 21, 28, 35, 42, and 49, and water intake (ml), food consumption (g), together with urine volume (ml) and composition (sodium, potassium, chloride, and osmotic pressure), were measured over that 24-h interval.

After 7 wk, all rats were euthanized and blood was collected. Both kidneys were excised immediately and cut longitudinally. Half of one kidney was fixed in 10% formol saline for immunohistochemistry. The remaining three halves were snap frozen in liquid nitrogen and stored at −80°C.

Tissue analysis. The osmolality of urine and plasma samples was determined using a vapor pressure osmometer (Wescor 5500, Logan, UT). Sodium, lithium, and potassium concentrations in the urine and plasma were determined by flame photometry (Radiometer FLM3), and lithium levels were confirmed by inductively coupled plasma (ICP) mass spectrometry (Thermo-Jarrell Ash Atomscan 25). Creatinine concentrations in urine and plasma were measured, after appropriate dilution, using Jaffé’s method, to confirm normal renal function. Amiloride concentration in urine was detected by HPLC using a Waters 5-μm Bondapak C18 column (44). Urine samples were passed through a 0.22-μm filter unit (Millipore) to remove particulate matter and then separated using a water-methanol mixture (75:25; vol/vol, adjusted to pH 3.6 with acetic acid) at a flow rate of 1 ml/min and a column temperature of 70°C. Amiloride was detected by fluorescence (Econo UV monitor, Bio-Rad, Richmond, CA) at 286 nm, identified by retention time of pure standards, and by coelution with added solute, and quantified by measurement of peak height and comparison with a series of standards from 0.1 to 0.3 mmol/l amiloride. Over this range, the response was linearly related to concentration.

Immunohistochemistry. For immunohistochemistry studies, kidneys were wax-embedded and sections cut at 4 μm. After rehydration, endogenous peroxidase activity was blocked with 3% H₂O₂ in PBS and the sections were preincubated in 1% BSA (Sigma) in PBS to block nonspecific binding. They were then labeled with the appropriate primary antibody. Antibodies used were rabbit anti-AQP1 (Alpha Diagnostics); rabbit anti-AQP2 (Chiron Mimotopes, Melbourne, Australia) (2); goat anti-AQP3 and 4 (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti-UT-A1 antibody (25). Labeling of the tissue was visualized using a horseradish peroxidase-coupled secondary antibody (goat anti-rabbit and rabbit anti-goat IgG, DAKO, Glostrup, Denmark), followed by incubation with diamobenzidine (Dako). After dehydration and clearing, sections were mounted in DPX, viewed using a Provis AX 70 Olympus microscope, and images of representative regions were recorded using a SPOT digital camera attached to a Macintosh computer running SPOT proprietary software. Later examination of the images was performed using ImageJ (National Institutes of Health).

Negative controls were carried out either by omitting the primary antibodies or by using appropriate blocking peptides (AQP1-BP, Alpha Diagnostics; AQP2-BP, Chiron Mimotopes; AQP3-BP and AQP4-BP, Santa Cruz Biotechnology).

SDS-PAGE and Western blotting. Frozen renal cortical and medullary tissue (50–100 mg) was homogenized (Ultra Turrex) in 0.5 ml, 0.3 mol/l sucrose, 0.25 mmol/l imidazole containing the protease inhibitors leupeptin (8.5 μmol/l) and PMSF (1 mmol/l). Following centrifugation to remove cell debris (5,000 g) for 15 min at 4°C, protein content of the supernatant was measured using BCA reagent (Pierce Chemical). A subsample was heated in Laemml buffer (1:2) for 4 min before being loaded on the gel. Equal amounts of protein samples per lane were separated electrophoretically (100 V for 5 min followed by 200 V for 30 min) on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride Immobilon membrane (Millipore) by electroblotting at 100 V for 1 h (Bio-Rad).

Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBS-T) followed by incubation with primary antibody at 4°C overnight. The membranes were washed, incubated with the secondary antibody, visualized with the luminescent substrate Super Signal West Pico (Pierce Chemical), and exposed to X-ray film. After development, films were scanned using a Bio-Rad (GS-700) densitometer using Molecular Analyst software.

Quantitative real-time RT-PCR. mRNA was extracted from 50- to 100-mg samples of renal cortex and medulla in 0.8 ml of TRIzol (Invitrogen). Equal amounts of mRNA were used for cDNA synthesis (Superscript First-Strand Synthesis System, Invitrogen). Real-time RT-PCR was carried out using an Assays-on-Demand Gene Expression kit (Applied Biosystems) in the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Reaction mixes [10 μl TaqMan Universal PCR MasterMix, No AmpErase (2X), 1 μl Assays-on-Demand Gene Expression Assay mix (20X, Applied Biosystems), 1 μl cDNA templates, or negative control (RNAse-free water), made up to 20 μl with RNase-free water] were loaded in triplicate, together with negative controls and two endogenous controls, β-actin and β2-microglobulin, onto a 96-well plate. Thermal cycling conditions set up in the ABI Prism 7300 Detection System were the following: an initial step of 50°C for 2 min, a denaturing step (95°C for 10 min), followed by 40 cycles of denaturing (95°C for 15 s) and annealing (60°C for 1 min). Fluorescence detection was by ABI Prism 7300 SDS Software (version 1.3.1. Applied Biosystems), and data analysis was performed using the comparative Ct method (5, 21).

Detection of renal osmolytes. Organic osmolytes [myo-inositol, glycerophosphorylcholine (GPC), sorbitol, urea and glycerine betaine] were analyzed in 50- to 100-mg samples of renal cortex and medulla by HPLC using a Sugar-Pak I column (Waters) (42). After perchloric acid extraction, samples were neutralized with potassium carbonate, and the supernatant obtained after centrifugation was passed through a SEP-PAK C18 filter. The centrifuged pellet was dissolved in 1 mol/l NaOH and used for determination of total protein using BCA reagent (Pierce Chemical).

Samples were separated using 0.13 mmol/l Ca-EDTA as a carrier, at a flow rate of 0.5 ml/min and a column temperature of 70°C. Osmolytes were detected by a Refractive Index detector (Jasco 830-HR). The data from the detector were digitized and integrated by a HP3395 Integrator (Hewlett-Packard), controlled by an IBM PC running the proprietary program PEAK 96 (Hewlett-Packard).
Presentation of data and statistical analysis. Quantitative data were presented as means ± SE. Statistical comparisons were accomplished using ANOVA followed by a Student-Newman-Keuls posttest or by Student’s two tailed t-tests, using Kaleidogaph software (Synergy Software, Reading, PA).

RESULTS

Physiological data. Rats fed the lithium diet ate less food and showed a decreased weight gain. Rats on a lithium-free diet, given amiloride only in their drinking water, showed similar weight gains to those of the control animals (data not shown). In the lithium group, polyuria and polydipsia were established within the first week and thereafter remained steady. Water intake was greatly increased with a corresponding increase in urine volume (Table 1). Plasma lithium at euthanasia was found to be 0.53 ± 0.05 mmol/l in the lithium-fed animals, but the other plasma values were not significantly different from controls, although plasma sodium was slightly lower. After 7 wk on the lithium diet, water intake was 206 ± 41 compared with 61 ± 6 μl·min⁻¹·kg body wt⁻¹ in the control animals. Urine output was correspondingly greatly increased over the controls, from 30 ± 3 to 168 ± 45 μl·min⁻¹·kg body wt⁻¹, while the urine-to-plasma osmolar concentration ratio fell from 4.1 ± 0.5 to 1.0 ± 0.3. Free water clearance was also greatly increased in the experimental group, from a mean of 0.11 ± 0.05 to 0.10 ± 0.03 l/kg body wt⁻¹·min⁻¹.

When amiloride (0.2 mmol/l) was added to the drinking water of rats fed the lithium diet, the polyuria and polydipsia rapidly diminished. Within 1 wk, the volume of water consumed was noticeably reduced and urine production decreased (data not shown). Analysis of the urine of these rats, at the end of the experimental period, revealed an amiloride concentration of 0.11 ± 0.01 mmol/l. Administration of amiloride and lithium, over a period of 3 wk, also resulted in a highly significant fall in urine production, to 108 ± 3 μl·min⁻¹·kg body wt⁻¹ compared with rats on the lithium diet (168 ± 45 μl·min⁻¹·kg body wt⁻¹), although this was still much greater than the control values. However, the rats in the lithium plus amiloride group were capable of concentrating their urine, up to a U/P ratio of 2.0, about twice that of the rats maintained on the lithium diet alone. Plasma creatinine levels were within the normal range in all groups.

Western blots of AQP1–4. When rats were fed a diet containing lithium there was a substantial fall, of >50%, in the AQP2 content of the renal medulla. Additionally, there was a fall of ~50% in the medullary abundance of AQP3 (Fig. 1, A and B). It has long been established that one of the principal effects of lithium on the kidney is a reduction in the expression of AQP2 in the collecting duct. When rats, already experiencing lithium-induced NDI, were given amiloride (0.2 mmol/l) in their drinking water, both aquaporins increased toward their original value over the subsequent 3-wk period, AQP2 reaching 80% of control values and AQP3 recovering to 100% of its control value, as judged by semiquantitative densitometry of Western blots. In contrast, AQP1 and -4, in the renal medulla, were apparently unaffected either by the lithium diet or by subsequent treatment with amiloride (Fig. 2, A and B).

Immunohistochemistry. Results of Western blotting were confirmed by immunohistochemistry. Figure 3, G–I, shows representative sections of the medullas of rats from the lithium and lithium plus amiloride groups probed to reveal the presence of AQP2, compared with control rats. AQP2 was barely detectable histochemically in the lithium-treated rats. However, after 3 wk of exposure to amiloride in their drinking water, rats in the lithium plus amiloride group showed an intensity of stain close to that of the controls. Similarly, AQP3 appeared to be considerably reduced in the renal medullas of the lithium group rats, while typically increasing after amiloride (Fig. 3, J–L).

In contrast, neither AQP1 (Fig. 3, A–C) nor AQP4 (Fig. 3, D–F) abundance appeared to be affected by either treatment.

Table 1. Physiological data of rats fed a lithium diet and rats with lithium-induced NDI given drinking water containing 0.2 mmol/l amiloride

<table>
<thead>
<tr>
<th></th>
<th>Control (60 mmol Lithium (7 wk))</th>
<th>60 mmol Lithium (7 wk) + 0.2 mmol/l Amiloride (3 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>416 ± 8</td>
<td>384 ± 7*</td>
</tr>
<tr>
<td>Water intake, μl·min⁻¹·kg BW⁻¹</td>
<td>61 ± 6</td>
<td>206 ± 41*</td>
</tr>
<tr>
<td>Urine output, μl·min⁻¹·kg BW⁻¹</td>
<td>30 ± 3</td>
<td>168 ± 45*</td>
</tr>
<tr>
<td>Osmplasma, mmol/kg H₂O</td>
<td>295 ± 4</td>
<td>298 ± 4</td>
</tr>
<tr>
<td>Na₂plasma, mmol/l</td>
<td>143 ± 2</td>
<td>139 ± 3</td>
</tr>
<tr>
<td>K₂plasma, mmol/l</td>
<td>5.5 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>L₂plasma, mmol/l</td>
<td></td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Osmurine, mmol/kg H₂O</td>
<td>1.21 ± 0.9</td>
<td>287 ± 19*</td>
</tr>
<tr>
<td>(U/P)osm</td>
<td>4.10 ± 0.5</td>
<td>0.96 ± 0.3*</td>
</tr>
<tr>
<td>C₀₂deo, μl·min⁻¹·kg BW⁻¹</td>
<td>−93 ± 7</td>
<td>+7 ± 17*</td>
</tr>
<tr>
<td>N₂urine, mmol/l</td>
<td>36 ± 2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>N₂urine, mmol·kg BW⁻¹·day⁻¹</td>
<td>1.7 ± 0.2</td>
<td>5.4 ± 2.6</td>
</tr>
<tr>
<td>K₂urine, mmol/l</td>
<td>154 ± 10</td>
<td>28 ± 2*</td>
</tr>
<tr>
<td>K₂urine, mmol·kg BW⁻¹·day⁻¹</td>
<td>6.6 ± 0.5</td>
<td>6.7 ± 1.7</td>
</tr>
<tr>
<td>Amilorideurine, mmol/l</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE. NDI, nephrogenic diabetes insipidus; BW, body wt; Osm, osmolality; ND, not determined. *P < 0.05 control value compared with control. †P < 0.05 lithium treated rats compared with those administered lithium and amiloride.
Quantitative real-time RT-PCR. Analysis of mRNA abundance by quantitative RT-PCR showed the same general trends as those revealed by Western blotting and immunohistochemistry (Fig. 4). AQP2 was greatly reduced by treatment with lithium but largely restored by administration of amiloride, and the same appeared to be true for AQP3. AQP4 mRNA abundance was seemingly unaffected by lithium exposure and remained at the same level in all experimental treatments. Anomalously, AQP1 mRNA appeared to undergo a very significant reduction on lithium exposure, which was reversed in the presence of amiloride, even though the abundance of AQP1 protein, as assessed by Western blotting and immunohistochemistry, appeared unaffected.

Fig. 1. Abundance of aquaporin-2 (AQP2) and aquaporin-3 (AQP3) protein in kidney inner medullas from control rats, rats fed LiCO₃ for 7 wk and rats with lithium-induced nephrogenic diabetes insipidus (NDI) given amiloride (0.2 mmol/l) in their drinking water for 3 wk. A and B: densitometric measurements of AQP2 (A) and AQP3 (B) on gels. Bottom: representative Western blots showing AQP2 (C) and AQP3 (D) of control, lithium-fed, and lithium-fed with amiloride rats. Each lane represents a sample from a single rat. Both aquaporins are substantially reduced in the lithium-fed rats and recover after drinking water contained amiloride. Values are means ± SE; N = 4 rats/group. * P < 0.05 compared with controls.

Fig. 2. Abundance of AQP1 and AQP4 protein in kidney inner medullas from control rats, rats fed LiCO₃ for 7 wk, and rats with lithium-induced NDI given amiloride in their drinking water for 3 wk. A and B: densitometric measurement of AQP1 (A) and AQP4 (B) on gels. Bottom: representative Western blots showing AQP1 bands (C) and AQP4 (D) of control, lithium-fed, and lithium-fed with amiloride rats. Each lane represents a sample from a single rat. Both aquaporins are substantially unchanged by either treatment. Values are means ± SE; n = 4 rats/group.
Figure 5 showed that in rats after 7 wk on a lithium diet, the urea transporter UT-A1, which was relatively weakly staining in the collecting duct cells of the inner renal medulla, had become almost undetectable in this region. In contrast, in the rats given amiloride for 3 wk, concurrently with lithium, UT-A1 expression was greatly enhanced in the inner medulla. It is interesting that although the amount of UT-A1 protein present is greatly increased on administration of amiloride to the rats with lithium-induced NDI (Fig. 6), this is not reflected in an abundant increase in the immunohistochemical localization (Fig. 5).

**Osmolytes in the kidney.** After 7 wk with animals on the lithium diet, medullary urea had fallen to ~30% of the control values, and this was associated with corresponding highly
significant decreases in the osmolytes inositol, sorbitol, betaine, and GPC (Table 2). Urea, inositol, sorbitol, GPC, and betaine all decreased in the presence of 60 mmol lithium, and apart from sorbitol, are partially, or completely, restored toward control values following amiloride treatment (Table 2). Thus amiloride treatment essentially restores the medullary osmotic gradient with the formation of a urine almost as concentrated as in the untreated control rats.

Amiloride (0.2–0.5 mmol/l) in the drinking water by itself was found to have no significant effect on the osmolytes in the renal medulla (data not shown). It is interesting that although the amount of UT-A1 protein present had greatly increased in the rats with lithium-induced NDI, when these rats were administered amiloride this was not reflected in an increase in the accumulation of urea. Although substantially augmented, urea content still remained significantly less that in the control animals.

DISCUSSION

This study has clearly demonstrated that in lithium-induced NDI there is a substantial reduction in the concentration of renal medullary organic osmolytes (urea, inositol, sorbitol, GPC, betaine, Table 2) with reduced expression of AQP2, AQP3, and UT-A1, which shows that the inability of these animals to concentrate the urine is due not only to a loss of the ability to modulate the water permeability of the apical membrane of the principal cells of the renal collecting duct, but also to the severe reduction of the medullary osmotic gradient. Although it has been known for many years that chronic lithium administration, in both rats and humans, induces an extensive diuresis (9, 17, 29), by preventing the osmotic retrieval of water from the collecting duct, this is the first time that it has been demonstrated that this treatment also greatly reduces the medullary osmotic gradient, as evidenced by the

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Fig. 4. Quantitative RT-PCR analysis of AQP mRNAs from medullas from control rats, rats fed LiCO3 for 7 wk, and rats with lithium-induced NDI given amiloride (0.2 mmol/l) in their drinking water for 3 wk. Results are expressed as C_t values relative to the control (1.0). mRNA abundance of AQP1, AQP2, and AQP3 falls in the presence of lithium and is partially restored with amiloride treatment.

Fig. 5. Immunohistochemical detection of an urea transporter (UT-A1) in the inner stripe of the outer medullas and inner medullas of control rats, rats fed LiCO3 for 7 wk, and rats with lithium-induced NDI given amiloride (0.2 mmol/l) in their drinking water for 3 wk. A–C: inner medullas of control rats (A) reveal weak staining of cells of the collecting ducts, and this is further diminished in lithium-fed rats (B). In contrast, rats given amiloride in their drinking water show a greatly increased intensity of staining (C). Magnification ×40.

Fig. 6. Abundance of urea transporter UT-A1 protein in kidney inner medullas from control rats, rats fed LiCO3 for 7 wk, and rats with lithium-induced NDI given amiloride (0.2 mmol/l) in their drinking water for 3 wk. A: densitometric summary of the sum of the glycosylated and nonglycosylated UT-A1 bands on the gels. B: representative Western blots showing UT-A1 bands of control, lithium-fed, and lithium-fed with amiloride rats. Each lane represents a sample from a single rat. UT-A1 is substantially reduced in the lithium-fed rats but greatly enhanced after drinking water contained amiloride. Values are means ± SE; n = 4 rats/group. *P < 0.05 compared with controls.
diminution of intracellular organic osmolytes. In this respect, it resembles the consequences of diuresis induced by other means, such as water loading (43), treatment with diuretics such as frusemide (36), or by inhibition of vasopressin action (24). Furthermore, treatment with amiloride results in a substantial restoration of the concentrating power of the kidney, and in the amounts of intracellular organic osmolytes, even with the ongoing administration of lithium. Treatment with amiloride resulted in a significantly enhanced expression of UT-A1 in the medullary collecting ducts despite ongoing lithium therapy. The effect of lithium on the urea transporter UT-A1 appears to be similar to its action in preventing the mobilization and membrane insertion of AQP2 via a cAMP-dependent pathway. Klein and colleagues (14) demonstrated that lithium-treated rats had greatly reduced levels of the urea transporter UT-A1, which was confirmed in the present work. Sands and coworkers (32) had previously shown that vasopressin increased urea permeability in rat collecting ducts, and Star and colleagues (37) showed that this was via a cAMP-dependent pathway. This finding was further developed by Zhang and coworkers (45), who reported that the action of vasopressin on UT-A1 was through PKA-dependent phosphorylation, while Klein and colleagues (14) showed that suspensions of inner medullary collecting duct cells of lithium-fed rats, exposed to physiological concentrations of vasopressin, did not have the anticipated increased levels of phosphorylated UT-A1.

Semiquantitative analysis of protein abundance by Western blotting of extracts of the renal medulla revealed that AQP2 and AQP3 are reduced to about half their relative abundance after lithium treatment, while the amount of AQP1 and AQP4 is unchanged. After amiloride treatment, the amount of all four aquaporins returned to near control values. Immunohistochemical probing of the medulla showed a similar pattern. Both AQP2 and AQP3 were substantially reduced in the collecting ducts of lithium-treated rats and were increased in the presence of amiloride. In contrast, AQP1 and AQP4 did not show any marked changes with either treatment.

This result was confirmed, with respect to AQP2 and AQP3, by analysis of mRNA abundance by quantitative RT-PCR. However, although mRNA encoding AQP1 was found to have been substantially decreased in the lithium-treated rats, the expression of this protein appeared unchanged. AQP1 is constitutively expressed in the proximal tubule and descending limb of the loop of Henle. It is also expressed on the vasa recta to facilitate the diffusion of water back into the intravascular compartment. We have previously shown the AQP1 expression is modified in many forms of renal injury (2). Following the administration of amiloride, aquaporin mRNA levels in the renal medulla returned to control levels, with the exception of that encoding AQP3, which was increased but which remained significantly below control values.

ENaC is also affected by lithium. Nielsen and colleagues (26) reported that in the cortical collecting duct of lithium-treated rats, expression of the α-ENaC subunit and its trafficking is no longer mediated by aldosterone, and they suggested that this may be a principal cause of the increased salt wasting associated with lithium treatment. Thus failure of the kidney to retain both urea and salt in the renal medulla contributes to the loss of the medullary gradient. It has been known for many years that lithium uptake by collecting duct cells is inhibited by a high-salt diet (35). It has been postulated that this arises because, even though ENaC, the entry channel for lithium, is preferentially selective for lithium, the exit pathway is still obscure, although the best candidate is the sodium hydrogen exchanger NHE1 (40). In the presence of high salt concentrations in the collecting duct, depolarization of the apical membrane of the principal cells lowers the driving force for lithium entry. This may in part at least explain why not all patients taking lithium experience NDI. Amiloride, however, has long been known to inhibit very effectively the uptake of lithium into the collecting duct and has been used clinically to alleviate or ameliorate lithium-induced polyuria (8), as confirmed in two earlier clinical studies which demonstrated its effectiveness (1, 15).

Vasopressin-mediated water uptake in the collecting duct only accounts for 5–10% of water reabsorption. Therefore, if the lithium-induced loss of the medullary concentration gradient was due to polyuria induced by blocking vasopressin-mediated insertion of the water channel protein AQP2 into the apical membranes of the principal cells of the renal collecting ducts (17, 18, 22, 30) causing washout of the medulla, one would have to postulate that the inner medullary collecting cells have become permeable to water in the absence of aquaporins, which would seem unlikely. Similarly, the modulating actions of amiloride were evident despite ongoing administration of lithium. In addition, Ottosen and colleagues (27) have demonstrated that morphological and enzyme histological changes induced by lithium are not due to the accompanying polyuria.

Since the concentration of amiloride in the drinking water was sufficient to ensure that its concentration in the collecting duct (0.11 ± 0.01 mmol/l in the urine) was well above that required to ensure complete inhibition of ENaC, it may be concluded that lithium entry to the principal cells of the collecting duct has been inhibited or at least greatly reduced and that this is the principal cause of the partial recovery from NDI shown by the amiloride-treated rats. Rats in the present work excreted amiloride at a rate of ∼1.1 mmol·min⁻¹·100 g body wt⁻¹, on the same order as that of Shalmi and coworkers (33), who estimated that the concentration of amiloride in the proximal tubules was well below that reported to block the Na⁺/H⁺ exchange mechanism on the proximal tubule (the inhibitory constant, $K_i > 50–100 \text{ mmol/l}$) but, at ∼100 mmol/l in the collecting duct, well above that needed to prevent movement of sodium through the epithelial sodium channel ($K_i < 1 \text{ mmol/l}$) (4). At these concentrations, Thomsen and others (38) found that amiloride also inhibited lithium and sodium reab-

### Table 2. Osmolytes in renal medulla of rats fed a lithium diet and in rats with lithium-induced NDI given drinking water containing 0.2 mmol/l amiloride

<table>
<thead>
<tr>
<th>Osmolyte</th>
<th>Control</th>
<th>60 mmol Lithium</th>
<th>60 mmol Lithium + 0.2 mmol/l amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol, mmol/kg protein</td>
<td>221 ± 35</td>
<td>85 ± 10*</td>
<td>179 ± 8†</td>
</tr>
<tr>
<td>Sorbitol, mmol/kg protein</td>
<td>35 ± 9</td>
<td>3 ± 1*</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>GPC, mmol/kg protein</td>
<td>352 ± 80</td>
<td>91 ± 20*</td>
<td>231 ± 38†</td>
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<tr>
<td>Betaine, mmol/kg protein</td>
<td>69 ± 11</td>
<td>38 ± 38*</td>
<td>84 ± 10</td>
</tr>
<tr>
<td>Urea, mmol/kg protein</td>
<td>2,868 ± 624</td>
<td>480 ± 117*</td>
<td>2,132 ± 184†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with control values. †P < 0.05 compared with lithium only values.
amiloride and lithium-induced nephrogenic diabetes insipidus

F819

sorption in the proximal tubules of conscious rats and suggested that the reason such a dose of amiloride showed no effect on sodium reabsorption by the proximal tubule in microperfusion studies was due to the activation of sodium-retaining mechanisms by acute anesthesia or surgery (39). In the present study, we conclude that the effect of amiloride is predominantly in preventing the uptake of lithium into the principal cells of the collecting ducts.

Lithium is transported preferentially into cells via sodium channels and presumably needs to achieve adequate intracellular concentrations to have a deleterious effect on cellular function, including inhibition of adenyl cyclase. This study and others have clearly demonstrated an effect of lithium on aquaporins, urea transporters, and organic osmolytes in the inner medulla. Similarly, amiloride at the concentrations used in the study inhibits ENaC and is unlikely to modify other sodium channels. It seems possible that the reduction of cortical collecting duct uptake of lithium by amiloride could preserve cortical salt and water reabsorption, leading to delivery of a reduced water load to the medulla, with consequent effects on expression of medullary transporters. Amiloride administration in control animals produced a small degree of sodium wasting, as would be expected, but no significant changes in urinary concentrating ability or medullary organic osmolytes. Therefore, further studies are required to elucidate the actions of amiloride.

Data presented here provide additional information as to how amiloride may be an effective means of restoration of the impaired renal concentrating mechanism after lithium-induced NDI is established, as has been demonstrated in earlier clinical studies (1, 15). Given that amiloride’s effect appears to be greater than what would be expected from merely competitively inhibiting lithium uptake via ENaC in the principal cells of the collecting tubule, further studies are required to fully elucidate the mechanisms of protection. It also remains to be established as to whether amiloride therapy will also reduce the chronic interstitial fibrosis that is associated with long-term lithium exposure (29). Studies are currently underway in our laboratory investigating these potential pathways.

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REFERENCES