Changes in cellular composition of kidney collecting duct cells in rats with lithium-induced NDI

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Changes in cellular composition of kidney collecting duct cells in rats with lithium-induced NDI. Am J Physiol Cell Physiol 286: C952–C964, 2004. First published November 12, 2003; 10.1152/ajpcell.00266.2003.—Lithium treatment for 4 wk caused severe polyuria, dramatic downregulation in aquaporin-2 (AQP-2) expression, and marked decrease in AQP-2 immunoreactivity with the appearance of a large number of cells without AQP-2 labeling in the collecting ducts after lithium treatment. Surprisingly, this was not all due to an increase in AQP-2-negative principal cells, because double immunolabeling revealed that the majority of the AQP-2-negative cells displayed [H+]ATPase labeling, which identified them as intercalated cells. Moreover, multiple [H+]ATPase-labeled cells were adjacent, which was never seen in control rats. Quantitation confirmed a significant decrease in the fraction of collecting duct cells that exhibited detectable AQP-2 labeling compared with control rats: in cortical collecting ducts, 40% of intercalated (H+/ATPase) cells was shown.

Urine output, whole kidney AQP-2 expression, cellular organization, and the fractions of principal and intercalated cells in cortex and inner medulla returned to control levels after 4 wk on a lithium-free diet following 4 wk on a lithium-containing diet. In conclusion, lithium treatment not only decreased AQP-2 expression, but dramatically and reversibly reduced the fraction of principal cells and altered the cellular organization in collecting ducts. These effects are likely to be important in lithium-induced nephrogenic diabetes insipidus.

Nephrogenic diabetes insipidus (NDI) is characterized by the inability of the kidney to concentrate urine in response to vasopressin. In rare cases, NDI can occur as an inherited disorder, which is due to mutations in the vasopressin-V2-receptor or AQP2 genes (31, 34, 42). However, most commonly the disease is acquired, and often it occurs as a side effect in humans subjected to different drug treatments. Lithium is widely used for treatment of bipolar affective disorders and is known to cause NDI or urinary-concentrating defects in as many as 40% of patients who take the medication (37). Lithium-induced NDI as well as other acquired forms of NDI (e.g., hypokalemia, hypercalcemia, and postobstructive NDI) have been shown to be associated with a downregulation in aquaporin-2 (AQP-2) protein expression (9, 13, 25, 29, 30, 35). AQP-2 is a vasopressin-regulated water channel that is present in kidney collecting duct principal cells and connecting tubule cells. The mechanism by which lithium inhibits the expression of AQP-2 is not clearly understood. It may inhibit the activity of adenylyl cyclase and thus prevent the production of cAMP and subsequently the activity of protein kinase A, which are involved in the regulation of AQP-2 expression as well as vesicular trafficking of AQP-2 (7).

It was previously shown that lithium induces structural changes and affects cell proliferation in kidneys (12, 17, 19). In addition, lithium has effects on both inhibition and stimulation of cell proliferation in other systems. Lithium treatment of nephrectomized rats was shown to cause increased proliferation of neural lobe astrocytes in vivo (26). In contrast, lithium decreased cell proliferation of primary bovine aortic endothelial cells by causing sustained G2/M cell cycle arrest without affecting cell viability. This effect was partly reversible after lithium cessation (28). In another study, lithium also caused accumulation of HL-60 cells in the G2/M phase, and this was associated with the onset of apoptosis (27). Aside from having an effect on AQP-2 protein levels, one can hypothesize that lithium may also affect cellular composition in kidney collecting ducts. Indeed, the cellular composition of adult rat kidney collecting ducts was previously shown to be remodeled by chronic carbonic anhydrase inhibition (3). Kidney collecting ducts are composed of principal and intercalated cells. In rat cortical collecting ducts (CCDs), the percentage of principal cells is ~60% (22), and this number increases slightly along the outer and inner stripes of outer medullary collecting ducts; in the proximal part (IM-1) of inner medullary collecting ducts (IMCDs), ~90% of the cells are principal cells. In the middle part of the inner medulla only a very small amount of intercalated cells is found, and no intercalated cells are located in the distal part of the inner medulla. The intercalated cells are involved in acid-base balance, whereas the principal cells are responsible for hormonally regulated sodium and water reabsorption.

In this study, we investigated whether the cellular composition of collecting ducts changes during chronic lithium treatment and whether the effects were reversible after a 4-wk recovery period.

MATERIALS AND METHODS

Experimental Animals and Protocols

These experiments conform to the guidelines of the National Institutes of Health.

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Wistar rats were obtained from M & B (Ejby, Denmark). Lithium bicarbonate was solubilized in water and a small amount of hydrogen chloride, and the lithium chloride solution was added to food to yield a lithium concentration of 40 or 60 mmol/kg of dry food as previously described (29).

Protocol 1. In protocol 1, rats received food that contained a lithium concentration of 40 mmol/kg of dry food for the first 7 days, and the next 3 wk, the lithium concentration was increased to 60 mmol/kg of dry food \((n = 10)\). This protocol was previously shown to result in therapeutic plasma lithium levels (0.8–1.3 mM; Ref. 29). All rats on lithium therapy had access to a sodium chloride block to ensure adequate intake of sodium chloride and thereby prevent lithium intoxication and a fatal outcome \((40)\) and also to avoid negative sodium balance. Control rats received normal food \((n = 10)\). All rats had free access to water and food.

Protocol 2. In a second protocol, 10 rats were placed on a lithium diet as described above for 4 wk, and 10 rats received normal food. After the 4-wk treatment, half of the lithium-treated rats and half of the control rats were killed \((n = 5)\). The remaining rats \((n = 5)\) were fed a lithium-free diet and were studied for an additional 4 wk.

During the last part of the experiments, rats were housed in metabolic cages for 3–7 days to measure urine output and osmolality.

**Immunocytochemistry**

**Fixation.** In protocol 1, both kidneys were perfusion fixed via the abdominal aorta with either PLP (2% paraformaldehyde, 0.01 M NaNO\(_3\), 0.075 M L-lysine, 0.0375 M Na\(_2\)HPO\(_4\), pH 6.2; for light microscopy; \(n = 4\)) or 0.1% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate, pH 7.4 (for immunoelectron microscopy; \(n = 5\)). The perfusion-fixed kidneys were postfixed for 30 min in 0.1 M sodium cacodylate, pH 7.4, and were subjected to paraffin embedding or cryostabilization. In protocol 2, one kidney was removed for homogenization and immunoblottting before fixation. The other kidney was perfusion fixed in 3% paraformaldehyde in 0.1 M cacodylate, pH 7.4, and after postfixation, kidneys were subjected to paraffin embedding.

**Preparation of tissue for light and laser confocal microscopy.** Before paraffin embedding was performed, tissue blocks from whole kidneys were dehydrated in a graded series of ethanol (2 h each in 70, 96, and 99%, respectively) and xylene (overnight). Paraffin sections (2-µm thick) were cut on a Leica RM 2126 microtome and dried overnight at 37°C.

**SINGLE LABELING.** Sections were incubated with affinity-purified polyclonal antibodies against AQP-2 (LL127, 1:10,000 or 1:15,000 dilution, raised to a COOH-terminal peptide of the bovine 31-kDa subunit of vacuolar \(H^+\)ATPase, kindly provided by Stephen Gluck). Labeling was visualized with Alexa 488- and Alexa 546-conjugated goat anti-rabbit IgG as well as on sections labeled with either polyclonal AQP-2 or \(\text{[H}^+\text{]}\)ATPase antibodies and peroxidase-conjugated goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles. Grids were counterstained with uranyl acetate for 10 min and lead citrate for 5 s.

**Double LABELING.** Three different double-labeling experiments were performed as follows: (1) rabbit polyclonal AQP-2 antibody (LL127, 1:1,000 dilution) and rabbit polyclonal \(\text{[H}^+\text{]}\)ATPase antibody (LL615, 1:200 dilution); (2) \(\text{band 3-like Cl}^-/\text{HCO}_3^-\) exchanger anion exchanger isoform 1 (AE-1, 1:30,000 dilution, kindly provided by Dr. Philip S. Low, Purdue University) and rabbit polyclonal \(\text{[H}^+\text{]}\)ATPase antibody (LL615, 1:200 dilution); and (3) rabbit polyclonal AQP-4 antibody (LL182, 1:300 dilution, raised to a COOH-terminal peptide of rat AQP-4) and rabbit polyclonal \(\text{[H}^+\text{]}\)ATPase antibody (LL615, 1:200 dilution). Sections were incubated overnight at 4°C with either AQP-2, AE-1, or AQP-4 antibodies before undergoing incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualization by DAB (brown color) as described above. Sections were then incubated in 3.5% \(\text{H}_2\text{O}_2\) in methanol to remove any remaining peroxidase from the first staining. Sections were incubated overnight at 4°C with \(\text{[H}^+\text{]}\)ATPase antibody before being incubated with horseradish peroxidase-conjugated secondary antibody. For detection of \(\text{[H}^+\text{]}\)ATPase, Vector SG substrate (Vector Laboratories) was used as the chromogen to produce a gray-blue label, which is easily distinguished from the brown label produced by DAB in the first immunolabeling procedure for AQP-2, AE-1, and AQP-4, respectively. The sections were washed with distilled water and counterstained with hematoxylin.

Double labeling was also performed with polyclonal antibodies against rat AQP-2 (LL127, 1:5,000 dilution) and monoclonal antibodies against \(\text{[H}^+\text{]}\)ATPase (E11, 1:300 dilution, raised to a COOH-terminal peptide of the bovine 31-kDa subunit of vacuolar \(\text{[H}^+\text{]}\)ATPase, kindly provided by Stephen Gluck). Labeling was visualized with Alexa 488- and Alexa 546-conjugated secondary antibodies (Alexa 488 anti-rabbit and Alexa 546 anti-mouse, respectively). The polyclonal \(\text{[H}^+\text{]}\)ATPase antibody only labels intercalated cells similar to an antibody raised against a shorter sequence of the vacuolar \(\text{[H}^+\text{]}\)ATPase 56-kDa subunit (32), whereas the monoclonal \(\text{[H}^+\text{]}\)ATPase antibody also stains the brush border and subvillar invaginations of proximal tubules (16).

For both double-labeling procedures, relatively high concentrations of AQP-2 antibody were used to ensure that cells that expressed even very low levels of AQP-2 were detected. Light microscopy was carried out using a Leica DMRE microscope or a Leica DMIRE2 laser confocal microscope.

**Preparation of tissue for immunoelectron microscopy.** Tissue blocks prepared from the proximal parts of kidney inner medulla and cortex were infiltrated with 2.3 M sucrose for 30 min, mounted on holders, and rapidly frozen in liquid nitrogen. Frozen tissue blocks were subjected to cryostablization and Lowicryl HM20 embedding. Cryostablization was performed as previously described (33). Ultrathin (80-nm thick) Lowicryl sections were cut on a Reichert Ultracut FCS microtome and were preincubated with 0.05 M Tris-HCl (pH 7.4) with 0.1% Triton X-100 that contained 0.1% sodium borohydride and 0.05 M glycine and were then incubated with identical concentrations of Tris-HCl and Triton X-100 but with 0.2% skimmed milk. The preincubation was followed by incubation with affinity-purified polyclonal antibodies against AQP-2 (LL358, raised to a COOH-terminal peptide of human AQP-2, inner medulla) or \(\text{[H}^+\text{]}\)ATPase (LL615, cortex), and labeling was visualized with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles. Grids were counterstained with uranyl acetate for 10 min and lead citrate for 5 s.

**Length Measurements of Apical Plasma Membrane Domains**

Electronic images were taken of whole kidney sections labeled with polyclonal anti-AQP-2 antibody and peroxide-conjugated secondary antibody (4 pictures per rat in each zone). Photomicrographs were imported into CoreDraw software (Corel), and the apical plasma membranes of collecting ducts and connecting tubules were traced using a polylne. This outline was copied, and in the copy the linear sections corresponding to the AQP-2-negative regions were deleted. The micrograph was then deleted, and the remaining lines were exported to Scion Image software (National Institutes of Health), where the total length of the outline and the lengths of the sections corresponding to AQP-2-positive cells were determined.

**Quantification of Principal and Intercalated Cells using Anti-AQP-2 and/or Anti-\(\text{[H}^+\text{]}\)ATPase Antibodies**

Cell counting was performed on kidney sections that were single labeled with either polyclonal AQP-2 or \(\text{[H}^+\text{]}\)ATPase antibodies and peroxide-conjugated goat anti-rabbit IgG as well as on sections double labeled with polyclonal AQP-2 and \(\text{[H}^+\text{]}\)ATPase antibodies and peroxide-conjugated secondary antibodies. Counting was performed either on the microscope or on electronic images taken with a \(\times 25\) objective. The numbers of positive (labeled) and negative (un-
labeled) cells with a distinct nucleus were counted. On kidney sections from rats subjected to protocol 1, cell counting was performed on sections labeled with either AQP-2 or [H\(^{38}\)]ATPase. The counting was performed in cortex, inner stripe of outer medulla (ISOM), and IM-1, and no distinction was made between collecting ducts and connecting tubules. The cell counting on double-labeled sections was performed only in collecting ducts in cortex, ISOM, and IM-1.

In the recovery study (protocol 2), counting was performed on collecting duct in cortex and IM-1 on AQP-2-single-labeled sections. The fractions of AQP-2- and [H\(^{38}\)]ATPase-labeled cells as well as nonlabeled cells, respectively, were calculated from the numbers of positive and negative cells divided by the total number of cells counted in each animal. To calculate the fractional increase or decrease, this number was then divided by the mean control value, and the average fractional values in the two respective groups were calculated.

The total numbers of cells counted on AQP-2-single-labeled sections (protocol 1) were 1,843 in IM-1, 1,597 in ISOM, and 1,524 in cortex of control animals (single-labeled sections), the numbers were 2,535 in IM-1, 2,410 in ISOM, and 2,119 in cortex of lithium-treated rats (n = 4). On double-labeled sections (protocol 1), the numbers were 962 in IM-1, 794 in ISOM, and 5,579 in cortex of control animals (n = 3) and 1,809 in IM-1, 1,498 in ISOM, and 6,485 in cortex of lithium-treated animals (n = 4). In the recovery study (protocol 2), the total numbers of counted cells in cortex were 1,995 cells in controls (n = 4) and 1,552 cells in recovered animals (n = 4); in IM-1, 1,810 cells were counted in controls (n = 5) and 2,714 cells were counted in recovered animals (n = 4).

Quantification of Pendrin-Positive Intercalated Cells

Cell counting was performed in CCDs of kidney sections single labeled with pendrin and peroxidase-conjugated secondary antibodies. Counting was performed directly on the microscope. The numbers of positive (labeled) and negative (unlabeled) cells with a distinct nucleus were counted, and the fraction of pendrin-labeled cells was calculated from the number of positive cells divided by the total number of cells counted for each animal. The total number of cells counted were 3,480 for the control animals (n = 4) and 3,409 for the lithium-treated rats (n = 4).

Immunoblotting

Whole kidney was homogenized in dissecting buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2) that contained the protease inhibitors leupeptin (8.5 μM) and phenylmethylsulfonyl fluoride (1 mM) and the phosphatase inhibitors okadaic acid (100 nM), sodium orthovanadate (1 mM), and sodium fluoride (25 mM). This homogenate was centrifuged in a Beckman L8M centrifuge at 4,000 g for 15 min at 4°C to remove nuclei, mitochondria, and any remaining large cellular fragments. Laemmli sample buffer was added to the supernatant, and samples were run in duplicate. One gel was Coomassie stained to ensure that loading in the lanes was consistent, whereas the other was subjected to immunoblotting (protocol 1, 60 μg/lane; protocol 2, 80 μg/lane). After transfer by electroblotting to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (that contained 80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated with anti-AQP-2 antibody (LL127, serum, 1:3,000 dilution). The labeling was visualized with a peroxidase-conjugated secondary antibody using an enhanced chemiluminescence system (Amersham).

Films were scanned using an AGFA scanner (Arcus II), and the labeling density was quantitated using specially written software (29). AQP-2 labeling in the samples from the experimental animals was calculated as a fraction of the mean control for that film.

Statistical Analyses

Values are presented as means ± SE. Comparisons between groups were made by unpaired t-test with equal or unequal variances. P values < 0.05 were considered significant.

RESULTS

Lithium Increases Urine Output and Decreases Whole Kidney AQP-2 Expression

Consistent with previous results, chronic lithium treatment resulted in severe polyuria (804 ± 45 vs. 24 ± 2 μl/min kg−1 in controls, n = 19; Table 1) and a corresponding decrease in urine osmolality (141 ± 5 vs. 1,779 ± 85 mosmol/kgH2O, n = 20; Table 1). Immunoblotting of whole kidney samples from the present experiment confirmed that the expression of AQP-2 protein was dramatically reduced to 6 ± 2% of controls (100 ± 21%, n = 5; P < 0.05; not shown) as previously reported (25, 29).

Immunocytochemistry using paraffin-embedded kidney sections confirmed a marked decrease in AQP-2 protein expression in cortex, outer medulla, and proximal and middle parts of inner medulla in lithium-treated rats (Fig. 1). In CCDs, the downregulation of AQP-2 was more pronounced in the inner parts compared with the outer parts. After lithium treatment in outer CCDs, AQP-2 labeling was seen in apical, cytoplasmic, and basolateral domains, whereas the labeling was mainly observed in apical and cytoplasmic domains in the inner parts of CCDs (see Fig. 5D). In IMCDs of lithium-treated rats, a considerable amount of AQP-2 labeling was still confined to the apical plasma membrane domains (see Figs. 1F and 5E), whereas basolateral labeling was markedly reduced in IM-1 areas. Immunoelectron microscopy from IM-1 regions revealed AQP-2 labeling of both the apical plasma membrane and intracellular vesicles in controls (Fig. 2A). This was reduced in kidneys from lithium-treated animals where very sparse labeling of the cytoplasm was observed, but there was still some remaining AQP-2 labeling in the apical plasma membrane (Fig. 2B).

Table 1. Functional data for rats treated with protocols 1 and 2

<table>
<thead>
<tr>
<th>Protocol 1</th>
<th>Protocol 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Lithium Treatment</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>416±11 (18)</td>
</tr>
<tr>
<td>Urine output, μl/min kg−1</td>
<td>24±2 (18)</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH2O</td>
<td>1,779±85 (20)</td>
</tr>
</tbody>
</table>

Lithium Treatment Control Recovery

| Body wt, g | 482±18 (5) | 427±18 (5) |
| Urine output, μl/min kg−1 | 19±2 (5) | 23±2 (5) |
| Urine osmolality, mosmol/kgH2O | 2,048±199 (5) | 1,857±80 (5) |

Values are means ± SE; n (in parentheses), no. of rats; *P < 0.05.
Lithium Changes Fraction of AQP-2-Labeled Cells in Rat Kidney Collecting Ducts

Kidney collecting ducts are composed of principal and intercalated cells. Principal cells constitute the majority of cells, accounting for ~60% in cortex and outer medulla and ~90% in IM-1. Within inner medulla, only a few intercalated cells are found in the middle part, and none are located in the distal part. As shown in Fig. 1, not only was the expression of AQP-2 decreased, but the fraction of cells labeled for AQP-2 was also decreased in lithium-treated compared with control rats. In particular, long rows of cells without AQP-2 labeling accounted for 60% in cortex and outer medulla and 60% in the inner stripe of outer medulla; IM-1, proximal part of inner medulla; magnification, ×450.

To quantify the decrease in the fraction of AQP-2-labeled cells, the lengths of the apical plasma membrane domains that exhibited AQP-2 labeling and the total lengths of the apical plasma membrane domains in connecting tubules or collecting ducts were measured. There was a significant decrease in the average fraction of the apical surface labeled for AQP-2 in lithium-treated rats (Fig. 3); values for lithium-treated rats vs. control rats were as follows: in cortex, 38 ± 2.2 vs. 78 ± 2.5% (P < 0.05; n = 4); in ISOM, 52 ± 8.5 vs. 81 ± 2.6% (P < 0.05; n = 4); and in IM-1, 68 ± 4.4 vs. 87 ± 1.5% (P < 0.05; n = 4).

These results indicated that there was a decrease in the fraction of AQP-2-positive cells and an increase in the fraction of AQP-2-negative cells after lithium treatment. However, it cannot be ruled out that the increased length of apical plasma membrane domains negative for AQP-2 may just have been due to enlargement of the AQP-2-negative cells. To further examine this, the fractions of AQP-2-positive and -negative cells were determined. The results showed a decrease in the fraction of AQP-2-positive cells and a corresponding increase in the fraction of AQP-2-negative cells in lithium-treated rats compared with control rats. In cortex, the fraction of AQP-2-positive cells was reduced to 66 ± 0.6% of control values (100 ± 2.0%, n = 4; P < 0.05), and in IM-1, the fraction was reduced to 75 ± 4.8% of control values (100 ± 1.3%, n = 4; P < 0.05). There was no significant difference in ISOM. Thus lithium treatment decreased the fraction of AQP-2-positive cells, i.e., principal cells, and the results are consistent with an increase in the fraction of intercalated cells.

Lithium Changes Fraction of [H\(^+\)]ATPase-Labeled Cells in Collecting Ducts

To further investigate whether the apparent increase in the fraction of AQP-2-negative cells was associated with an increase in the number of intercalated cells, sections were labeled for the B\(\_\) subunit of the vacular [H\(^+\)]ATPase that is known to be a marker for intercalated cells (Fig. 4). Immunolabeling demonstrated that the density of [H\(^+\)]ATPase-labeled cells appears to be increased in kidneys from lithium-treated rats compared with control rats. Inner medulla, which in control rats contains only few intercalated cells, especially exhibited a marked increase in the density of intercalated cells in response to lithium treatment. This was particularly prominent in IM-1 (Fig. 4F) as well as the middle part of inner medulla (not shown). The densities of [H\(^+\)]ATPase-positive and -negative cells were determined. The results showed a significant increase in the fraction of [H\(^+\)]ATPase-labeled cells in kidney cortex and inner medulla from lithium-treated rats compared with control rats. The values were as follows: cortex, 153 ± 3.9% (100 ± 2.3%, n = 4; P < 0.05) and IM-1, 204 ± 10.2% (100 ± 5.8%, n = 4; P < 0.05). No significant difference was observed in ISOM. Thus quantification of AQP-2- and [H\(^+\)]ATPase-labeled cells, respectively, revealed a significant decrease in the fraction of principal cells in parallel with an increased fraction of [H\(^+\)]ATPase-positive intercalated cells in cortex and inner medulla. The increase in the fraction of [H\(^+\)]ATPase-labeled cells is consistent with a lithium-induced increase in [H\(^+\)]ATPase protein expression as shown by immunoblot studies using a slightly different protocol (40 mmol lithium/kg of dry food for 4 wk; Ref. 23).

Double Labeling with Anti-AQP-2 and Anti-[H\(^+\)]ATPase

To confirm the changes in cellular composition after lithium treatment and to investigate whether any cells in the lithium-treated animals labeled either positive or negative for both AQP-2 and [H\(^+\)]ATPase, double labeling was performed with polyclonal AQP-2 and monoclonal [H\(^+\)]ATPase (Fig. 5, A, B, D, and E) using an immunofluorescence method as well as with polyclonal AQP-2 and polyclonal [H\(^+\)]ATPase using horseradish peroxidase immunolabeling (Fig. 5, C and F). The results revealed long rows of [H\(^+\)]ATPase-labeled cells in lithium-treated rats (Fig. 5, D-F), which were never seen in control rats. The changes in cellular composition were further confirmed via cell counting on the sections double labeled with...
polyclonal AQP-2 and polyclonal [H\textsuperscript{+}]ATPase (Fig. 5, C and F). The results showed a significant decrease in the fraction of AQP-2-positive cells and a corresponding increase in the fraction of [H\textsuperscript{+}]ATPase cells in CCDs and IMCDs in the lithium-treated rats compared with control rats (Table 2). In the distal part of CCDs, the effect was more pronounced, whereas in the proximal part of CCDs, the distributions of AQP-2- and [H\textsuperscript{+}]ATPase-labeled cells were more similar to those of control rats. Similar to the single-labeling results, there were no statistical differences in ISOM between lithium-treated and control rats (Table 2).

Quantitation of the double-labeled sections revealed that 10% of the cells in CCDs were negative for both AQP-2 and [H\textsuperscript{+}]ATPase in lithium-treated rats (Fig. 5, D and F, arrows, and Table 2). Whether these cells are principal cells with no AQP-2 expression or undetectable levels of AQP-2 (despite the high antibody concentration used) or intercalated cells with no [H\textsuperscript{+}]ATPase expression is not known. Even if the negative cells are principal cells, the fraction of principal cells was still significantly decreased in CCDs of lithium-treated rats compared with control rats (51\% vs. 62\%, n = 4; P < 0.05). In inner medulla, the fraction of completely negative cells was very low in lithium-treated rats and was not statistically different from controls. In very rare cases (<1%), it was not possible to determine whether a cell was labeled or not.

The double labeling also showed that cells were almost exclusively positive for only one of the proteins, and only very occasionally did cells appear to stain positive for both AQP-2 and [H\textsuperscript{+}]ATPase (Fig. 5D, arrowhead). This was the case in kidneys from both control and lithium-treated rats.

\[\text{[H}^+\text{]ATPase-Positive Cells Were Costained with AE-1 but Not with AQP-4}\]

To obtain additional evidence that the increased fraction of [H\textsuperscript{+}]ATPase-positive cells in IM-1 after lithium treatment
of dry food for 4 wk (23) and the present protocol, not
istics as type A intercalated cells [using 40 mmol lithium/kg
microscopy to show that the adjacent \( \text{H}^+ \)
cells. This was also further supported by immunoelectron
in IMCDs after lithium treatment appeared to be intercalated
AQP-4 was not present in any of the \( \text{H}^+ \)
cells in IMCDs (Fig. 6B) or in the rest of the collecting ducts.

Ultrastructure of Types A and B Intercalated Cells in
Lithium-Treated Rats

In the present study, all intercalated cells with \( [\text{H}^+] \)ATPase
in the inner medulla of lithium-treated rats had the same
ultrastructural characteristic of type A intercalated cells, which
is consistent with the previous report by Kim et al. (23).
Immunoelectron microscopy of kidney cortex demonstrated
that compared with the normal configuration of type A
intcalated cells in control rats (Fig. 8), the majority of the adjacent
A intercalated cells from lithium-treated rats were more
flattened and elongated (Fig. 9, A and C). The type A interca-
lated cells in the control animals had apical microvilli and
numerous mitochondria in the cytoplasm (see Fig. 8A) as well
as \( [\text{H}^+] \)ATPase labeling in the apical plasma membrane (see
Fig. 8B). Aside from having a more elongated cytoplasm,
the adjacent A cells of lithium-treated rats also had fewer
microvilli and mitochondria than control rats. In some cells
apical \( [\text{H}^+] \)ATPase labeling was seen (Fig. 9B), whereas in
other cells the labeling was mainly observed in the subapical
region of the cells (Fig. 9D).

Immunoelectron microscopy confirmed that the density of
the type B intercalated cells was not altered. Similar to type B
cells of control rats (Fig. 10), the ultrastructure of type B cells
in lithium-treated rats (Fig. 11) was characterized by a few
apical microprojections and denser cytoplasm than in type A
cells. The cells contained \( [\text{H}^+] \)ATPase in the basolateral
membrane and cytoplasm, and there were no differences between
control and lithium-treated rats in the distribution and density
of \( [\text{H}^+] \)ATPase in type B cells.

Four-Week Recovery Period Reverses AQP-2 Expression
and Cellular Profiles to Control Levels

To investigate whether the lithium effects were reversible,
rats were allowed to recover on a lithium-free diet for 4 wk
after lithium treatment (protocol 2). Urine-output measure-
ments (23 ± 2 vs. 19 ± 2 \( \mu \)L/min•kg\(^{-1}\)) in lithium-treated
and control rats, respectively, \( n = 5 \); see Table 1) and the
corresponding urine osmolality values (1,857 ± 80 vs. 2,048 ±
199 mosmol/kH\(_2\)O in lithium-treated and control rats, respec-
tively, \( n = 5 \); see Table 1) returned to levels that were not
significantly different from control animals. Immunoblotting of

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**Fig. 4.** Immunocytochemistry using whole kidney sections from control (A-C) and lithium-treated (D-F) rats. Sections were incubated with anti-[H\(^{+}\)]ATPase antibody and peroxidase-conjugated secondary antibody. Density of [H\(^{+}\)]ATPase-positive cells appeared to be increased in the lithium-treated rats. Strikingly, rows of [H\(^{+}\)]ATPase-positive cells were observed in these rats that were not present in control rats. In cortex, some of the [H\(^{+}\)]ATPase cells appeared more flattened (arrows). Magnification, ×600.
whole kidney samples prepared from recovered animals and corresponding control rats showed that AQP-2 protein levels returned to control levels (95 ± 15% vs. 100 ± 29%, n = 5; P not significant; Fig. 12). The density values and distributions of AQP-2-positive cells were also similar to control rats as shown by immunocytochemistry (Fig. 13). Quantitation of AQP-2-labeled cells in CCDs and IM-1s showed that there were no significant differences in the fraction of AQP-2-positive cells between the two groups (Fig. 14); values were as follows: cortex, 60 ± 1.5% vs. 62 ± 1.3% in controls (n = 4; P not significant) and IM-1, 79 ± 5.5 vs. 92 ± 1.3% in controls (n = 4; P not significant). Thus the fractional decrease in principal cells and the fractional increase in intercalated cells, respectively, reversed to control levels after lithium-treated rats had recovered for 4 wk.

DISCUSSION

In this study, AQP-2 protein levels were severely downregulated in rats with lithium-induced NDI, which is consistent...
with previous studies (25, 29). Interestingly, quantitation of cells revealed a marked decrease in the fraction of collecting duct cells that exhibited detectable AQP-2 labeling and an increase in the fraction of AQP-2-negative cells in cortex and inner medulla compared with control rats. Surprisingly, the majority of the AQP-2-negative cells displayed significant \( \left[H^+\right] \text{ATPase} \) labeling, thereby identifying them as intercalated cells. This was further confirmed with double labeling using \( \left[H^+\right] \text{ATPase} \) and AE-1. In particular, multiple \( \left[H^+\right] \text{ATPase} \)-labeled cells were connected, and quantitation revealed an increase in intercalated cell density parallel to the decrease in principal cells. Urine output, whole kidney AQP-2 expression, and fractions of principal and intercalated cells in cortex and inner medulla returned to control levels after a 4-wk recovery period.

The mechanisms behind the changes in the cellular profile in response to lithium and the observed reversibility after lithium cessation are not known. Potential explanations are discussed here.

**Cellular Profile of Collecting Duct Changed Dramatically in Response to Lithium**

Under normal conditions, collecting duct cells in the mature kidney divide at a very slow rate, i.e., the turnover is slow (43). However, an early study from 1982 showed that lithium treatment of rats (using 40 mmol lithium/kg of dry food for 3, 7, and 21 days) can cause an increase in the mitotic rate in the collecting duct cells as shown by \( ^3\text{H}\)thymidine incorporation (determined after 7 days). In that study, the investigators showed that the hyperplasia and marked increase in DNA synthesis in the collecting ducts seen after lithium treatment were most pronounced at the border region between outer and inner medulla (19). Although they made no distinction between intercalated and principal cells, this study supports our results that show the most marked increase in the number of intercalated cells in the initial and middle parts of IMCD. In another study, lithium also caused accumulation of HL-60 cells in the G2/M cell phase, and this was associated with the onset of

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### Table 2. Fraction of AQP-2 and \( \left[H^+\right] \text{ATPase} \)-labeled cells in control and lithium-treated animals

<table>
<thead>
<tr>
<th></th>
<th>Control, %</th>
<th>Lithium Treatment, %</th>
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<tbody>
<tr>
<td><strong>Cortex/CCD</strong></td>
<td></td>
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<tr>
<td>AQP-2</td>
<td>62±1.8</td>
<td>40±3.4*</td>
</tr>
<tr>
<td>( \left[H^+\right] \text{ATPase} )</td>
<td>38±1.7</td>
<td>50±2.0*</td>
</tr>
<tr>
<td>Negative</td>
<td>0.1±0.07</td>
<td>10±2.1*</td>
</tr>
<tr>
<td><strong>ISOM</strong></td>
<td></td>
<td></td>
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<tr>
<td>AQP-2</td>
<td>61±4.1</td>
<td>59±4.9</td>
</tr>
<tr>
<td>( \left[H^+\right] \text{ATPase} )</td>
<td>36±4.2</td>
<td>39±4.6</td>
</tr>
<tr>
<td>Negative</td>
<td>2.3±1.9</td>
<td>1.6±0.7</td>
</tr>
<tr>
<td><strong>IM-1</strong></td>
<td></td>
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<tr>
<td>AQP-2</td>
<td>81±1.3</td>
<td>58±1.6*</td>
</tr>
<tr>
<td>( \left[H^+\right] \text{ATPase} )</td>
<td>18±1.3</td>
<td>42±1.6*</td>
</tr>
<tr>
<td>Negative</td>
<td>0.2±0.1</td>
<td>0.06±0.03</td>
</tr>
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Values are means ± SE; \( n = 4 \) lithium-treated rats or 3 control rats. Fractions of cells with a distinct nucleus and immunoreactivity for aquaporin-2 (AQP-2) and \( \left[H^+\right] \text{ATPase} \) and cells negative for AQP-2 and \( \left[H^+\right] \text{ATPase} \) were counted on double-labeled sections. CCD, cortical collecting duct; ISOM, inner stripe of outer medulla; IM-1, proximal part of inner medulla. * \( P < 0.05 \).
apotosis (27). Therefore, there could be several possible ways to explain the changes in cell composition in kidneys of lithium-treated rats. First, we could speculate that with lithium treatment, intercalated cells may divide faster than principal cells and lead to an increased fraction of intercalated cells. It is also possible that lithium has no effect on the turnover of principal cells but causes the proliferation of intercalated cells only. In addition, lithium treatment could lead to a selective depletion of principal cells, e.g., by apoptosis followed by increased proliferation mainly of intercalated cells. Lithium may also cause apoptosis of principal cells that then leads to increased proliferation of the remaining principal cells and differentiation to both principal and intercalated cells. It is noteworthy that there were ~10% of cells without both AQP-2 and [H\(^+\)]ATPase in CCDs of lithium-treated rats, and this percentage was significantly higher than in control rats (as demonstrated in RESULTS). Therefore, there might be more cells undergoing mitosis and/or apoptosis in response to lithium treatment. In fact, there has been no conclusion regarding the origination of principal and intercalated cells in the kidney. Some cell culture and morphological studies suggest that intercalated cells originate from principal cells or some undifferentiated precursor cells (1, 20). Other cell culture studies suggest that β-intercalated cells can give rise to both α-intercalated cells and principal cells, but that principal cells cannot convert to intercalated cells (11). Therefore, additional studies are warranted.

In the present study, we would have expected to see some cells with both AQP-2 and [H\(^+\)]ATPase if cell conversion had occurred between principal and intercalated cells. However, the double-labeling results showed that only very few cells from lithium-treated rats stained positive for both AQP-2 and [H\(^+\)]ATPase, and some of these rare events may not have been due to true double-labeled cells but rather may have reflected the sectioning and staining of two different cells. The ultra-structural organization of principal and intercalated cells may lead to labeling of the apical part of the principal cell and labeling of the cytoplasm of an underlying intercalated cell with an apical part that does not show in that particular section. Thus the very few double-labeled cells speak against interconversion. Furthermore, we observed the same results from the double labeling with antibodies against AQP-4 and [H\(^+\)]ATPase. However, it cannot be ruled out that a potential interconversion between principal and intercalated cells may not occur progressively during the 4-wk treatment period but may take place at an earlier stage during treatment, and a cell converting to another cell type may therefore not be visible after 4 wk of treatment. Interconversion could also explain that the situation reverses after cessation of lithium, i.e., the intercalated cells convert back to principal cells. Thus there are no data in the present study that support interconversion, but it cannot be ruled out entirely.

Whether the observed changes in cellular composition of the collecting ducts represent a direct effect of lithium or are instead due to the side effect of lithium treatment has not been established. It has been suggested that a rise in intracellular sodium can induce cell proliferation of renal epithelial cells with sodium acting as a mitogen (36). Lithium-treated rats exhibit natriuresis (25), and competing lithium uptake by the cells inhibits sodium reabsorption in these rats. It could be speculated that lithium can act as a mitogen. It may also be caused by an indirect effect of lithium treatment, e.g., due to the high vasopressin concentration that is known to be present in lithium-treated animals (2, 4, 15, 38). Vasopressin has been shown to stimulate mesangial cell proliferation (14). It could also be hypothesized that the very high urine flow observed in lithium-treated rats could cause the observed changes in cellular composition. In this regard, it is noteworthy that sustained hyposmotic stress induces cell death in human kidney tubule cells (18). However, Brattleboro rats, which have urine production that is similar to lithium- and furosemide-treated rats (which are also polyuric, although not to the same extent as lithium-treated rats), do not display dramatic changes in cellu-
lar composition (B. M. Christensen and S. Nielsen, unpublished observations).

In several previous studies, investigators showed that lithium causes an acidification defect by decreasing the favorable electric gradient for hydrogen ion secretion and also has an inhibitory effect on the hydrogen ion pump (8). In addition, we have demonstrated in a recent study that in chronic lithium-treated rats, the [H⁺]ATPase (B₁ subunit) protein expression and the density of cells with [H⁺]ATPase expression were significantly increased (23). Therefore, it can be speculated that the distal renal tubular acidosis induced either by reduced lumen negative transepithelial potential or decreased [H⁺]ATPase activity causes the increase of intercalated cells in the kidney, especially in the inner medulla. However, the mechanism behind the increased number of intercalated cells in the inner medulla in response to chronic lithium treatment is not yet established.

The cellular profile of kidney collecting ducts has recently been shown to be remodeled in another animal model, i.e., in adult rats treated with acetazolamide, which inhibits carbonic anhydrase activity (3). The authors speculated that the changes in intercalated cells may be adaptive processes that tend to correct or stabilize the metabolic acidosis that would otherwise ensue after systemic carbonic anhydrase inhibition (3).

Several factors may contribute to the severe polyuria that was observed in lithium-treated rats. The dramatic downregulation of AQP-2 expression and the subsequent reduced
amount of AQP-2 in the apical plasma membrane play a key role. This correlates with the fact that lithium is able to inhibit adenylylate cyclase activity and thus the production of cAMP. AQP-2 expression in basolateral plasma membrane domains in IM-1 appeared to be decreased after lithium treatment. The role of AQP-2 in the basolateral plasma membrane is not known, but the decreased expression of AQP-2 in basolateral plasma membrane domains in this part of the inner medulla may potentially also contribute to the polyuria. There have been several reports regarding the role of vasopressin on basolateral AQP-2 targeting. We have recently shown that acute V2-receptor agonist treatment of Brattleboro rats did not cause an increase in basolateral AQP-2 expression, and similarly, acute V2-receptor antagonist treatment did not cause a retrieval of AQP-2 from the basolateral plasma membrane, which suggests that basolateral AQP-2 is probably not regulated by short-term vasopressin stimulation (6). However, van Balkom et al. (41) recently showed that acute vasopressin stimulation of dissected kidney slices from normal rats induced insertion of AQP-2 into basolateral plasma membrane domains in the distal part of inner medulla.

AQP-3 is present in the basolateral plasma membrane of collecting duct principal cells, where it functions as an exit pathway for water entering through AQP-2 in the apical plasma membrane. AQP-3 protein expression has previously been shown to be downregulated to the same extent as AQP-2 in whole kidney preparations from lithium-treated rats, and the density of AQP-3 labeling in the basolateral plasma membrane in inner medulla was decreased after lithium treatment (25). Thus the decrease in AQP-3 expression probably contributes to the marked diuresis. Transport of AQP-3 to the basolateral plasma membrane is apparently not regulated by vasopressin, because only a very small
proportion of AQP-3 is present in intracellular vesicles in normal rats (10). In contrast, AQP-3 appears to be regulated by vasopressin on a long-term basis (39). Another key factor in the development of polyuria may be the fractional reduction in principal cells observed after lithium treatment. Thus the decrease in water reabsorption is probably a combination of the reduced fraction of AQP-2-containing cells and the decrease in AQP-2 expression in the remaining principal cells.

In conclusion, the decreased fraction of principal cells in parallel with the increased fraction of intercalated cells observed in this study are likely to be important in lithium-induced NDI. Additional investigations are needed to clarify the mechanisms behind these major changes in cell composition in renal collecting ducts.

Recovery from Lithium-Induced NDI

Both urine output and AQP-2 protein levels returned completely to control levels 4 wk after cessation of lithium treatment. A previous experiment where rats were treated with lithium for 35 days showed that after 7 days of recovery, rats had increased their AQP-2 expression incompletely to 40% of control levels (29). The recovery of patients after lithium therapy is also slow. The urinary concentrating ability improves significantly during the first 2 mo after removal of lithium. However, 17 out of 27 patients had persistent concentrating defects 1 yr after stopping lithium (5). In the present study, there were no significant differences in the fraction of AQP-2-positive cells between the recovered animals and the control animals after 4 wk of recovery. Moreover, the cellular profile reverted to the normal configuration, i.e., the long rows of intercalated cells seen in the lithium-treated rats were no longer visible, and collecting duct intercalated cells were almost exclusively separated from other intercalated cells by principal cells. The recovery could be explained by interconversion of some of the intercalated cells to principal cells. It is also possible that intercalated cells are removed in a similar way as seen in the developing kidney. Here intercalated cells are depleted from the inner medulla by two distinct mechanisms: simple extrusion from the epithelium and by apoptosis followed by phagocytosis by neighboring principal cells during the first 2 wk after birth (21). However, the mechanisms involved in the removal of intercalated cells in kidneys of rats treated for 4 wk with lithium followed by normal diet for 4 wk remains to be established.

Summary. This study demonstrated a dramatic change in the composition of cell types in renal collecting ducts in response to prolonged lithium treatment. There is a major decrease in the density of principal cells in parallel with a decrease in AQP-2 expression and an increase in the number of intercalated cells. This may have profound importance for the development of lithium-induced NDI. Moreover, this study raises the possibility that the cellular composition of collecting ducts undergoes substantial modification in response to metabolic changes in the body, which adds another layer of complexity to the web of mechanisms regulating its function.

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Fig. 13. Immunocytochemistry using whole kidney sections from control rats (A-C) and rats treated with lithium for 4 wk followed by a 4-wk recovery period (recovery, D-F). Sections were incubated with anti-AQP-2 antibody and peroxidase-conjugated secondary antibody. Both AQP-2 expression and the density of AQP-2-positive cells were similar in the control and recovery groups. Magnification, ×380.
REFERENCES


