Segment-specific ENaC downregulation in kidney of rats with lithium-induced NDI

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1The Water and Salt Research Center, University of Aarhus, DK-8000 Aarhus C, Denmark; 2Department of Physiology, School of Medicine, Dongguk University, Kyungju 780-714, Korea; and 3Laboratory of Kidney and Electrolyte Metabolism, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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Nielsen, Jakob, Tae-Hwan Kwon, Jeppe Praetorius, Young-Hee Kim, Jørgen Frøkiær, Mark A. Knepper, and Søren Nielsen. Segment-specific ENaC downregulation in kidney of rats with lithium-induced NDI. Am J Physiol Renal Physiol 285: F1198–F1209, 2003. First published August 19, 2003; 10.1152/ajprenal.00118.2003.—Lithium-induced nephrogenic diabetes insipidus is associated with increased renal sodium excretion in addition to severe urinary concentrating defects. However, the molecular basis for this altered renal sodium excretion remains undefined. The amiloride-sensitive sodium channel (ENaC) is expressed in the renal connecting tubule and collecting duct and is essential in renal regulation of body sodium balance and blood pressure. We hypothesized that dysregulation of ENaC subunits may be responsible for the increased sodium excretion associated with lithium treatment. Lithium treatment for 28 days resulted in severe polyuria, increased fractional excretion of sodium, and increased plasma aldosterone concentration. Immunoblotting revealed that lithium treatment induced a marked decrease in the protein abundance of β-ENaC and γ-ENaC in the cortex and outer medulla. Moreover, immunohistochemistry and laser confocal microscopy demonstrated an almost complete absence of β-ENaC and γ-ENaC labeling in cortical and outer medullary collecting duct, which was not affected by dietary sodium intake. In contrast, immunohistochemistry showed increased apical labeling of all ENaC subunits in the connecting tubule and inner medullary collecting duct in rats on a fixed sodium intake but not in rats with free access to sodium. Except for a modest downregulation of the thiazide-sensitive Na–Cl cotransporter, the key renal sodium transporters upstream from the connecting tubule (including the α1-subunit of Na-K-ATPase, type 3 Na/H exchanger, and Na-K-2Cl cotransporter) were unchanged. These results identify a marked and highly segment-specific downregulation of β-ENaC and γ-ENaC in the cortical and outer medullary collecting duct, chief sites for collecting duct sodium reabsorption, in rats with a lithium-induced increase in fractional excretion of sodium.

aldo–; collecting duct; epithelial sodium channel; hypertension; nephrogenic diabetes insipidus; sodium reabsorption; sodium wasting

LITHIUM IS AN IMPORTANT DRUG widely used in the management of bipolar affective mood disorders (50). Unfortunately, lithium treatment is associated with decreased urinary concentrating ability in up to 50% of patients receiving lithium therapy and overt polyuria in 20% of patients (3, 8). The impaired urinary concentration ability and polyuria have been associated with impaired V2 receptor-mediated vasopressin responsiveness (12) and decreased protein abundances of aquaporin-2 (AQP2) and aquaporin-3 (AQP3) in collecting duct principal cells (26, 30).

Lithium treatment is also associated with a concomitant increase in urinary sodium excretion (5, 39, 47). However, the mechanism for sodium wasting is still incompletely understood and the molecular basis remains undefined. Studies have implicated the aldosterone-responsive distal renal tubule and collecting duct segments in the increased urinary sodium excretion in response to lithium treatment. Specifically, physiological studies have shown that chronic lithium treatment induces decreased responsiveness of sodium reabsorption to mineralocorticoids and amiloride (5, 46, 48). Because the amiloride-sensitive sodium channel (ENaC) is a known target for aldosterone-stimulated sodium reabsorption in the renal collecting duct (31), these studies raise the possibility that ENaC is also the target for the natriuretic effects of lithium. Compatible with distal effects of lithium, Kwon et al. (26) demonstrated that key apical sodium transporters expressed in the renal tubule segments proximal to the connecting tubule were not downregulated, including transporters in the proximal tubule [type 3 Na/H exchanger (NHE3) and the α1-subunit of Na-K-ATPase] and thick ascending limb (Na-K-2Cl cotransporter; NKCC2) despite an increased urinary sodium excretion. We therefore hypothesize that altered expression and regulation of ENaC subunits are importantly involved in the increased urinary sodium excretion in rats chronically treated with lithium.
ENaC is expressed in connecting tubule cells and principal cells of the collecting duct (19). It is the principal transporter of sodium across the apical plasma membrane and reabsorbs a large fraction of the sodium delivered from the distal convoluted tubule in the connecting tubule and cortical collecting duct (2, 43). ENaC is important in the regulation of sodium balance, extracellular fluid volume, and blood pressure (17). ENaC is a heteromeric protein with three homologous subunits, i.e., α-, β-, and γ-ENaC (9), and is characterized by a cation selectivity for sodium and lithium (37). Expression of ENaC subunits in Xenopus laevis oocytes and the generation of gene knockouts of the individual subunits in mice have demonstrated that altered expression of any of the three subunits has significant effects on the multimeric ENaC protein sodium transport capacity (6, 9, 22, 34). Accordingly, regulation of sodium reabsorption by ENaC mediated by hormones such as vasopressin and aldosterone (17) is associated with characteristic alterations in the expression of the individual ENaC subunits (15, 31). Chronic vasopressin infusion in naturally vasopressin-deficient Brattleboro rats resulted in significantly increased abundances of all three ENaC subunits, whereas 7-day water restriction in Sprague-Dawley rats results in significantly increased abundances of only β-ENaC and γ-ENaC (15). Chronic aldosterone infusion to Sprague-Dawley rats increases the protein abundance of α-ENaC. Moreover, aldosterone causes a mobility shift of γ-ENaC from an 85- to a 70-kDa band without a change in total γ-ENaC protein abundance (31). The appearance of the 70-kDa form of γ-ENaC in response to aldosterone is putatively due to a channel-activating proteolytic cleavage (51). The same changes are observed in chronically sodium-restricted rats in addition to a significant downregulation of the β-ENaC subunit (32). ENaC is also subjected to regulation by trafficking. In normal rat kidney, we have demonstrated that immunolabeling of α-ENaC is mainly present at the apical domains of the principal cells, whereas the labeling of β-ENaC and γ-ENaC is associated with intracellular vesicles dispersed in the entire cytoplasm with sparse labeling of the apical plasma membrane (19). Elevated plasma aldosterone concentration is associated with a markedly increased apical expression of β-ENaC and γ-ENaC, with a lesser effect on α-ENaC already present in the apical domain (28, 31). This suggests that there are also differences in the regulation of the subcellular localization of ENaC subunits. In addition to the described regulatory mechanisms, ENaC is also subjected to regulation by the tubular sodium load, intraluminal flow rate, intracellular pH, and angiotensin II (7, 14, 36, 38, 40).

The purpose of this study was to directly investigate the effect of chronic lithium treatment on the regulation of ENaC subunit protein abundance and the segmental and subcellular localization to elucidate the underlying molecular mechanisms responsible for the increased urinary sodium excretion associated with prolonged lithium treatment.

**METHODS**

Animal protocol 1: low-dose lithium treatment with a fixed sodium intake. Male Wistar rats (n = 12, body wt 190–210 g, Møllegaard Breeding Center, Skensved, Denmark) received daily food rations of a food mix consisting of 20 g of ground rat chow (Altromin 1320, Petersen, Ringsted, Denmark), 20 ml tap water, and an additional 0.25 mmol NaCl (total content of sodium 2.5 mmol Na/day). The lithium-treated rats received 0.8 mmol LiCl (L 4408, Sigma) added to the food mixture. In addition to the water in the food mixture, the rats had free access to a water bottle. For the first 16 days, the rats were housed individually in normal rat cages, and each received 40 g of the food mix/day. During the last 12 days, the rats were housed in metabolic cages and pair fed. The controls were offered only the amount (of the 40 g offered) that the lithium-treated rats ate, which averaged 33 g of the food mix. The daily water intake and food intake were monitored throughout the study. Urine was analyzed for concentrations of sodium, potassium, creatinine, and urea and for the measurement of urinary osmolality. During the entire experiment, there was a 12:12-h artificial light-dark cycle, a temperature of 21 ± 2°C, and humidity of 55 ± 2%. The food was administered daily 1 h before the dark cycle began. On day 28, the rats were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ), and a large laparotomy was made. Blood was collected from the inferior vena cava. The blood was prepared to be analyzed for sodium, potassium, creatinine, urea, osmolality, and plasma aldosterone concentration. The right kidney was rapidly removed, dissected into regions (cortex, inner stripe of outer medulla and inner medulla), and processed for immunoblotting as described below. The left kidney was perfusion fixed as described below.

Animal protocol 2: high-dose lithium treatment with free access to sodium. Male Wistar rats were used (n = 12, Møllegaard Breeding Center). Lithium chloride was added to rat chow to give a concentration of 40 or 60 mmol lithium/kg dry food (Altromin 1320, Petersen) as previously described (12, 30). Rats received food containing 40 mmol lithium/kg dry food for the first 7 days and thereafter 60 mmol lithium/kg dry food. All rats had access to NaCl lacking-block to replace sodium loss and avoid lithium intoxication. This protocol has been reported to give a plasma lithium concentration of 0.70 ± 0.09 mM (12) and a similar protocol with 80 mmol lithium/kg dry food and free access to sodium resulted in a plasma lithium concentration of 0.89 ± 0.13 mM (49). The rats had free access to a water bottle. The rats were housed individually in normal rat cages for the first 20 days and then in metabolic cages for the last 7 days of the study to allow measurements of urine production. On the last day of the study, the rats were killed and kidneys were perfusion fixed as described below. The studies complied with the Danish regulations (Danish Ministry of Justice) for the care and use of experimental animals.

Semi quantitative immunoblotting. The procedure was similar to what has been described in detail previously (24, 45) and is summarized briefly in the following. The dissected renal cortex, inner stripe of the outer medulla, and inner medulla were homogenized (Ultra-Turrax T8 homogenizer, IKA Labortecnik, Staufen, Germany) in ice-cold isolation solution containing 0.5 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.2. To remove large cellular debris and nuclei, the homogenates were centrifuged at 4,000 g for 15 min at 4°C, and the supernatant was pipetted off and kept on ice for further processing. The total protein concentration was mea-
sured (Pierce BCA protein assay reagent kit, Pierce, Rock-
ford, IL), and all samples were adjusted with an isolation
solution to reach the same final protein concentrations, sol-
ubilized at 65°C for 15 min in Laemmli sample buffer, and
then stored at −20°C. To confirm equal loading of protein, an
initial gel was stained with Coomassie blue as described
previously (45). SDS-PAGE was performed on 9 or 12%
polyacrylamide gels. The proteins were transferred from the
gel electrophoretically (Bio-Rad Mini Protein II) to nitrocel-
lulose membranes (Hybond ECL RPN3032D, Amersham
Pharmacia Biotech, Little Chalfont, UK). After transfer, the
blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4,
20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for
1 h and incubated overnight at 4°C with primary antibodies.
The sites of antibody-antigen reaction were visualized with
horseradish peroxidase-conjugated secondary antibodies
(P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark)
with an enhanced chemiluminescence (ECL or ECL+) sys-
tem and exposed to photographic film (Hyperfilm ECL,
RPN5103K, Amersham Pharmacia Biotech). The band den-
sities were quantitated by scanning the films and normaliz-
ing sections prepared for immunofluorescence, a secondary
results are listed as the relative, and not absolute, band den-
sities between the groups, hence the term semiquantitative
immunoblotting.

Immunohistochemistry. A perfusion needle was inserted
into the abdominal aorta, and the inferior vena cava was cut
to establish an outlet after blood sampling. Blood was flushed
from the kidneys with cold 0.01 M PBS (pH 7.4) for 15 s,
before a switch to cold 3% paraformaldehyde in 0.1 M cac-
dylylate buffer (pH 7.4) for 3 min. The kidney was removed,
and the midregion was sectioned into 2- to 3-mm transverse
sections and immersion fixed additionally for 1 h, followed
by 3 × 10-min washes with 0.1 M cacodylylate buffer, pH 7.4.
The tissue was dehydrated in graded ethanol and left over-
night in xylene. After the tissue was embedded in paraffin,
2-μm sections were cut on a rotary microtome (Leica Micro-
systems, Herlev, Denmark).

For immunolabeling, the sections were dewaxed with xy-
lene and rehydrated with graded ethanol. Sections had en-
dogenous peroxidase activity blocked with 0.5% H2O2 in
absolute methanol for 10 min. Using a microwave oven, the
sections were boiled in a target-retrieval solution (1 mM Tris,
pH 9.0, with 0.5 mM EGTA) for 10 min. After cooling, non-
specific binding was blocked with 50 mM NH4Cl in PBS for
30 min followed by 3 × 10 min with PBS blocking buffer
containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The
sections were incubated with primary antibody (diluted in
PBS with 0.1% BSA and 0.3% Triton X-100) overnight at 4°C.
The sections were washed 3 × 10 min with PBS wash buffer
containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin and
incubated with horseradish peroxidase-conjugated secondary
antibody (DAKO P448, goat anti-rabbit IgG) for 1 h at room
temperature. After 3 × 10-min rinses with PBS wash buffer,
the sites of antibody-antigen reaction were visualized with a
brown chromogen produced within 10 min by incubation with
0.05% 3,3′-diaminobenzidine tetrachloride (Kemen Tek,
Copenhagen, Denmark) dissolved in distilled water with
0.1% H2O2. Mayer’s hematoxylin was used for counterstain-
ing, and after dehydration coverslips were mounted with
hydrophilic medium (EuKitt, Kindler, Freiburg, Germany).
For immunofluorescence microscopy, an immunofluorescence,
fluorescent antibody was used (goat anti-rabbit IgG, Alexa
Fluor 488, 11008; and goat anti-mouse IgG, Alexa Fluor 546,
11003, Molecular Probes, Eugene, OR). After a 1-h incuba-
tion at room temperature, coverslips were mounted with
hydrophilic mounting media containing an antifading re-
agent (n-propyl-gallat, P-3101, Sigma, St. Louis, MO). Light
microscopy was carried out with a Leica DMRE (Leica Mi-
crosystems). Laser confocal microscopy was carried out with
a Leica TCS-SP2 laser confocal microscope (Leica, Heidel-
berg, Germany).

Antibodies. Rabbit polyclonal antibodies to the following
renal sodium transporters were utilized: NHE3 of the prox-
imal tubule (16); NKCC2 of the thick ascending limb (24); the
thiazide-sensitive NaCl cotransporter (NCC) of the distal
collected tube (25); and the ENaC subunits α-ENaC, β-ENaC,
and γ-ENaC in the connecting tube and collecting
duct (31). The antisera were affinity purified against the
immunizing peptides as previously described (24, 25). Spe-
cificity of the antibodies has been demonstrated by showing
unique peptide-abloatalable bands on immunoblots and a
specific labeling by immunocytochemistry. Additional antisera
against α-ENaC used for immunocytochemistry was kindly
provided and characterized by Dr. D. M. Fambrough (Johns
Hopkins Univ. Medical School) (23).

Presentation of data and statistical analyses. Quantitative
data are presented as means ± SE. Statistical comparisons
were accomplished by unpaired t-test (when variances were
the same) or by Mann-Whitney rank-sum test (when vari-
ances were significantly different between groups). P values
<0.05 were considered statistically significant.

RESULTS

Lithium-treated rats had increased fractional excre-
tion of sodium. Consistent with previous observations
(26, 30), the lithium-treated rats (see METHODS) devel-
oped polyuria (156 ± 24 vs. 24 ± 1 ml/24 h, P < 0.05)
and low urinary osmolality (178 ± 22 vs. 895 ± 42
mosmol/kgH2O, P < 0.05, Table 1). The lithium-treated
and control rats consumed equal amounts of food (i.e.,
the same sodium and potassium intake) and, as ex-
pected, urinary sodium and potassium excretion rates

Table 1. Physiological data of effect of
lithium treatment

<table>
<thead>
<tr>
<th>Control</th>
<th>Lithium Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td></td>
</tr>
<tr>
<td>221 ± 3</td>
<td>196 ± 10</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td></td>
</tr>
<tr>
<td>16.5 ± 0.5</td>
<td>17.2 ± 1.0</td>
</tr>
<tr>
<td>Sodium intake, mmol/24 h</td>
<td></td>
</tr>
<tr>
<td>2.15 ± 0.05</td>
<td>2.24 ± 0.24</td>
</tr>
<tr>
<td>Lithium intake, mmol/24 h</td>
<td></td>
</tr>
<tr>
<td>0.68 ± 0.04*</td>
<td></td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td></td>
</tr>
<tr>
<td>42.1 ± 1.0</td>
<td>186 ± 24*</td>
</tr>
<tr>
<td>P, mosmolality, mosmol/kgH2O</td>
<td></td>
</tr>
<tr>
<td>301 ± 4</td>
<td>303 ± 3</td>
</tr>
<tr>
<td>PNa, mM</td>
<td></td>
</tr>
<tr>
<td>141.2 ± 0.5</td>
<td>138.2 ± 1.0*</td>
</tr>
<tr>
<td>PK, mM</td>
<td></td>
</tr>
<tr>
<td>4.03 ± 0.21</td>
<td>4.60 ± 0.23</td>
</tr>
<tr>
<td>P, ura, mM</td>
<td></td>
</tr>
<tr>
<td>5.0 ± 0.4</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>P, creatinine, mM</td>
<td></td>
</tr>
<tr>
<td>30.5 ± 1.1</td>
<td>33.6 ± 1.4</td>
</tr>
<tr>
<td>P, aldosterone, nM</td>
<td></td>
</tr>
<tr>
<td>0.35 ± 0.06</td>
<td>11.5 ± 2.2*</td>
</tr>
<tr>
<td>UVol, ml/24 h</td>
<td></td>
</tr>
<tr>
<td>24 ± 1</td>
<td>156 ± 24*</td>
</tr>
<tr>
<td>P, sodium, mosmol/kgH2O</td>
<td></td>
</tr>
<tr>
<td>895 ± 42</td>
<td>178 ± 22*</td>
</tr>
<tr>
<td>UNa,V, mmol/24 h</td>
<td></td>
</tr>
<tr>
<td>2.23 ± 0.05</td>
<td>2.35 ± 0.16</td>
</tr>
<tr>
<td>UK,V, mmol/24 h</td>
<td></td>
</tr>
<tr>
<td>4.09 ± 0.07</td>
<td>4.13 ± 0.27</td>
</tr>
<tr>
<td>P, excretion, µmol/min</td>
<td></td>
</tr>
<tr>
<td>1.86 ± 0.08</td>
<td>1.49 ± 0.12</td>
</tr>
<tr>
<td>Filtered Na*, µmol/min</td>
<td></td>
</tr>
<tr>
<td>262 ± 11</td>
<td>207 ± 18*</td>
</tr>
<tr>
<td>FEK*, %</td>
<td>0.60 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; P, plasma; U,
urinary; UNa,V and UK,V, urinary excretion rate of Na+ and K+, respec-
tively; FE, fractional excretion. *Significantly different, P < 0.05.
were not different (Table 1). However, the lithium-treated rats had a marked increase in the fractional excretion of sodium (0.80 ± 0.02 vs. 0.60 ± 0.03%, P < 0.05). Moreover, the lithium-treated rats had decreased creatinine clearance (1.49 ± 0.12 vs. 1.86 ± 0.08 ml/min, P < 0.05) and a decreased filtered load of sodium (207 ± 18 vs. 262 ± 11 μmol/min, P < 0.05). This indicates that lithium treatment is associated with an impairment of the tubular reabsorption of filtered sodium, thereby producing natriuresis and extracellular fluid volume contraction despite markedly increased plasma aldosterone concentrations (11.5 ± 2.2 vs. 0.35 ± 0.06 nM, P < 0.05).

Lithium-treated rats had significantly reduced expression of β-ENaC and γ-ENaC in cortical and outer medullary collecting duct principal cells. To investigate the effect of lithium treatment on ENaC subunit protein abundances in the cortex, inner stripe of the outer medulla, and inner medulla, semiquantitative immunoblotting was carried out. Using protein from cortical, medulla, and inner medulla, semiquantitative immunoblotting was carried out. Using protein from cortical homogenates, immunoblots revealed markedly decreased band densities of β-ENaC and γ-ENaC (both 85- and 70-kDa bands of γ-ENaC) in the lithium-treated rats compared with control rats (Fig. 1). On the other hand, the band density of α-ENaC was not significantly changed. The analyses of normalized band densities are shown in Table 2. The changes in the inner stripe of the outer medulla were similar to the changes in the cortex, namely, markedly downregulated β-ENaC and γ-ENaC (only the 85-kDa band was observed on the immunoblot shown, and a negligible 70-kDa band was observed with longer film exposure) and no change in α-ENaC protein expression despite the marked elevation of plasma aldosterone concentration (Fig. 2).

In addition to the changes in protein abundances of the ENaC subunits, the sodium channel is also regulated by intracellular trafficking. Increased plasma aldosterone concentration mediates a change in the subcellular localization of ENaC from a dispersed cytoplasmic distribution to the apical plasma membrane domains (28, 31). To investigate whether lithium treatment affected this response, we carried out immunoperoxidase labeling and immunofluorescence labeling of tissue sections for microscopic analysis. Striking

### Table 2. Densitometric analysis of immunoblots from lithium-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lithium Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ENaC</td>
<td>1.00 ± 0.07</td>
<td>0.99 ± 0.05</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00 ± 0.04</td>
<td>0.41 ± 0.04 a*</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>1.00 ± 0.08</td>
<td>0.30 ± 0.02 a*</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>1.00 ± 0.10</td>
<td>0.63 ± 0.06 a*</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.00 ± 0.12</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00 ± 0.19</td>
<td>1.04 ± 0.11</td>
</tr>
<tr>
<td>NCC</td>
<td>1.00 ± 0.06</td>
<td>0.58 ± 0.10 a*</td>
</tr>
<tr>
<td>Na-K-ATPase α1</td>
<td>1.00 ± 0.04</td>
<td>0.97 ± 0.01</td>
</tr>
<tr>
<td>AQP1</td>
<td>1.00 ± 0.13</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>AQP2</td>
<td>1.00 ± 0.17</td>
<td>0.12 ± 0.01 a*</td>
</tr>
<tr>
<td><strong>ISOM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00 ± 0.14</td>
<td>0.86 ± 0.10</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00 ± 0.10</td>
<td>0.31 ± 0.04 a*</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>1.00 ± 0.07</td>
<td>0.24 ± 0.03 a*</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>1.00 ± 0.07</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>Na-K-ATPase α1</td>
<td>1.00 ± 0.08</td>
<td>1.19 ± 0.05</td>
</tr>
<tr>
<td><strong>IM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00 ± 0.10</td>
<td>3.34 ± 0.33 a*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00 ± 0.03</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>γ-ENaC (85 kDa)</td>
<td>1.00 ± 0.07</td>
<td>1.08 ± 0.12</td>
</tr>
<tr>
<td>γ-ENaC (70 kDa)</td>
<td>1.00 ± 0.07</td>
<td>3.47 ± 0.26 a*</td>
</tr>
<tr>
<td>Na-K-ATPase α1</td>
<td>1.00 ± 0.27</td>
<td>1.87 ± 0.22 a*</td>
</tr>
<tr>
<td>AQP2</td>
<td>1.00 ± 0.06</td>
<td>0.42 ± 0.04 a*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 rats in both groups for cortex and inner stripe of the outer medulla (ISOM) and 4 rats in both groups for inner medulla (IM). ENaC, epithelial Na channel; NHE3, type 3 Na/H exchanger; NKCC2, Na-K-2Cl cotransporter; NCC, Na-Cl co-transporter; AQP1 and AQP2, aquaporin-1 and aquaporin-2, respectively. The band density of 70-kDa γ-ENaC in ISOM was negligible and only observed with longer exposure. *Significantly different, P < 0.05.

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![Fig. 1. Semiquantitative immunoblots using protein prepared from cortical homogenates from control and lithium-treated rats.](http://ajprenal.physiology.org/)

![Fig. 2. Semiquantitative immunoblots using protein prepared from inner stripe of outer medulla homogenates from control and lithium-treated rats.](http://ajprenal.physiology.org/)
findings were observed for β-ENaC and γ-ENaC. In the lithium-treated rats, the cortical collecting ducts showed markedly reduced labeling of β-ENaC and γ-ENaC with no apparent apical labeling (Fig. 3, B and D, respectively). In control rats, there was dispersed cytoplasmic labeling of both β-ENaC and γ-ENaC in the collecting duct principal cells (Fig. 3, A and C, respectively). Similarly, in the outer medullary collecting duct (both inner and outer stripe), labeling of both β-ENaC (not shown) and γ-ENaC was decreased in lithium-treated rats and dispersed in control rats (Fig. 3, E and F). To confirm that the tubule segments were cortical collecting duct and not connecting tubule, double labeling with γ-ENaC and calbindin-D_{28k} was carried out and analyzed by laser scanning confocal microscopy. As described below, calbindin-D_{28k} is expressed in connecting tubule cells. The tubule segments showing dispersed γ-ENaC labeling (green) in control rats (Fig. 3G) and no labeling in lithium-treated rats (Fig. 3H) were all negative for calbindin-D_{28k} (red), thus confirming the tubule to be the cortical collecting duct. In contrast, there were no major changes in the subcellular localization of α-ENaC immunolabeling in the cortical and outer medullary collecting duct in lithium-treated rats compared with control rats (not shown). The labeling of α-ENaC remained dispersed in the cytoplasm, with some apical labeling in the cortex but not in the outer medulla of both control rats and lithium-treated rats.

Fig. 3. Immunoperoxidase and immunofluorescence microscopy of β- and γ-ENaC in collecting duct. Labeling of β- and γ-ENaC is strong and dispersed in the cytoplasm of principal cells of the cortical collecting duct (CCD; A and C) in control rats. However, the labeling intensity is strikingly reduced in the cytoplasm of principal cells in the same segments of lithium-treated rats (B and D). In the outer medullary collecting duct (OMCD), similar changes were observed (E and F). With double labeling, the tubule segment with dispersed γ-ENaC labeling (green) in control rats (G) does not show any calbindin-D_{28k} labeling (red), confirming that the tubule is CCD. In the lithium-treated rats, the tubule segment is unlabeled with both γ-ENaC and calbindin-D_{28k} (H), thus confirming that the tubule is CCD. (See Fig. 4 for examples of positive calbindin-D_{28k} labeling.) The tubular structures are visualized by differential interference contrast (DIC). Arrows indicate dispersed intracellular labeling. Scale bars, 10 μm.
Lithium-treated rats had increased apical ENaC subunit expression in connecting tubule cells. In contrast to the significant downregulation of β-ENaC and γ-ENaC in the cortical and outer medullary collecting duct, immunocytochemistry revealed a distinct apical labeling of β-ENaC and γ-ENaC (Fig. 4, B and D, respectively) in the connecting tubule cells in lithium-treated rats. Labeling of β-ENaC and γ-ENaC in control rats was dispersed throughout the cytoplasm of connecting tubule cells (Fig. 4, A and C, respectively). The labeling of α-ENaC was unchanged and showed both apical and dispersed labeling in control and lithium-treated rats (not shown). To confirm that the distinct apical labeling observed was located in connecting tubule cells, double labeling with γ-ENaC and calbindin-D28k was carried out and analyzed by laser scanning confocal microscopy. Calbindin-D28k was used as a marker for connecting tubule, where it is abundantly expressed, although it is also expressed at much lower levels in late distal convoluted tubule and initial collecting duct in rat kidney (27, 44). Figure 4 shows the colocalization of γ-ENaC (green) and calbindin-D28k (red) in the same cells (Fig. 4, E and F). In the lithium-treated rats, most of the cells displaying distinct apical γ-ENaC labeling in cortex were strongly colabeled with calbindin-D28k, demonstrating that these cells are connecting tubule cells (Fig. 4, I and J). In control rats, the labeling of γ-ENaC was dispersed in cells coexpressing high levels of calbindin-D28k (Fig. 4, G and H).

Lithium-treated rats had increased expression of α-ENaC and γ-ENaC in inner medullary collecting duct cells, but unchanged β-ENaC expression. Immunoblotting of protein from kidney inner medulla revealed a significantly increased protein abundance of α-ENaC and the 70-kDa form of γ-ENaC in lithium-treated rats compared with controls (Fig. 5). These two changes are compatible with changes seen in response to increased plasma aldosterone concentrations (31). In

![Image of immunoperoxidase and immunofluorescence microscopy](image-url)

**Fig. 4.** Immunoperoxidase and immunofluorescence microscopy of β- and γ-ENaC in connecting tubule (CNT). In the CNT, β- and γ-ENaC labeling is dispersed in the cytoplasm of CNT cells in the control rats (A and C), whereas lithium-treated rats show a distinct apical labeling (B and D). Calbindin-D28k was used as a marker for the CNT, where it is abundantly expressed. The colocalization of γ-ENaC and calbindin-D28k in the same cells is observed for both control (E) and lithium-treated rats (F). The γ-ENaC labeling (green) is dispersed in CNT cells of control rats (G and H) and distinctly apical in the lithium-treated rats (I and J). Calbindin-D28k labeling (red) is dispersed in the cells labeling with γ-ENaC (G and I). Arrows indicate dispersed intracellular labeling. Arrowheads indicate apical labeling. Scale bars, 10 μm.
Lithium-treated rats with free access to sodium also showed markedly downregulated \( \beta \)-ENaC and \( \gamma \)-ENaC in cortical and outer medullary collecting duct. In previous studies with chronic lithium treatment, the rats had free access to NaCl to compensate for increased renal sodium excretion caused by lithium (26, 48). To confirm that the downregulation of \( \beta \)-ENaC and \( \gamma \)-ENaC in cortical and outer medullary collecting duct is not related to secondary changes caused by a fixed sodium intake, we analyzed tissue sections labeled with \( \beta \)-ENaC and \( \gamma \)-ENaC from lithium-treated rats with free access to sodium (see METHODS). These rats also showed markedly reduced labeling intensity of \( \beta \)-ENaC (not shown) and \( \gamma \)-ENaC in cortical (Fig. 7B) and outer medullary collecting duct (Fig. 7D) compared with control rats (Fig. 7, A and C, respectively). These findings are identical to the labeling patterns seen in lithium-treated rats with a fixed sodium intake (Fig. 3) and support the view that the downregulation of \( \beta \)-ENaC and \( \gamma \)-ENaC in these segments is a primary effect of lithium treatment. Interestingly, there was much less distinct apical labeling of ENaC in the connecting tubule and inner medullary collecting duct in the lithium-treated rats with free access to sodium.

Lithium clearance and fractional excretion of lithium in lithium-treated rats. To evaluate how the differences in ENaC regulation affected the renal handling of lithium, we measured plasma and urinary parameters to calculate the renal clearance of creatinine and lithium and the fractional excretion of lithium in two different sets of rats. Rats treated with a low dose of lithium and with a fixed sodium intake (less sodium intake; protocol 1) had plasma lithium concentrations of 0.61 \( \pm \) 0.02 mM. Rats treated with a high dose of lithium and with free access to sodium (more sodium intake; protocol 2) had a similar plasma lithium concentration of 0.61 \( \pm \) 0.09 mM. The creatinine clearance in lithium-treated rats with a fixed sodium intake was

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**Fig. 5.** Semiquantitative immunoblots using protein prepared from inner medullary homogenates prepared from control and lithium-treated rats. There is a significant increase in \( \alpha \)-ENaC protein abundance, whereas \( \beta \)-ENaC and the 85-kDa band of \( \gamma \)-ENaC are unchanged. However, the 70-kDa band of \( \gamma \)-ENaC is significantly increased in the lithium-treated rats. *P < 0.05.

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**Fig. 6.** Immunoperoxidase microscopy of \( \alpha \)-ENaC and \( \gamma \)-ENaC in inner medulla. The labeling of \( \alpha \)-ENaC is weak and dispersed in the cytoplasm of inner medullary collecting duct cells in control rats (A), whereas the lithium-treated rats show distinct apical labeling (B) in addition to dispersed cytoplasmic labeling. The labeling of \( \gamma \)-ENaC is weak and dispersed in the cytoplasm in control rats (C) but distinctly apical in the lithium-treated rats (D). The apical labeling of ENaC subunits was predominant only in the middle region of the inner medulla. Arrows indicate dispersed intracellular labeling. Arrowheads indicate apical labeling. Scale bars, 10 \( \mu \)m.
1.32 ± 0.19 ml/min, and lithium clearance was 0.59 ± 0.03 ml/min. The ratio of lithium clearance to creatinine clearance as a measure of the fractional excretion of lithium was 0.51 ± 0.09. In contrast, rats with free access to sodium had a creatinine clearance of 1.92 ± 0.11 ml/min, lithium clearance of 1.57 ± 0.29 ml/min, and fractional excretion of lithium of 0.82 ± 0.14. Therefore, the decreased clearance and fractional excretion of lithium in rats with a fixed sodium intake (less sodium intake; protocol 1) may contribute to the similar plasma lithium concentrations between the two groups, despite the fact that their lithium intake was lower. The presence of similar plasma lithium concentrations suggests that the differences in ENaC labeling observed in the connecting tubule (Fig. 4) and inner medullary collecting duct (Fig. 6) of lithium-treated rats with a fixed sodium intake and free sodium intake are largely dependent on the differences in sodium intake.

Lithium-treated rats showed decreased NCC expression, whereas expression of NHE3, NKCC2, and α1-subunit of Na-K-ATPase was unaffected or increased. To confirm that the increased fractional excretion of sodium in lithium-treated rats was not associated with decreased protein expression of other major cortical sodium transporters located proximal to ENaC expression sites (i.e., connecting tubule and collecting duct), additional immunoblots were carried out using antibodies against NHE3, NKCC2, NCC, and the α1-subunit of the Na-K-ATPase (Fig. 8; summary of normalized band densities in Table 2). As expected, these transporters were not significantly changed except for NCC in the cortex, which was significantly downregulated in lithium-treated rats. In the inner stripe of the outer medulla, there was no change in NKCC2 or Na-K-ATPase α1-subunit protein expression. In the inner medulla, the Na-K-ATPase α1-subunit was significantly upregulated. Immunolabeling of tissue sections with the α1-subunit of the Na-K-ATPase showed upregulation in the same region where ENaC was expressed in the apical cell domain of inner medullary collecting duct cells (not shown). Consistent with previous studies, the AQP2 water channel was also significantly downregulated in both the cortex and inner medulla of lithium-treated rats, whereas AQP1 was unchanged in the cortex (summary of normalized band densities in Table 2; immunoblots not shown).

**DISCUSSION**

The results of this study demonstrate a highly segment-specific downregulation of β-ENaC and γ-ENaC in cortical and outer medullary collecting duct in lithium-treated rats with reduced tubular reabsorption of filtered sodium. These changes, affecting chief sites of collecting duct sodium reabsorption, are consistent with previous studies showing reduced amiloride-sensitive sodium reabsorption in the collecting duct in rats treated with lithium (46). Moreover, NCC expression in the distal convoluted tubule was also reduced, which may participate in the reduction in distal tubular sodium reabsorption. Increased apical ENaC expression was noted in connecting tubule and inner medullary collecting duct in rats with fixed sodium intake but not with free access to sodium and is therefore likely to be a compensatory mechanism limiting renal sodium loss. There was no reduction in the expression of other major renal sodium transporters. Thus the marked downregulation of β-ENaC and γ-ENaC is likely to represent the key molecular basis for the frequently observed sodium wasting associated with lithium treatment of common bipolar affective disorders.

Significant downregulation of β-ENaC and γ-ENaC in the cortical collecting duct and outer medullary collecting duct. Lithium-induced nephrogenic diabetes insipidus (NDI) is associated with polyuria, decreased
ENaC channel activity is regulated by a number of mechanisms, including aldosterone-stimulated intracellular trafficking of the ENaC α-, β-, and γ-subunits from the cytoplasm to the apical plasma membrane (29, 31, 35). Because the synthesis of α-ENaC has been suggested to be a rate-limiting factor of the multimeric ENaC complex (33), sodium transport could be expected to be proportional to the abundance of the α-ENaC protein levels. Recent studies have documented, however, that β-ENaC and γ-ENaC are also important factors in the regulation of sodium transport. First, studies of ENaC subunit expression in X. laevis oocytes demonstrated that elimination of either β-ENaC or γ-ENaC reduced sodium transport to ~10% of that when all three subunits were expressed (9), and coexpression of β-ENaC and γENaC with αENaC in Xenopus oocytes increased plasma membrane expression about threefold (4). Second, β-ENaC- or γ-ENaC-deficient mice have very high urinary sodium excretion, low urinary potassium excretion, severe hyperkalemia, and die before adulthood, indicating that β-ENaC and γ-ENaC are essential for sodium reabsorption in the distal nephron (6, 34). Moreover, the importance of the β-ENaC or γ-ENaC subunits in volume regulation has been emphasized in studies that have identified mutations in β-ENaC or γ-ENaC as the basis of the pathogenesis of Liddle’s syndrome, a disorder characterized by volume expansion and hypertension (21, 41). Therefore, downregulation of any of the ENaC subunits would be predicted to have a severe impact on collecting duct sodium reabsorption and regulation of extracellular fluid volume. Thus the selective downregulation of β-ENaC and γ-ENaC in lithium-induced NDI is likely to play a significant role in renal sodium wasting.

Evidence for impaired aldosterone and vasopressin regulation of ENaC subunits in lithium-treated rats. In the present study, we demonstrate physiological and biochemical changes that are compatible with impaired ENaC regulation by aldosterone and vasopressin in the cortex and outer medulla of lithium-treated rats. Physiological analysis demonstrated that lithium treatment was associated with increased fractional excretion of sodium despite increased plasma aldosterone concentration, decreased glomerular filtration rate, and a decreased filtered load of sodium. It has long been recognized that circulating levels of aldosterone regulate renal sodium reabsorption. The increased plasma aldosterone concentration was possibly caused by sodium depletion and extracellular fluid volume contraction in lithium-treated rats. The observed increase in the fractional excretion of sodium indicated that 1) lithium treatment is associated with impairment of the tubular reabsorption of filtered sodium and 2) increased plasma aldosterone was ineffective in increasing sodium reabsorption in the aldosterone-responsive renal tubule segments for maintaining sodium balance. In support of this are findings by Thomsen et al. (46, 48) demonstrating an inability of aldosterone to decrease the consumption of hypertonic NaCl in lithium-treated adrenalectomized rats and no
significant effect of aldosterone or amiloride on sodium metabolism in conscious, catheterized (venous, arterial, and urinary bladder) lithium-treated rats with computer-controlled servo replacement of urinary water and sodium loss. Moreover, the lithium-treated rats were severely polyuric with low urine osmolality consistent with decreased vasopressin effects.

Biochemically, the impaired aldosterone response was evidenced by unchanged α-ENaC expression in the cortex and outer medulla and decreased NCC expression in the cortex, both of which are normally strongly induced by increases in plasma aldosterone seen in response to lithium treatment (25, 31). The mechanism for the possible lithium interaction with the aldosterone-signaling pathway is not known. The lithium-induced inhibition of adenyly cyclase (12, 13) is also likely to play a role in the lack of vasopressin effects on ENaC in the cortex and outer medulla, as evidenced by markedly reduced β-ENaC and γ-ENaC protein abundances, as well as decreased AQP2 expression. All three of these proteins are normally strongly upregulated by increases in vasopressin levels such as those induced by lithium treatment (18). Immunocytochemical analysis of ENaC subunits showed decreased labeling in the cortical and outer medullary collecting duct confirmed a dysregulation of β- and γ-ENaC subunits independent of sodium intake (Figs. 3 and 7).

In contrast, ENaC regulation in the connecting tubule and inner medullary collecting duct in rats with a fixed sodium intake was consistent with normal actions mediated by increased plasma aldosterone and vasopressin concentrations. The increase in α-ENaC and the 70-kDa form of γ-ENaC is consistent with the effects of aldosterone (31). The increase in γ-ENaC (sum of both 85- and 70-kDa forms) in the inner medulla is consistent with an effect of vasopressin (15). Furthermore, there was increased apical labeling of ENaC subunits in both the connecting tubule and inner medullary collecting duct, consistent with redistribution of ENaC mediated by elevated aldosterone concentrations. In contrast, no apparent apical labeling of ENaC subunits was seen in lithium-treated rats with free access to sodium (more sodium intake). Thus the redistribution of ENaC in the connecting tubule and inner medullary collecting duct was dependent on sodium intake, whereas plasma lithium was not different, therefore likely to be compensating for reduced sodium reabsorption in the cortical collecting duct and outer medullary collecting duct. In addition, the increased α1-subunit of the Na-K-ATPase in the inner medulla is supporting evidence of a compensatory effect to prevent urinary sodium loss. Thus lithium-induced impaired aldosterone- and vasopressin-mediated regulation of ENaC is specific to the cortical and outer medullary collecting duct and is likely to play an important role in the development of natriuresis and decreased urinary concentration ability in rats with lithium-induced NDI. It remains unknown whether the putative compensatory changes in the connecting tubule and inner medulla represent direct effects of aldosterone and vasopressin or whether other signal-
REFERENCES


