Maintained ENaC trafficking in aldosterone-infused rats during mineralocorticoid and glucocorticoid receptor blockade

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1The Water and Salt Research Center, University of Aarhus, Aarhus; 2Institute of Anatomy, University of Aarhus, Aarhus; 3Institute of Clinical Medicine, University of Aarhus, Aarhus, Denmark; 4Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; and 5Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

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Nielsen J, Kwon T-H, Frøkiær J, Knepper MA, Nielsen S. Maintained ENaC trafficking in aldosterone-infused rats during mineralocorticoid and glucocorticoid receptor blockade. Am J Physiol Renal Physiol 292: F382–F394, 2007. First published August 15, 2006; doi:10.1152/ajprenal.00212.2005.—Aldosterone induces redistribution of epithelial sodium channel (ENaC) to the apical plasma membrane from intracellular vesicles in renal connecting tubule (CNT) and cortical collecting duct (CCD). The role of the classical mineralocorticoid receptor (MR) in ENaC trafficking is still debated. We examined whether the MR antagonist spironolactone affects ENaC regulation in the kidney cortex of aldosterone-infused rats. Aldosterone infusion for 7 days resulted in a plasma aldosterone concentration in the high physiological range (3 to 4 nM). Aldosterone infusion decreased plasma K+ concentration compared with untreated control rats. Cotreatment with spironolactone completely blocked the aldosterone-induced decrease in plasma K+. Immunoblotting and immunohistochemistry showed increased protein abundance of Na+-K-ATPase α1-subunit and NCC in the kidney cortex, in response to aldosterone infusion that was blocked by spironolactone. In contrast, aldosterone-induced redistribution of ENaC subunits from the cytoplasm to the apical plasma membrane domain in CNT and CCD was unaffected by spironolactone. Immunoblotting of αENaC showed increased protein abundance in aldosterone-infused rats that was not blocked by spironolactone treatment. To exclude possible glucocorticoid receptor (GR)-mediated effects of aldosterone, we treated aldosterone-infused rats with both spironolactone and the GR antagonist RU486. Combined MR and GR blockade prevented neither ENaC trafficking nor the upregulation of αENaC protein abundance in aldosterone-infused rats. We provide new evidence for ENaC trafficking occurring independent of MR and GR activation in aldosterone-infused rats.

Expression of ENaC at the apical plasma membrane is required for sodium transport and the importance of this aspect of ENaC regulation is illustrated by Liddle’s syndrome. In this familial autosomal dominant genetic disease, a COOH-terminal truncation of either βENaC or γENaC results in a loss of the PY motif required for ENaC complex removal from the apical plasma membrane producing a salt-sensitive hypertensive pseudohypoaldosteronism (22, 37).

A key regulator of ENaC trafficking is the adrenocorticotrophic hormone aldosterone (30). Physiological studies have shown that aldosterone treatment to adrenalectomized rats increases amiloride-sensitive sodium reabsorption after 30 min (13), and immunohistochemistry showed increased ENaC expression after 2 h (29). The role of the mineralocorticoid receptor (MR) in aldosterone-mediated ENaC redistribution is not clear. We have shown that blockade of the MR by spironolactone did not prevent the increased apical ENaC expression induced by dietary sodium restriction. Recently, it was suggested that aldosterone-mediated glucocorticoid receptor (GR) activation may be involved in ENaC regulation (16), which could explain the lack of effect of spironolactone.

To further study the aldosterone-mediated regulation of ENaC redistribution, we directly investigated the effect of MR and GR blockade in chronically aldosterone-infused rats on controlled dietary sodium and water intake. This protocol provides a direct test for effects of MR and GR blockade on protein expression and trafficking of ENaC in the CNT and cortical collecting duct (CCD), in addition to effects on other key sodium transporter proteins.

METHODS

Animal protocol: aldosterone infusion rate. The infusion rate of aldosterone was tested in preliminary sets of male Sprague-Dawley rats (n = 24, Taconic Farms, Germantown, NY). Twenty-four rats had osmotic minipumps delivering 250 μg·kg body wt−1·day−1 (n = 12) or 50 μg·kg body wt−1·day−1 (n = 12) implanted subcutaneously. Six of the rats receiving 50 μg·kg body wt−1·day−1 aldosterone were also treated with 120 mg·kg body wt−1·day−1 spironolactone in the food. In addition, six vehicle-treated control rats were included. All rats received daily food rations of 15 g rat food (formula code 53140000; Zeigler Bros., Gardners, PA). 30 ml tap water, and were housed individually in metabolic cages. The food contained 1.7 mmol Na+·200 g body wt−1·day−1 and 3.8 mmol K+·200 g body wt−1·day−1 by addition of extra NaCl and KCl. After 10 days blood was collected and the plasma aldosterone concentration was deter-
mined. The infusion rate of 250 and 50 μg·kg body wt\(^{-1}\)·day\(^{-1}\) resulted in a plasma aldosterone concentration of 14 ± 2 and 3.6 ± 0.4 nM, respectively. Spironolactone cotreatment did not change the plasma aldosterone concentration (3.2 ± 0.3 vs. 3.6 ± 0.4 nM, not significant), and vehicle-treated control rats had significantly lower plasma aldosterone concentrations of 0.6 ± 0.1 nM. The plasma aldosterone concentrations achieved by 50 μg·kg body wt\(^{-1}\)·day\(^{-1}\) are similar to the high physiological plasma concentration during chronic dietary sodium restriction (30) and were therefore used in animal protocols 1, 2, and 3.

Animal protocols 1 and 2: chronic aldosterone infusion and MR antagonist (spironolactone) treatment. We carried out two studies, an initial study (protocol 1, \(n = 14\)) and a confirmatory study (protocol 2, \(n = 12\)). Male Sprague-Dawley rats (190–210 g body wt, Taconic, Skensved, Denmark) were kept individually in metabolic cages. The rats received daily food rations of 15 g rat chow and 30 ml tap water. The food used in protocol 1 (Altroniin 1320, Chr. Petersen A/S, Ringsted) was different from the food used in protocol 2 (formula code 53140000; Zeigler Bros.); however, the daily sodium intake in both studies was adjusted to 1.7 mmol Na\(^+\)·200 g body wt\(^{-1}\)·day\(^{-1}\) and the daily potassium intake was adjusted to 3.8 mmol K\(^+\)·200 g body wt\(^{-1}\)·day\(^{-1}\) by addition of extra NaCl and KCl. All rats ate all of the food given. 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%.

After 3 days of equilibration, rats had osmotic minipumps (model 2002; Alzet, Palo Alto, CA) implanted subcutaneously under 3.5% halothane anesthesia (Halocarbon Laboratories), while bupivacaine was injected subcutaneously for pain relief. The osmotic minipumps delivered 50 μg·kg body wt\(^{-1}\)·day\(^{-1}\) aldosterone (Sigma A6628) dissolved in DMSO (25% vol/vol) and sterile saline (75% vol/vol). The control rats received pumps with vehicle only. Rats also treated with spironolactone in addition to aldosterone infusion had 120 mg·kg body wt\(^{-1}\)·day\(^{-1}\) spironolactone (Sigma 3378) added to the food. This spironolactone dose is markedly higher than what has previously been shown to block the MR more than 95% (12). Urine was collected daily and analyzed for concentrations of Na\(^+\), K\(^+\), creatinine, urea, and for the measurement of the urine osmolality.

After 7 days, the rats were anesthetized with isoflurane and a large abdominal incision was made. Two milliliters of blood were collected from the inferior vena cava and rapidly transferred into a lithium-heparin or sodium-EDTA-coated tube (Vacutte, Greiner bio-one, Austria). Next, the right kidney was rapidly removed and dissected into three regions: 1) cortex and outer stripe of outer medulla, 2) inner strip of the outer medulla, and 3) inner medulla, for immunoblotting as described under semiquantitative immunoblotting.

Immediately after removal of the right kidney a large perfusion needle was inserted in the abdominal aorta from the aortic bifurcation. The inferior vena cava was immediately cut open to establish an outlet for the fixative. Blood was flushed from the kidneys with cold 0.01 M PBS (pH 7.4) for 15 s, before switching to cold 3% paraformaldehyde in 0.01 M PBS buffer (pH 7.4) for 3 min. The rat was euthanized during the process of fixation by the rapid bleeding. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for additionally 1 h, followed by 3 × 10-min washes with 0.1 M PBS buffer, pH 7.4. Further processing is described in Immunohistochemistry.

Animal protocol 3: chronic aldosterone infusion and combined MR antagonist (spironolactone) and GR antagonist (RU486) treatment. We used 24 male Sprague-Dawley rats (190–210 g body wt; Taconic) that were housed and fed as described in the animal protocol for protocol 1. After equilibration in cages, osmotic minipumps delivering aldosterone were implanted in 18 rats as described in animal protocol for protocol 1. Six rats received only aldosterone infusion. Six aldosterone-infused rats were cotreated with 15 mg·kg body wt\(^{-1}\)·day\(^{-1}\) RU486 (Sigma M8046) in the food. This dose of RU486 has previously been shown to effectively block muscle proteolysis induced by 200 mg·kg body wt\(^{-1}\)·day\(^{-1}\) corticosterone (44). Six aldosterone-infused rats we cotreated with both 15 mg·kg body wt\(^{-1}\)·day\(^{-1}\) RU486 and 120 mg·kg body wt\(^{-1}\)·day\(^{-1}\) spironolactone in the food. Six rats were untreated control rats (no aldosterone infusion). Urine was collected daily and analyzed for concentrations of Na\(^+\), K\(^+\), creatinine, urea and for the measurement of the urine osmolality. After 7 days of treatment, the rats were euthanized as described for protocol 1 and 2.

Approval of animal protocols. The animal protocols described above have been approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus according to the licenses for use of experimental animals issued by the Danish Ministry of Justice and animal protocol (9KE-5) approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

Analysis of plasma and urine biochemistry. The blood samples were centrifuged for 15 min at 4,000 g in table top centrifuge and plasma was transferred to Eppendorf tubes. Measurements of plasma and urinary concentrations of Na\(^+\), K\(^+\), creatinine and urea were determined by Vitros 950 (Johnson and Johnson). Measurement of plasma and urine osmolality was carried out by freezing-point depression (Advanced Osmometer, model 3900, Advanced Instruments, Norwood, MA, and Osmomat 030-D, Gonotec, Berlin, Germany). Plasma concentrations of aldosterone and angiotensin II were measured using commercially available radioimmunoassay kits: Coat-A-Count Cat. TKAL-1, Diagnostic Products, Los Angeles, CA, and Angiotensin II RIA kit Cat. RK-A22, Bühlmann Laboratories AG, Schönenbuch, Switzerland. Creatinine clearance was calculated as

\[
C_{cr} = \frac{U_{c}\text{ Creatine conc} \times U_{v}\text{ volume/24 h}}{P_{\text{creatinine conc}}}
\]

Semiquantitative immunoblotting. The dissected renal cortex, inner stripe of the outer medulla (ISOM), and inner medulla were homogenized in isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.2. Homogenates were centrifuged at 4,000 g for 15 min at 4°C to remove cellular debris. Supernatant protein was solubilized at 65°C for 15 min in Laemmli sample buffer. To confirm equal loading of protein, an internal gel was stained with Coomassie Blue dye as described previously (40). SDS-PAGE was performed on 9 or 12% polyacrylamide gels, and proteins were transferred electrophoretically (Bio-Rad Mini Protein II) to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were blocked with 5% milk in PBS-T (80 mM Na\(_2\)HPO\(_4\), 20 mM NaH\(_2\)PO\(_4\), 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 (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) with an enhanced chemiluminescence (ECL or ECL+plus) system and exposure to photographic film (Hyperfilm ECL, RPN3103K, Amersham Pharmacia Biotech). The band densities were quantified by computer scanning of films and normalizing the densitometry values to facilitate comparisons. We used Scion Image (Scion, Frederick, MD) for computer analysis of band pixel intensities. Results are listed as the relative band densities between the groups and not absolute, hence the term semiquantitative immunoblotting.

Immunohistochemistry. Kidneys were perfusion fixed by insertion of a 17-gauge needle in the abdominal aorta. Blood was flushed from the kidneys with cold 0.01 M PBS (pH 7.4) for 15 s, before switching to cold 3% paraformaldehyde in 0.01 M PBS buffer (pH 7.4) for 3 min. The kidney was euthanized during the process of fixation by the rapid bleeding. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for additionally 1 h, followed by 3 × 10-min washes with 0.1 M PBS buffer, pH 7.4. Further processing is described in Immunohistochemistry.
was blocked by 0.5% H2O2 in absolute methanol for 10 min. Sections were microwave boiled in a target retrieval solution (1 mmol/l Tris, pH 9.0, with 0.5 mM EGTA) for 10 min, before nonspecific binding was blocked with 50 mM NH4Cl in PBS for 30 min, and 3 × 10-min blocking with PBS blocking buffer (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 HRP-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO P448, DAKO A/S) 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 tetra-chloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2. Mayer’s hematoxylin was used for counterstaining, and after dehydration coverslides were mounted with a hydrophilic mounting media containing antifade (Eukitt, O. Kindler GmbH, Freiburg, Germany). For sections prepared for immunofluorescence, a secondary fluorescent antibody was used (goat anti-rabbit IgG, Alexa Fluor 488 11008, Molecular Probes, Eugene, OR). After 1-h incubation at room temperature, coverslides were mounted with a hydrophilic mounting media containing antifading reagent (n-propyl-gallat, P-3101, Sigma, St. Louis, MO). Light microscopy was carried out with Leica DMRE (Leica Microsystems A/S). Laser confocal microscopy was carried out on a Leica TCS-SP2 laser confocal microscope (Heidelberg, Germany).

Semiquantitative confocal laser-scanning microscopy. For semiquantitative analysis, microscope settings (light intensity, PMT offset and gain, sampling period, and averaging) were identical for all rats, and the observer was blinded for the treatment of the individual rats. From each rat five images of tubule segments were identified as DCT cells by the following criteria: tall cuboidal cells, apically located nucleus, marked basolateral striation, only one cell type in tubule segment, and negative for NKCC2 labeling. The dynamic range was set such that the tissue with the most intense fluorescence signal only had few saturated pixels. The results are given as mean density.

Antibodies. Rabbit polyclonal antibodies to the following renal transporter proteins were utilized: the type 3 Na-H exchanger (NHE3) of the proximal tubule (15), the Na-K-2Cl cotransporter (NKCC2) of the thick ascending limb (27), the thiazide-sensitive Na-CI cotransporter (NCC) of the distal convoluted tubule (28), the ENaC subunits αENaC, βENaC, and γENaC in the CNT and collecting duct (30, 33).

Table 1. Physiological data from protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Aldo (n = 5)</th>
<th>Aldo+ Spiro (n = 5)</th>
<th>Control (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>193±3</td>
<td>198±3</td>
<td>193±2</td>
</tr>
<tr>
<td>P-Na, mmol/l</td>
<td>138.8±0.8</td>
<td>139.4±0.8</td>
<td>140.0±1.0</td>
</tr>
<tr>
<td>P-K, mmol/l</td>
<td>3.1±0.1†</td>
<td>4.0±0.2</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>P-Creatinine, μmol/l</td>
<td>30.0±0.9</td>
<td>28.0±1.7</td>
<td>29.3±2.1</td>
</tr>
<tr>
<td>P-Urea, mmol/l</td>
<td>4.1±0.6</td>
<td>3.5±0.6</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>P-Osmol, mosmol/kgH2O</td>
<td>302±1</td>
<td>303±1</td>
<td>304±1</td>
</tr>
<tr>
<td>U-Osmol, mosmol/kgH2O</td>
<td>818±2</td>
<td>837±22</td>
<td>877±47</td>
</tr>
<tr>
<td>U-Vol, ml/24 h</td>
<td>19.4±0.9</td>
<td>18.6±0.7</td>
<td>18.3±0.9</td>
</tr>
<tr>
<td>UVNa, mmol/24 h</td>
<td>1.68±0.09</td>
<td>1.53±0.03</td>
<td>1.65±0.06</td>
</tr>
<tr>
<td>UVo2, mmol/24 h</td>
<td>3.64±0.20</td>
<td>3.55±0.07</td>
<td>3.62±0.11</td>
</tr>
<tr>
<td>C4o, mmol/min·kg−1·h−1</td>
<td>5.0±0.28</td>
<td>5.3±0.53</td>
<td>5.1±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significantly different from control and †significantly different from Aldo + Spiro (P < 0.05). Aldo, aldosterone; Spiro, spironolactone; Osmol, osmolality; P, Plasma; U, urine, VNa+, urinary sodium excretion; V, urinary potassium excretion; C4o, creatinine clearance.

RESULTS

Spironolactone treatment blocks aldosterone-induced decrease in plasma potassium. Chronic infusion of aldosterone producing a plasma aldosterone concentration in the high physiological range (see METHODS) was associated with significantly decreased plasma potassium concentration compared with untreated control rats (3.1 ± 0.1 vs. 4.3 ± 0.2 mM, P < 0.05; Table 1). The decrease in plasma potassium concentration was effectively blocked and normalized to control levels by administration of spironolactone to the aldosterone-infused rats (4.0 ± 0.2 vs. 4.3 ± 0.2 mM, not significant; Table 1). The urinary excretion rates of sodium and potassium were not significantly different between groups consistent with equal dietary sodium intake and steady state. There was no evidence of changes in glomerular filtration rate, indicated by unaffected creatinine clearance. Additional physiological data are summarized in Table 1.

Aldosterone-induced increase in the protein expression of NCC and Na-K-ATPase α1-subunit in the kidney cortex is spironolactone sensitive. Immunoblotting using protein from the renal cortex revealed that protein expression of the Na-K-ATPase α1-subunit, and NCC was markedly increased in the aldosterone-infused rats compared with control rats (Fig. 1). The aldosterone-induced increases in Na-K-ATPase α1-subunit and NCC were completely blocked by spironolactone treatment of the aldosterone-infused rats. A summary of densitometry analysis of immunoblots is shown in Table 2. Immunohistochemical analysis of tissue sections from kidney cortex labeled for Na-K-ATPase α1-subunit showed an increased labeling intensity of Na-K-ATPase α1-subunit in the basolateral plasma membrane domain in the CDD (Fig. 2A) and in the CNT (not shown) in aldosterone-infused rats compared with untreated control rats (Fig. 2C). Treatment with spironolactone largely blocked the aldosterone-induced increase in Na-K-ATPase α1-subunit labeling intensity in the CDD (Fig. 2B) and CNT (not shown). The expression levels of the Na-K-ATPase α1-subunit in the DCT were examined using semiquantitative immunofluorescence confocal microscopy. To distinguish cortical thick ascending limb (cTAL) from DCT, the sections were labeled for NKCC2 (green labeling). Five images from each rat kidney were recorded for analysis. In contrast to the CDD, the results showed no significant changes in the average immunofluorescence density of the Na-K-ATPase α1-subunit (red labeling; Fig. 2, D-F) in the DCT cells when comparing the three groups (in arbitrary units, aldosterone-infused rats 44 ± 5, aldosterone-infused rats given spironolactone 37 ± 4, untreated control rats 41 ± 1, not signifi-
completely, and these rats showed labeling intensity similar to untreated control rats (Fig. 2F). Similar changes in immunoperoxidase labeling were found in protocol 2 (not shown). These results support that the aldosterone-induced upregulation of Na-K-ATPase α1-subunit and NCC is mediated by activation of the classical MR and that the dose of spironolactone was sufficient to block the MR.

Aldosterone-induced apical targeting of ENaC in CNT and CCD is not blocked by spironolactone cotreatment. The purpose of this study was to determine whether aldosterone-induced ENaC trafficking is sensitive to spironolactone blockade of the MR. To study this, we labeled tissue sections of kidney cortex with antibodies specific for the αENaC or γENaC subunits. The intensity of αENaC immunoperoxidase labeling was stronger in the CNT and CCD of aldosterone-infused rats compared with untreated control rats (Fig. 3, A and D vs. C and F). The distribution of αENaC labeling in aldosterone-infused rats was mainly localized to the apical cell domain and plasma membrane. In contrast, labeling in untreated control rats was mainly present in the cytoplasm with less intense labeling of the apical plasma membrane domain. Cotreatment with spironolactone to the aldosterone-infused rats did not alter the αENaC immunolabeling pattern in CNT and CCD (Fig. 3, B and E) compared with aldosterone-infused rats (Fig. 3, A and D). The αENaC labeling was strong and predominantly seen in the apical cell domain and plasma membrane (Fig. 3, B and E) compared with untreated control rats. Tissue sections labeled with γENaC antibodies also showed increased apical targeting in aldosterone-infused rats and in the aldosterone-infused rats cotreated with spironolactone compared with untreated control rats (Fig. 4). However, the overall labeling intensity was unchanged between the groups (Fig. 4). βENaC showed changes similar to γENaC (not shown). The lack of effect of spironolactone on aldosterone-induced ENaC trafficking was confirmed in protocol 2 (images not shown). These results suggest that aldosterone-mediated trafficking of ENaC (α-, β-, and γ-subunits complex) is spironolactone insensitive.

The downregulation to control levels of Na-K-ATPase protein abundance in response to spironolactone was confirmed in a repeat study (protocol 2; Table 2). The repeat study also confirmed the changes in plasma potassium concentration in the aldosterone-infused rats compared with untreated control rats (3.0 ± 0.1 vs. 3.9 ± 0.1 mM, P < 0.05) and normalization to control levels with spironolactone treatment (3.6 ± 0.1 vs. 3.9 ± 0.1 mM, not significant).

Consistent with immunoblotting results, the labeling intensity of NCC in the apical plasma membrane domain of DCT cells was increased by aldosterone treatment (Fig. 2G) compared with untreated control rats (Fig. 2H). Spironolactone blocked the aldosterone-induced NCC upregulation completely, and these rats showed labeling intensity similar to untreated control rats (Fig. 2F). Similar changes in immunoperoxidase labeling were found in protocol 2 (not shown). These results support that the aldosterone-induced upregulation of Na-K-ATPase α1-subunit and NCC is mediated by activation of the classical MR and that the dose of spironolactone was sufficient to block the MR.

**Table 2. Immunoblotting results from renal cortex in protocol 1 and protocol 2**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Aldo (n=5)</th>
<th>Aldo + Spiro (n=5)</th>
<th>Control (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protocol 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHE3</td>
<td>0.94 ± 0.11</td>
<td>1.12 ± 0.12</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.01 ± 0.06</td>
<td>1.18 ± 0.04</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>NCC</td>
<td>1.54 ± 0.11</td>
<td>1.07 ± 0.14</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>αENaC</td>
<td>1.55 ± 0.12</td>
<td>1.59 ± 0.05</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>βENaC</td>
<td>1.07 ± 0.05</td>
<td>0.96 ± 0.09</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>γENaC total</td>
<td>1.02 ± 0.07</td>
<td>0.90 ± 0.05</td>
<td>1.00 ± 0.10</td>
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<tr>
<td>γENaC 85 kDa</td>
<td>0.73 ± 0.04</td>
<td>0.65 ± 0.05</td>
<td>1.00 ± 0.10</td>
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<tr>
<td>γENaC 70 kDa</td>
<td>4.84 ± 0.55</td>
<td>4.26 ± 0.43</td>
<td>1.00 ± 0.14</td>
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<tr>
<td>Na-K-ATPase</td>
<td>1.77 ± 0.08</td>
<td>1.11 ± 0.09</td>
<td>1.00 ± 0.14</td>
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<td><strong>Protocol 2</strong></td>
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<tr>
<td>NHE3</td>
<td>0.84 ± 0.06</td>
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<td>1.00 ± 0.08</td>
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<td>NKCC2</td>
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<td>0.87 ± 0.09</td>
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<td>αENaC</td>
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<td>βENaC</td>
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<td>γENaC total</td>
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<td>γENaC 85 kDa</td>
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<td>γENaC 70 kDa</td>
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<td>1.00 ± 0.05</td>
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<td>1.41 ± 0.04</td>
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Values are means ± SE. *Significantly different (P < 0.05) from control and †significantly different (P < 0.05) from Aldo + Spiro (P < 0.05).
Aldosterone-induced ENaC trafficking is insensitive to combined blockade of MR and GR. In addition to the activation of MR, elevated plasma aldosterone concentrations may also activate the GR and thereby regulate ENaC activity (16). To determine whether GR activation could play a role in the spironolactone-insensitive ENaC trafficking, we examined the effect of RU486, a GR antagonist, in aldosterone-infused rats cotreated with spironolactone (protocol 3).

Fig. 3. Immunoperoxidase microscopy of the αENaC subunit in the kidney cortex in protocol 1. In the aldosterone-infused rats, the immunolabeling was strong in the apical cell domain and there was distinct labeling of the apical plasma membrane domain (arrow) in connecting tubule cells of the CNT (A) and principal cells of the CCD (D). In rats treated with both aldosterone infusion and oral spironolactone (B and E), the labeling intensity and labeling distribution were similar to the rats treated only with aldosterone. In the untreated control rats, the labeling intensity was reduced and mainly dispersed in the cytoplasm (arrowhead) with no distinct labeling of the apical plasma membrane domain (C and F). Scalebar = 10 μm (A–C) and 20 μm (D–F).

Fig. 2. Immunoperoxidase microscopy and immunofluorescence confocal microscopy of Na-K-ATPase α1-subunit and NCC in the kidney cortex in protocol 1. Na-K-ATPase α1-subunit labeling is seen in the basolateral cell domain (arrows) in the cortical collecting duct (CCD) and is markedly increased in the aldosterone-infused rats (A) compared with rats receiving spironolactone (B) and untreated control rats (C), which appeared similar. To identify distal convoluted tubule (*), we used immunofluorescence double labeling with Na-K-ATPase α1-subunit (red) and NKCC2 (green), which identifies cTAL. In NKCC2 negative tubule segments with distal convoluted tubule (DCT) morphology, there was no overall difference in the labeling intensity, although the labeling in aldosterone-treated rats appeared marginally stronger (D) and in spironolactone-treated rats marginally weaker (E) compared with untreated control rats (F). NCC is expressed in the apical plasma membrane (arrows) of the DCT and is increased in the aldosterone-infused rats (G). The increased labeling was blocked by spironolactone to the level of untreated control rats (H and I). Scalebar = 10 μm (A–C and G–I) and 50 μm (D–F).
Immunoperoxidase labeling with βENaC and γENaC antibodies showed apical targeting in the CNT and CCD in aldosterone-infused rats (Fig. 5, A and E), as observed in protocol 1 (Figs. 3 and 4). Cotreatment of aldosterone-infused rats with RU486 alone (Fig. 5, B and F) or in combination with spironolactone (Fig. 5, C and G) had no effect on aldosterone-induced ENaC trafficking to the apical plasma membrane and ENaC labeling in CNT and CCD. In contrast, untreated control rats showed dispersed labeling without apical targeting (Fig. 5, D and H). The plasma potassium was decreased in aldosterone-infused rats and aldosterone-infused rats cotreated with RU486 but normalized to control levels by addition of spironolactone (Table 3). Additional physiological data are summarized in Table 3.

Spironolactone did not block αENaC upregulation in aldosterone-treated rats. It has previously been shown that aldosterone treatment increases αENaC protein abundance (30). Immunoblotting against the ENaC subunits using protein prepared from kidney cortex in both protocol 1 and protocol 2 confirmed increased protein expression of αENaC (Fig. 6 and Table 2). Surprisingly, aldosterone-infused rats cotreated with spironolactone did not decrease the protein abundance of αENaC compared with rats treated only with aldosterone (Fig. 6 and Table 2). Moreover, cotreatment with both spironolactone and RU486 in aldosterone-infused rats (protocol 3) also
Fig. 5. Immunoperoxidase microscopy of the βENaC in CNT (A–D) and γENaC in CCD (E–G) in the kidney cortex in protocol 3. In the aldosterone-infused rats, the immunolabeling is concentrated toward the apical plasma membrane domain (arrows) in connecting tubule cells of the CNT (A) and principal cells of the CCD (E). In aldosterone-infused rats receiving RU486 (B and F) and RU486 and spironolactone (C and G), the labeling remained in the apical plasma membrane domain. In the untreated control rats (D and H), the labeling intensity was similar but dispersed in the cytoplasm (arrowheads). Scalebar = 20 μm.
did not block the increased αENaC protein expression compared with untreated control (Fig. 7A and Table 4), despite normalization of the plasma potassium concentration (Table 3). The protein expression of αENaC in aldosterone-infused rats cotreated with RU486 alone was similar to aldosterone-infused rats and significantly elevated compared with untreated control rats (Table 4).

The mechanism mediating the elevated αENaC protein abundance in the presence of MR and GR blockade is unclear. It may be speculated that MR blockade may lead to increased ANG II levels that, in turn, could upregulate αENaC protein expression directly (6, 9). Measurements of plasma ANG II in protocol 3 showed more than a 10-fold increase in ANG II in aldosterone-infused rats cotreated with spironolactone and RU486 compared with aldosterone-infused rats and aldosterone-infused rats cotreated with RU486 (Table 4). ANG II was not significantly lower in aldosterone-infused rats and aldosterone-infused rats cotreated with RU486 compared with untreated control rats. Measurement of plasma aldosterone concentration in the same animals showed significantly elevated plasma aldosterone in all aldosterone-infused rats compared with untreated controls but no significant difference between the groups of aldosterone-infused rats (Table 3).

Aldosterone is also known to cause a molecular weight shift of γENaC from an 85-kDa band to a 70-kDa band (30) which we confirmed (Fig. 6C and Tables 2 and 4). The shift in molecular mass from 85 to 70 kDa was not reversed by cotreatment with spironolactone (protocol I; Fig. 6C and Table 2) or cotreatment with both spironolactone and RU486 (protocol 3; Fig. 7C and Table 4). Interestingly, aldosterone-infused rats treated with RU486 alone showed a significantly decreased total abundance of γENaC compared with control rats and compared with the other aldosterone-infused groups (Table 4). For unknown reasons, the immunoblot for γENaC in protocol 2 showed only a weak and unchanged 70-kDa form of γENaC (Table 2). The protein abundance of βENaC was unchanged in all studies (Figs. 6B and 7B; Tables 2 and 4).

In addition to the findings for ENaC subunits, NCC and Na-K-ATPase α1-subunit, there was no change in the protein expression of the sodium transporter NHE3 expressed in the proximal tubule and thick ascending limb of Henle, and of NKCC2 expressed in the thick ascending limb in either protocol 1 (Fig. 8 and Table 2) or protocol 2 (Table 2, blots not shown).

### DISCUSSION

We investigated the effects of MR and GR blockade on the regulation of ENaC trafficking in aldosterone-infused rats. ENaC trafficking to the apical plasma membrane in CNT and CCD of aldosterone-infused rats could not be blocked by cotreatment with MR and GR antagonists. Thus we provide evidence for ENaC trafficking mediated by a mechanism independent of the classical MR and GR in rats receiving aldosterone infusion. In contrast, we showed that aldosterone upregulates the protein expression of NCC and Na-K-ATPase α1-subunit in kidney cortex through MR activation, consistent with previous studies. These results extend our previous findings of spironolactone-insensitive ENaC trafficking induced by dietary sodium restriction (33).

**Regulation of ECF volume, NCC, and Na-K-ATPase.** In the present study, we investigated the direct effect of aldosterone on ENaC trafficking. Aldosterone infusion constitutes a physiological state prone to expansion of the ECF volume and hypertension that could be mediated by the regulation of aldosterone-sensitive renal sodium transporters and channels (e.g., NCC, Na-K-ATPase, and ENaC). However, it was previously shown that 2 wk of aldosterone infusion at a similar dose combined with saline drinking water did not cause weight gain or hypertension (34). The plasma aldosterone concentration in the high physiological range, combined with a normal daily sodium intake used in the present study, is therefore not expected to lead to massive ECF volume expansion, weight gain, or hypertension.

It should also be noted that the model used in this study is not an aldosterone escape model. During aldosterone escape, the kidney increases urinary sodium excretion despite elevated plasma aldosterone concentration when sodium intake is increased, preventing excessive fluid retention and blood pressure increase. Aldosterone escape is associated with a selective downregulation of NCC in aldosterone-infused rats when the daily sodium intake increased from 0.02 to 2 mmol Na+/day (43). It should be emphasized, however, that the protein abundance of NCC in the aldosterone-infused rats on a 2-mmol Na intake per day did increase compared with control rats as shown here and previously (28).

In this study, we show that the aldosterone-induced regulation of NCC is sensitive to blockade by spironolactone, which normalizes the expression level to control levels. This effect of
Fig. 6. Semiquantitative immunoblotting of protein prepared from the kidney cortex in protocol 1. αENaC (A) and βENaC (B) appears as single ~85-kDa bands. γENaC (C) appears as an upper 85-kDa band and a lower 70-kDa band, thought to be a proteolytic cleavage product of the 85-kDa band. Densitometry analysis revealed that αENaC was significantly increased in aldosterone-treated rats and rats treated with both aldosterone and spironolactone. The total γENaC was unchanged, although there was an increased expression of the 70-kDa band of γENaC. βENaC was unchanged. *P < 0.05 compared with untreated control rats.

Fig. 7. Semiquantitative immunoblotting of protein prepared from the kidney cortex in protocol 3. αENaC (A) was upregulated in all aldosterone-infused groups compared with control. βENaC (B) was unchanged. The 85-kDa form of γENaC (C) was downregulated in all aldosterone-infused groups while the lower 75-kDa band was increased. *P < 0.05 compared with untreated control rats.
spironolactone is consistent with previous observations in sodium-restricted rats (33) and supports the view that NCC is regulated by MR activation. Interestingly, it was recently shown that low-sodium intake is associated with redistribution of NCC in subapical vesicles to the apical plasma membrane (35). In our study, we are not able to determine whether the immunoperoxidase labeling of NCC is located in the apical plasma membrane or a subapical vesicle pool.

The Na-K-ATPase is also a well-known aldosterone target. Previously, aldosterone has been shown to increase the Na-K-ATPase activity in the CNT and CCD but not in the DCT (17), while adrenalectomized rats showed a 50% reduction in the Na-K-ATPase α1 mRNA expression in the CCD (14, 42). Consistent with this, we showed a markedly increased protein expression of the Na-K-ATPase α1-subunit in the kidney cortex in response to aldosterone, which was blocked by spironolactone. Moreover, immunohistochemistry revealed that the changes in Na-K-ATPase α1-subunit expression occurred in the CNT and CCD, while no changes were observed in the DCT. Our results support the previous studies showing changes in Na-K-ATPase activity and mRNA expression with changes in aldosterone levels (14, 17, 42). Interestingly, our findings are not consistent with the observations from the 8-day-old MR knockout mice, which showed no change in Na-K-ATPase α1-subunit mRNA (5), suggesting that the regulation of the Na-K-ATPase α1 is complex.

Finally, an important premise for the conclusion of the present study is the efficacy of the MR blockade by spironolactone. In addition to the complete blockade of the aldosterone-induced upregulation of NCC and Na-K-ATPase α1-subunit, the efficacy of MR blockade by spironolactone was confirmed in all three studies by a complete blockade of the aldosterone-mediated decrease in plasma potassium concentration.

ENaC trafficking in aldosterone-infused rats was insensitive to blockade of the MR and GR. It is well documented that aldosterone induces ENaC trafficking and increased ENaC activity at the apical plasma membrane (2, 4, 29). The MR is commonly thought to be involved in ENaC trafficking in vivo, although effects of MR antagonism on aldosterone-induced ENaC trafficking have not previously been studied. We previously showed that ENaC trafficking induced by dietary sodium restriction could not be blocked by spironolactone (33). In a novel CCD cell line (mCCDcl1) increased ENaC activity induced by aldosterone could be blocked by spironolactone, but the effect of spironolactone on aldosterone-induced ENaC trafficking was not directly examined (16). Since ENaC is regulated both by changes in open probability and trafficking, it is possible that spironolactone may affect the open probability but not ENaC trafficking. Decreased ENaC activity during spironolactone treatment is consistent with the potassium sparing effect of spironolactone and the changes on plasma potassium observed in the present study. The role of the MR in ENaC trafficking in vivo therefore remains unclear. Aldosterone has a lower affinity for the GR compared with the MR (Kd = 14 nmol/l vs. Kd = 0.5 nmol/l) (24). It has been suggested that aldosterone binding to the GR may play an

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**Table 4. Immunoblotting results from renal cortex in protocol 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aldo (n = 5)</th>
<th>Aldo + Spiro + RU (n = 5)</th>
<th>Aldo + RU (n = 5)</th>
<th>Control (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αENaC</td>
<td>2.41±0.34*</td>
<td>2.96±0.24*</td>
<td>2.33±0.33*</td>
<td>1.00±0.15</td>
</tr>
<tr>
<td>βENaC</td>
<td>1.17±0.15</td>
<td>1.25±0.09</td>
<td>1.00±0.07</td>
<td>1.00±0.14</td>
</tr>
<tr>
<td>γENaC total</td>
<td>0.75±0.08</td>
<td>0.82±0.04</td>
<td>0.64±0.05*</td>
<td>1.00±0.11</td>
</tr>
<tr>
<td>γENaC 85 kDa</td>
<td>0.41±0.04*</td>
<td>0.52±0.07*</td>
<td>0.41±0.05*</td>
<td>1.00±0.13</td>
</tr>
<tr>
<td>γENaC 70 kDa</td>
<td>1.92±0.24*</td>
<td>1.85±0.14*</td>
<td>1.42±0.09*</td>
<td>1.00±0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significantly different (P < 0.05) from control.

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Fig. 8. Semiquantitative immunoblotting of protein prepared from the kidney cortex in protocol 1. NHE3 (A) appears as single ~85-kDa bands and NKCC2 (B) appears as a ~165-kDa band. Densitometry analysis revealed no difference between any of the groups.
important role in obtaining maximal sodium transport during prolonged periods of elevated plasma aldosterone levels (16, 19). Our results show that aldosterone alone is sufficient to induce trafficking of ENaC and that ENaC trafficking in aldosterone-infused rats is not inhibited by cotreatment with MR antagonist and GR antagonist.

If the classical MR and GR are not mediating the aldosterone-induced ENaC trafficking, what then is the mechanism? It may be hypothesized that 1) aldosterone acts independent of the classical cytoplasmic MR and GR or 2) that increased ANG II levels in the spironolactone-treated rats regulate ENaC trafficking directly. Aldosterone has been shown to induce rapid changes in intracellular second messengers such as inositol triphosphate (IP3), diacylglycerol (DAG), cAMP, and intracellular Ca2+ in various tissues (for a review, see Ref. 7). In kidney cells, aldosterone has been shown to cause spironolactone-insensitive rapid increase in cAMP in primary cultures of inner medullary collecting duct cells (36) and transient increases in intracellular Ca2+—PKC activation, and increased Na/H exchange in Madin-Darby canine kidney cells (20, 23), via so-called nongenomic actions. The changes in second messengers may, in turn, regulate the serum- and glucocorticoid-regulated kinase (SGK) (for a review, see Ref. 31) known to be involved in ENaC trafficking (3, 11, 29, 32). SGK regulates ENaC through phosphorylation of the ubiquitin ligase Nedd4–2 and thereby decreases the ability of Nedd4–2 to bind and ubiquitylate ENaC, promoting longer retention of ENaC in the apical plasma membrane (38). Alternatively, the increased ANG II concentration in the spironolactone-treated rats could potentially explain the spironolactone-insensitive ENaC trafficking, although ANG II has not previously been associated with ENaC trafficking. ANG II induces a rise in intracellular IP3 and DAG leading to increased Ca2+ and PKC activation (8, 26) that, in turn, could regulate SGK and thus ENaC trafficking.

**Protein expression of αENaC and the 70-kDa γENaC form was not decreased by spironolactone treatment.** In our previous study, increased αENaC expression due to dietary sodium restriction could be blocked by spironolactone treatment (33). In this study, we observed no effect of spironolactone on the increased αENaC protein abundance in aldosterone-infused rats. One possible explanation for this difference could be the markedly increased plasma concentration of ANG II in the aldosterone-infused rats treated with spironolactone and RU486 compared with rats given either aldosterone infusion or RU486. ANG II has been shown to directly increase αENaC mRNA and protein expression through the AT1α receptor (6, 9). Blockade of the AT1α receptor with candesartan in the presence of high-dose spironolactone was associated with decreased αENaC mRNA and protein expression (6). Moreover, the AT1α receptor knockout mice, which showed elevated plasma aldosterone, had markedly decreased αENaC protein expression. Thus spironolactone treatment may lead to a compensatory increase in αENaC expression mediated by increased ANG II masking the effect of spironolactone. In contrast, the ANG II levels in both sodium-restricted rats and sodium-restricted rats receiving spironolactone are expected to be markedly elevated and will therefore not differentially affect the αENaC protein expression.

In two of the three protocols, we found no evidence that spironolactone could reverse the aldosterone-induced molecular mass shift of γENaC from 85 to 70 kDa. The lack of effect of spironolactone is in contrast to what was observed during sodium restriction (33). The molecular mass shift of γENaC associated with increased aldosterone (30) is thought to be the result of proteolytic cleavage by membrane-associated serine proteases (41). However, ENaC cleavage also occurs as a part of maturation in the Golgi apparatus by the protease furin (25). Common for both pathways of cleavage is that the regulatory mechanism remains unknown and we therefore cannot explain the lack of effect of spironolactone. It should, however, be emphasized that spironolactone treatment was associated with physiological renal changes (including plasma potassium) as well as blocking the aldosterone-induced increases in NCC and α1-subunit of the Na-K-ATPase expression, consistent with previous evidence documenting its effect. Based on this, it is speculated that the differences seen in this study compared with previous studies in relation to αENaC expression and γENaC proteolytic cleavage may be caused by different experimental conditions between sodium restriction and direct aldosterone infusion.

In summary, the present study provides new evidence of ENaC trafficking in aldosterone-infused rats independent of activation of the MR and GR. Furthermore, the study demonstrated an aldosterone-induced increase in NCC expression in the DCT and Na-K-ATPase α1 expression in CNT and CCD mediated by the classical MR.

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