Activation of epithelial Na channels during short-term Na deprivation

GUSTAVO FRINDT,1 SHYAMA MASILAMANI,2 MARK A. KNEPPER,2 AND LAWRENCE G. PALMER1
1Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, New York 10021; and 2Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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Frindt, Gustavo, Shyama Masilamani, Mark A. Knepper, and Lawrence G. Palmer. Activation of epithelial Na channels during short-term Na deprivation. Am J Physiol Renal Physiol 280: F112–F118, 2001.—The role of epithelial Na channels in the response of the kidney to short-term Na deprivation was studied in rats. Animals were fed either a control-Na (3.9 g/kg) or a low-Na (3.8 mg/kg) diet for 15 h. Urinary excretion of Na (μmol/min), measured in conscious animals in metabolic cages, was 0.45 ± 0.07 in controls and 0.04 ± 0.01 in Na-deprived animals. Glomerular filtration rate, measured as the clearance of creatinine, was unaffected by the change in diet, suggesting that the reduced Na excretion was the result of increased Na reabsorption. K excretion (μmol/min), increased after the 15-h period of Na deprivation from 0.70 ± 0.10 to 1.86 ± 0.19. Thus the decrease in urine Na was compensated for, in terms of electrical charge balance, by an increase in urine K. Plasma aldosterone increased from 0.50 ± 0.08 to 1.22 ± 0.22 nM. Principal cells from cortical collecting tubules isolated from the animals were studied by using the patch-clamp technique. Whole cell amiloride-sensitive currents were negligible in the control group (5 ± 4 pA/cell) but substantial in the Na-deprived group (140 ± 28 pA/cell). The abundance of the epithelial Na channel subunits, α, β, and γ in the kidney was estimated by using immunoblots. There was no change in the overall abundance of any of the subunits after the 15-h Na deprivation. However, the apparent molecular mass of a fraction of the γ-subunits decreased as was previously reported for long-term Na deprivation. Calculations of the rate of Na transport mediated by the Na channels indicated that activation of the channels during short-term Na deprivation could account in large part for the increased Na reabsorption under these conditions.

cortical collecting tubule; epithelial sodium channel; aldosterone; sodium transport; potassium transport

THE RENIN-ANGIOTENSIN-ALDOSTERONE axis is thought to play an important role in the regulation of Na balance and plasma volume (8). A reduction in plasma volume triggers the release of renin from juxtaglomerular cells, stimulating production of angiotensin from angiotensinogen and ultimately leading to increased circulating levels of aldosterone. An important target of aldosterone action on the kidney is the epithelial Na channel (ENaC) (23). Chronic elevation of plasma aldosterone has been shown to increase amiloride-sensitive Na transport in the isolated-perfused cortical collecting tubule (CCT) (21, 26, 29) and to increase amiloride-sensitive short-circuit current in the colon (2, 32). In the rat kidney, aldosterone increases the density of conducting Na channels in the apical membrane of the CCT (17). The increase can be dramatic, starting from an undetectably low number when animals are on a diet of normal rat chow and reaching three to five channels per patch when the rats are fed a low-salt diet for a week or more. More rapid effects of aldosterone at the single-channel level have been observed in vitro by using the A6 cell line (11). In this case increased channel activity is due at least in part to an increase in open probability.

The role of aldosterone-mediated activation of Na channels in the day-to-day regulation of sodium excretion is not so well documented. Infusion of aldosterone in vivo can reduce Na excretion within 1–2 h (10), although the mechanisms through which this occurs have not been delineated. In addition to activation of Na channels, aldosterone has been shown to stimulate NaCl cotransporter in the distal convoluted tubule (12) and to alter Na/H exchange in Madin-Darby canine kidney cells through a nongenomic pathway (15). In previous studies on the rat CCT, our laboratory could not document a clear elevation of either Na channel activity or plasma aldosterone levels for periods of Na deprivation of less than 48 h (17). Thus, although the regulation of Na channels by aldosterone has been extensively studied, the relevance of this pathway in the day-to-day maintenance of Na homeostasis is less well established.

In the present study we have reevaluated this question by measuring changes in Na and K excretion as well as levels of Na channel activity and plasma aldosterone in rats that have been fed a low-Na diet for short periods of time. We found that the appearance of
channel activity correlates well with the fall in Na excretion, and that the magnitude of the increase can account in large part for the increased rate of Na reabsorption by the nephron.

**METHODS**

**Animals.** Sprague-Dawley rats of either sex (100–150 g) raised free of viral infections (Charles River Laboratories, Kingston, NY) were fed with either a low-Na diet (Na content 3.8 mg/kg; K content 8.6 g/kg; ICN, Cleveland, OH) or a control diet (3.9 g/kg Na, 6.4 g/kg K, Harlan-Teklad, Madison, WI). The food intake per rat ranged between 6 and 15 g/day. A different group of animals were implanted subcutaneously with osmotic minipumps (model 2002 or 1007D Alza, Palo Alto, CA) to increase levels of circulating aldosterone. Aldosterone was dissolved in polyethylene glycol 300 at concentrations calculated to give the desired infusion rate according to the pumping rate specified by the manufacturer. These rats were fed a control diet.

To measure urinary excretion rates animals were kept in a metabolic cage (Nalgene Nunc International, Rochester, NY) for 2–3 h without food but with free access to drinking water, lightly sweetened with 3% sucrose, to increase water intake and urine flow.

**Analytical methods.** Rats were anesthetized with methoxyflurane, and blood was obtained from the abdominal aorta. Na and K were measured in plasma and in urine by flame photometry (model 943, Instrumentation Laboratory, Lexington, MA).

Creatinine was measured by an enzymatic method (6). Only female rats were used for creatinine clearance measurements because creatinine is not secreted by the kidney in the female rat (9).

Radioimmunoassay for plasma aldosterone was carried out with the ImmunoChem double antibody kit from ICN (ICN Pharmaceuticals, Costa Mesa, CA) as described previously (19).

**Whole cell currents.** After the animals were killed, the kidneys were removed, and CCTs were dissected free and opened manually to expose the luminal surface. Under these conditions the tissues retain their epithelial structure and the cells are presumed to remain polarized. The split tubules were attached to a small plastic rectangle coated with Cell-Tak (Collaborative Research, Bedford, MA) and placed in a perfusion chamber mounted on an inverted microscope. The chamber was continuously perfused with solution consisting of (in mM) 135 Na methane sulfonate, 5 KCl, 2 CaCl₂, 1 MgCl₂, 2 glucose, 5 mM BaCl₂, and 10 HEPES, adjusted to pH 7.4 with NaOH, and prewarmed to 37°C.

Principal cells of the tubule were identified visually. The patch-clamp pipettes were filled with solutions containing (in mM) 7 KCL, 123 aspartic acid, 20 CsOH, 20 tetraethylammonium hydroxide, 5 EGTA, 10 HEPES, 3 MgATP and 0.3 guanosine 5′-O-(2-thiodiphosphate) with the pH adjusted to 7.4 with KOH. Basic protocols for measuring whole cell amiloride-sensitive current were previously described (18, 20).

**Antibodies.** This study utilized rabbit polyclonal antibodies directed to the α-, β-, and γ-subunits of the rat ENaC (14) and the Na-K-2Cl cotransporter (BSC1/NKCC2) (12).

**Semi quantitative immunoblotting.** To compare sodium transporter protein abundance between groups of rats, semiquantitative immunoblotting was utilized. The procedure has been described in detail previously (12, 28). Briefly, the kidneys were homogenized intact, by using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) in ice-cold isolation solution containing 250 mM sucrose/10 mM triethanolamine (Calbiochem, La Jolla, CA) with 1 μg/ml leupeptin (Bachem California, Torrance, CA) and 0.1 mg/ml phenylmethylsulfonyl fluoride (US Biochemical, Toledo, OH). Total protein (Pierce BCA kit) was measured and the samples were solubilized at 60°C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on 7.5, 10, or 12% polyacrylamide gels. To confirm equal loading among samples, an initial gel was stained with Coomassie blue as described previously (28). For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes. The membranes were blocked with 5 g/dl nonfat dry milk for 30 min, probed with the respective primary antibodies overnight at 4°C, and then probed with secondary antibody (donkey anti-rabbit immunoglobulin G conjugated with horseradish peroxidase, Pierce no. 31458, diluted to 1:5,000) for 1 h at room temperature. The sites of antibody-antigen reaction were visualized by using enhanced chemiluminescence substrate (LumiGLO for Western blotting, Kirkegaard and Perry no. VC110) before exposure to X-ray film (Kodak no. 165–1579). The band densities were quantitated by laser densitometry (Molecular Dynamics model PDS1-P90). Image Quant version 5.0 software was used to determine the background correction and quantify the band densities. The densitometry values were normalized to facilitate comparisons, defining the mean for the control group as 100%. Statistical analysis used either an unpaired t-test or Welch t-test.

**RESULTS**

Rats were kept overnight on either a control or low-Na diet. After 15 h the animals were transferred to metabolic cages without food but with free access to drinking water. Normally the rats eat little food during the late morning hours, so we presume that the food deprivation has a minimal effect on overall salt balance. Urine was collected over a 2- to 3-h period. The effect of the low-sodium diet on excretion rates is shown in Table 1. Compared with matched controls, sodium excretion is reduced by more than 90%.

### Table 1. Effect of low-Na diet on renal function

<table>
<thead>
<tr>
<th></th>
<th>Control (15 h) (n = 11)</th>
<th>Low Na (15 h) (n = 11)</th>
<th>Control (10 days) (n = 6)</th>
<th>Low Na (10 days) (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR, ml/min</td>
<td>1.29 ± 0.11</td>
<td>1.31 ± 0.12</td>
<td>1.65 ± 0.14</td>
<td>1.65 ± 0.11</td>
</tr>
<tr>
<td>Plasma Na, mM</td>
<td>139 ± 1</td>
<td>140 ± 1</td>
<td>139 ± 1</td>
<td>136 ± 1</td>
</tr>
<tr>
<td>Plasma K, mM</td>
<td>3.62 ± 0.07</td>
<td>4.07 ± 0.10*</td>
<td>3.56 ± 0.10</td>
<td>4.02 ± 0.11*</td>
</tr>
<tr>
<td>( U_{NaV} ), μmol/min</td>
<td>0.45 ± 0.07</td>
<td>0.04 ± 0.01*</td>
<td>0.36 ± 0.11</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>( U_{KV} ), μmol/min</td>
<td>0.70 ± 0.10</td>
<td>1.86 ± 0.19*</td>
<td>0.61 ± 0.11</td>
<td>0.95 ± 0.09*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; GFR, glomerular filtration rate; \( U_{NaV} \), urinary sodium excretion rate; \( U_{KV} \), urinary potassium excretion rate. *Significantly different from controls (P < 0.05).
In principle, a reduction in Na excretion can result from either a reduction in the filtered Na load or from an increase in Na reabsorption along the nephron. To evaluate the impact of changes in filtered load we estimated GFR by using the creatinine clearance. Urinary creatinine was measured over the same 2- to 3-h period described above, whereas plasma creatinine was measured on a sample of blood obtained from the aorta when the rat was killed at the end of this period. The results are shown in Table 1. The low-Na diet did not significantly change GFR after 15 h. Therefore, the decreased Na excretion is most likely due to an increase in Na reabsorption by the kidney. There was a tendency for GFR to decrease after long-term Na deprivation (Table 1), but this did not reach statistical significance (P > 0.2).

In most nephron segments, reabsorption of Na is accompanied by a parallel reabsorption of Cl. The major exception is in the CCT in which uptake of Na is coupled electrically with the secretion of K and H ions. We therefore compared changes in K and Na excretion as a first test for whether the CCT might be involved in the increased reabsorption rate. As shown in Table 1, K excretion increased after 15 h on a low-Na diet. In fact, the increase in K excretion consistently exceeded the decrease in Na excretion. We interpreted these data as indicating that nephron segments in which Na is effectively exchanged for K, most likely including the CCT, are involved in the alteration of electrolyte handling under these conditions.

To evaluate whether increased mineralocorticoid secretion by the adrenals could play a role in the sodium retention, we measured levels of aldosterone in the plasma of rats that were Na depleted for 15 h. As indicated in Table 2, aldosterone levels increased modestly from 18 to 44 ng/dl or 0.50 to 1.22 nM. This increase was statistically significant but much smaller than that observed after chronic Na depletion (Table 2, see also Refs. 18, 20).

To test whether this increase in circulating aldosterone could be sufficient to activate Na channels, CCTs were dissected from control and 15-h Na-deprived rats. The tubules were split open, and Na channel activity was assessed as the whole cell amiloride-sensitive current. Figure 1 shows typical current-voltage relationship curves in the presence and absence of amiloride for the two conditions. In tubules from control animals there was little or no effect of amiloride on the electrical properties of the principal cells. Changes in current of >50 pA at a cell potential of −100 mV were observed in only 2 of 28 cells (Fig. 2). In contrast, in tubules from Na-deprived animals significant amiloride-sensitive currents (\( I_{Na} \)) were recorded in 18 of 27 cells. The mean value was 140 pA/cell at −100 mV. This represents one-third to one-fourth of that seen in tubules from chronically Na-depleted rats (Table 2). As shown in Fig. 2 there was a substantial variation in the \( I_{Na} \) values.

### Table 2. Effect of low-Na diet on Na channels and plasma aldosterone

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Na (15 h)</th>
<th>Low Na (10 days)</th>
<th>Aldosterone Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>( I_{Na}, ) pA/cell</td>
<td>5 ± 4 (28)</td>
<td>140 ± 28* (27)</td>
<td>510 ± 100* (17)</td>
<td>93 ± 29* (19)</td>
</tr>
<tr>
<td>Plasma aldosterone, nM</td>
<td>0.50 ± 0.08 (21)</td>
<td>1.22 ± 0.22* (11)</td>
<td>28 ± 2* (4)</td>
<td>1.72 ± 0.39* (3)</td>
</tr>
</tbody>
</table>

Values are means ± SE. \( I_{Na} \), amiloride-sensitive currents; nos. in parentheses, no. of cells. *Significantly different from control values (P < 0.05).
These data were compared with those obtained by infusing aldosterone through osmotic minipumps to give comparable levels of the hormone over a comparable period of time. The aldosterone infusion resulted in slightly higher hormone levels (1.72 vs. 1.22 nM), whereas the $I_{Na}$ values were somewhat lower (93 vs. 142 pA/cell). This suggests that increased aldosterone can account in large part for the observed Na channel activity.

Previous studies indicated that biochemical alterations in ENaC subunits were associated with stimulation of Na channel activity. These included an increase in the abundance of the $\alpha$-subunit protein, and a shift in the apparent molecular mass of the $\gamma$-subunit to lower levels (14). We tested whether either of these parameters correlated with the decreased Na excretion that we observed with the 15-h depletion protocol. Figure 3 shows an immunoblot in which samples of kidney from the same animals used for the excretion and electrophysiological measurements were probed with antibodies raised against the three different ENaC subunits. Similar blots made with kidneys from chronically Na-depleted rats are shown for comparison (Fig. 4). There was no significant change in the abundance of the $\alpha$-subunit in the 15-h depleted animals [$107 \pm 24\%$ of control; $P =$ not significant (NS)], whereas in those depleted for longer times higher levels were observed (164 $\pm 27\%$ of control; $P < 0.05$) as previously reported. For the $\gamma$-subunit there was an increase in the amount of the lower molecular mass species (60–70 kDa) (314 $\pm 57\%$ of control; $P < 0.05$). The percentage of the total densitometric signal in the lower molecular mass region was 6.2 $\pm 0.4\%$ in controls and 25 $\pm 7\%$ ($P < 0.05$ vs. controls) for the 15-h Na-depleted rats. Similar but more pronounced effects were seen in the chronically depleted animals. The increase in the lower molecular mass species was 885 $\pm 93\%$ of control ($P < 0.05$). The fraction of the signal in the lower molecular mass region was 2.2 $\pm 2.3\%$ in controls and 44 $\pm 2\%$ for the Na-depleted rats.

Thus the alterations in the $\gamma$-subunit correlate well with the activation of the channels. There was a significant heterogeneity evident in the blots. This also corresponded reasonably well with physiological measurements. The two animals that had the largest average $I_{Na}$ were also those that had the two largest effects on the $\gamma$-subunit (second and third lanes from left, low-NaCl, Fig. 3). The rat that had the largest percentage of low-molecular mass $\gamma$-subunit (middle lane, low NaCl, Fig. 3) also had the highest value of plasma aldosterone.

As a negative control, samples were also probed for the bumetanide-sensitive Na-K-2Cl cotransporter (BSC1/NKCC2). There was no significant change in the protein abundance for BSC1 in samples from 15-h depleted animals (85 $\pm 32\%$ of control; $P = NS$) or chronically depleted animals (113 $\pm 25\%$ of control; $P = NS$), as previously shown.

**DISCUSSION**

*Time course of aldosterone action.* Our results imply that the control of Na channels by aldosterone can participate in Na homeostasis during day-to-day variations in Na intake. This result is not surprising in light of the central role generally attributed to the renin-angiotensin-aldosterone axis in the control of blood volume and blood pressure. However, we consider its demonstration significant in that it is, to our knowledge, the first study to make a direct connection.
between channel activity and in vivo salt balance over this short time course.

In a previous study (17), our laboratory found little evidence for increased channel activity or plasma aldosterone until 48 h after the initiation of a low-Na diet regimen. We suspect that the difference may lie in the degree of Na deprivation. In the earlier protocol, several rats were housed in the same cage and during Na depletion were frequently observed drinking each other’s urine. This would allow a fair degree of Na “recycling,” delaying the time at which the renin-angiotensin-aldosterone system is activated. In the present study the animals were housed singly.

We cannot be certain that the observed activation of Na channels is completely accounted for by increased aldosterone secretion. The rise in plasma aldosterone is significant but small. Because the concentrations are close to the reported dissociation constant values for binding to mineralocorticoid receptors (0.5–3 nM (23)), this rise will also be accompanied by a significant increase in receptor occupancy. As shown in Table 2, infusion of exogenous aldosterone to achieve similar plasma levels had an effect on $I_{Na}$, which was not statistically different from that achieved with Na depletion. However, the effect was, if anything, a bit smaller, and we cannot rule out the possibility that other factors are involved in regulating the channels under these circumstances.

**K secretion vs. Na absorption.** One unexpected finding was the large increase in K excretion relative to the decrease in Na excretion during the short-term Na depletion (Table 1). It is likely that the enhanced K excretion is related to the increase in plasma K that was also observed. The reason for this increase is unclear, but it has been observed before during Na depletion (3). In the CCT, Na and K fluxes can be electrically coupled across the apical membrane and more directly coupled through the Na/K-ATPase across the basolateral membrane. It is unlikely that there can be an excess of K secretion over Na reabsorption in such a system. In the rabbit CCT, measurements over a large range of Na transport indicated a coupling ratio of K secretion to Na reabsorption of 0.74 (27). It is possible that, whereas Na/K exchange is activated in the CCT, NaCl reabsorption is inhibited at a more proximal site, increasing delivery of Na to the collecting duct. Alternatively, KCl secretion might be stimulated. The most likely site for the latter event would be the distal convoluted tubule where an electroneutral KCl cotransport is thought to contribute to transepithelial salt movement (30). Regardless of the mechanism, it is likely that enhanced K secretion is driven at least in part by the elevation in plasma K that was consistently observed with the low-Na diet (Table 1).

This could be due in part to the slightly higher K content of the low-Na diet (see METHODS). We cannot rule out a contribution due to efflux of K from intracellular stores.

**Biochemical events.** The cellular actions of aldosterone are complex and are thought to involve the increased synthesis of key transport and/or regulatory proteins on different time scales (31). These time scales are often divided into “early” (<3 h) and “late” (>3 h) phases based on the responses of in vitro systems to an instantaneous addition of a large concentration of hormone. It is not completely clear how this relates to the in vivo response to a change in salt intake, which in the case of a reduction of dietary Na will result in a gradually increasing concentration of circulating aldosterone levels.

In the rat kidney, two effects of aldosterone on the ENaC subunit proteins have been documented. The abundance of both mRNA (1, 5, 16, 22) and protein (14) of the $\alpha$-subunit increases with mineralocorticoid status, although the fractional increase is small and unlikely to account for the large stimulation of channel activity. A second event is the appearance of a lower molecular mass band of the $\gamma$-subunit of ENaC (60–70 kDa), consistent with cleavage of the subunit by a luminal protease (14). In this study, we have demonstrated that chronically depleted animals demonstrate an increase in the protein abundance of the $\alpha$-subunit of ENaC and showed an appearance of the lower molecular mass band of the $\gamma$-subunit of ENaC (60–79 kDa). In contrast, 15-h depleted animals did not show an increase in the protein abundance of the $\alpha$-subunit of ENaC but did show the appearance of the lower molecular mass band of the $\gamma$-subunit of ENaC (60–79 kDa). Thus we conclude that the $\alpha$- and $\gamma$-subunits of ENaC can be regulated independently. Further, our data imply that the shift in the molecular mass of the
γ-subunit is well correlated with the time course of appearance of channel activity in response to a reduction in Na intake. It is not known whether this effect is a cause or an effect of channel activation.

Quantitative assessment. To consider the quantitative impact of the stimulation of Na channel activity on the reduction of Na excretion by the kidney, we have made the following assumptions regarding transport by the CCT.

1) The apical membrane voltage is −80 mV (24, 25). At this voltage, the mean Na current into the cell under whole cell clamp conditions is 116 pA/cell = 7 × 10−14 mol Na/min.

2) There are 200 cells/mm tubeule, of which two-thirds are principal cells. This implies a Na reabsorption rate of 9 × 10−12 mol·min−1·mm tubeule−1.

3) There are 7,200 CCTs/kidney with a length of 2 mm (13). In addition, there are 36,000 initial collecting tubules/kidney with a length of 0.4 mm (4). These are assumed to have the same Na transport properties as the CCT. This gives a total length of 58,000 mm/2 kidneys, corresponding to a total reabsorption rate of 0.5 × 10−6 mol/min.

This estimate is comparable to the actual measured decrease in Na excretion of 0.4 × 10−6 mol/min (Table 1). The agreement is probably fortuitous, because the uncertainty of some of the assumptions is larger than the difference between estimated and measured values. In particular, we have assumed that the channel activity in vivo is the same as that measured in vitro. This is probably unlikely, because the channels are influenced by other hormones such as antidiuretic hormone (7, 21, 29), the effects of which on the cells are probably rapidly reversed when the kidneys are removed from the animal. On the other hand, if salt is reabsorbed in excess of water in the CCT, the luminal Na concentration could fall below that required for maximal transport rates (20). Finally, we have assumed that Na channel densities are similar both along the length of the CCT and in the initial collecting tubule. There is no experimental basis for this assumption. Nevertheless this simple calculation indicates that the increase in Na transport in the collecting tubule expected from the measured activation of Na channel activity could account for a large part of the reduced Na excretion under the conditions of short-term Na depletion.

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