Conservation of Na\textsuperscript{+} vs. K\textsuperscript{+} by the rat cortical collecting duct

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Regulation of transport by principal cells of the distal nephron contributes to maintenance of Na\textsuperscript{+} and K\textsuperscript{+} homeostasis. To assess which of these ions is given a higher priority by these cells, we investigated the upregulation of epithelial Na\textsuperscript{+} channels (ENaC) in the rat cortical collecting duct (CCD) during Na depletion with and without simultaneous K depletion. ENaC activity, assessed as whole cell amiloride-sensitive current in split-open tubules, was 260 ± 40 pA/cell in K-repleted but virtually undetectable (3 ± 1 pA/cell) in K-depleted animals. This difference was confirmed biochemically by the reduced amounts of the cleaved forms of both the α-ENaC and γ-ENaC subunits measured in immunoblots. In contrast, in K-depleted rats, simultaneously reducing Na intake did not affect the activity of ROMK channels, assessed as tertiapin-Q-sensitive whole cell currents, in the CCDs. The lack of Na current in K-depleted animals was the result of reduced levels of aldosterone in plasma, rather than a reduced sensitivity to the hormone. However, rats on a low-Na, low-K diet for 1 wk did not excrete more Na than those on a low-Na, control-K diet for the same period of time. Immunoblot analysis indicated increased levels of the thiazide-sensitive Na\textsuperscript{+}–Cl\textsuperscript{−} cotransporter and the apical Na–H exchanger NHE3. This suggests that with reduced K intake, Na balance is maintained despite reduced aldosterone and Na\textsuperscript{+} channel activity by upregulation of Na\textsuperscript{+} transport in upstream segments. Under these conditions, Na\textsuperscript{+} transport by the aldosterone-sensitive distal nephron is reduced, despite the low-Na intake to minimize K\textsuperscript{+} secretion and urinary K losses.

ENaC; ROMK; aldosterone; NCC; NKCC2; NHE3

Principal cells of the distal nephron help to maintain homeostasis of both Na and K. Decreases in Na intake upregulate Na\textsuperscript{+} reabsorption in the connecting tubule (CNT) and cortical collecting duct (CCD) to reduce urinary Na losses. In addition, increased dietary intake of K increases K\textsuperscript{+} secretion in these segments to help keep plasma levels of the ion within normal limits. The two systems share at least one common signaling factor—the adrenocortical steroid aldosterone. This hormone is secreted in response to both a reduction in plasma volume via the renin-angiotensin system and also directly in response to hyperkalemia (15, 23, 31). It interacts with distal segments of the nephron, called the aldosterone-sensitive distal nephron or ASDN, to promote both Na retention and K excretion by the kidney (42). In fact, both the natrieric and kaliuretic responses result at least in part from stimulation of the same membrane transport protein, the epithelial Na\textsuperscript{+} channel (ENaC). Increased numbers of conducting channels allow more Na\textsuperscript{+} to diffuse into the cell from the urine, the rate-limiting step for Na\textsuperscript{+} transport (42). At the same time, this inflow of Na\textsuperscript{+} depolarizes the apical cell membrane and increases the driving force for K\textsuperscript{+} efflux from the cell into the tubular fluid (24). Thus, the activity of Na\textsuperscript{+} channels can also be the limiting factor for K\textsuperscript{+} secretion (11, 14, 48).

In some cases, the needs of the organism to retain Na and excrete K will be in conflict. Under such conditions, transport by the ASDN of one of these ions may take precedence over that of the other. We studied the regulation of these systems in the rats fed a diet that is very low in both Na\textsuperscript{+} and K\textsuperscript{+}. In this instance, activation of Na\textsuperscript{+} channels will serve the animal well in terms of conserving Na, but it will be detrimental in that it will promote K excretion when this ion is also in short supply. We found that the principal cells of the CCD keep Na\textsuperscript{+} channel activity low under these conditions, even with Na intake near zero, apparently giving precedence to the minimization of K\textsuperscript{+} secretion. Na excretion may be minimized by increasing reabsorption by other transporters in other segments through mechanisms that do not require elevation of plasma aldosterone.

METHODS

Animals. All procedures using animals were approved by the Institutional Animal Care and Use Committee of Weill-Cornell Medical College. Sprague-Dawley rats (150–200 g) of either gender (Charles River Laboratories, Kingston, NY) raised free of viral infections were used for all experiments. Animals were fed for 6–8 days with a sodium-deficient diet (<0.005% Na and 1% KCl), a K-deficient diet (0.4% Na and 0.1% KCl), or a Na- and K-deficient diet (<0.005% Na and 0.1% KCl; Harlan-Teklad, Madison, WI).

Electrophysiology. Measurement of whole-cell currents in principal cells of the CCD followed procedures described previously (6, 9). Split-open tubules were superfused with solutions prewarmed to 37°C containing (in mM) 135 Na methanesulfonate, 5 KCl, 2 Ca methanesulfonate, 1 MgCl\textsubscript{2}, 2 glucose, and 10 HEPES adjusted to pH 7.4 with NaOH.

For measurements of Na\textsuperscript{+} currents through ENaC, the bath solution also contained 5 mM Ba methanesulfonate. Patch-clamp pipettes were filled with solutions containing (in mM) KCl, 123 aspartic acid, 20 CsOH, 20 TEAOH, 5 EGTA, 10 HEPES, 3 MgATP, and 0.3 NaGDP\textsubscript{β}S with the pH adjusted to 7.4 with KOH. The total concentration of K\textsuperscript{+} was 120 mM. Na\textsuperscript{+} currents were measured as the difference in current with and without 10–3 M amiloride in the bath.

For measurements of K\textsuperscript{+} currents through ROMK channels, the patch-clamp pipettes were filled with solutions containing (in mM) KCl, 123 aspartic acid, 5 EGTA, 10 HEPES, with the pH adjusted to 7.4 with KOH. The total concentration of K\textsuperscript{+} was 145 mM. Tertiapin-Q (TPNQ; Sigma, St. Louis, MO) was dissolved in H2O at a concentration of 100 µM and diluted into the bath solution to a final concentration of 100 nM. Ba acetate was added to the bath solution to a final concentration of 5 mM.

Pipettes were pulled from hematocrift tubing, coated with Sylgard, and fire-polished with a microforge. Pipette resistances ranged from 2 to 5 MΩ. Currents were measured with a List EPC-7 amplifier (Heka Elektronik, Lambrecht, Germany). Voltages were controlled and currents were recorded using Pulse software (Heka) and an Instrutech ITC-16 interface (Instrutech, Mineola, NY).

Antibodies. Polyclonal antibodies against the α-, β-, and γ-subunits of the rat ENaC were described previously (4, 5). Antibodies against

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the Na-CI cotransporter (NCC) were a generous gift of Dr. David Ellison (Oregon Health Sciences Univ.). Antibodies raised against Na-H exchanger type 3 (NHE3) and the Na-K-2Cl cotransporter type 2 (NKCC2) were purchased from Chemicon International (Millipore, Billerica, MA).

**Immunoblotting.** Whole kidneys were minced and homogenized in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 1,000 rpm for 10 min to sediment intact cells, nuclei, and debris. Protein in the low-speed supernatant was measured (BCA Kit, Pierce Biotechnology, Rockford, IL). Equal amounts of protein (40–60 μg/sample) were solubilized at 70°C for 10 min in Laemmli sample buffer and were resolved on 4–12% bis-Tris gels (Invitrogen, Carlsbad, CA) by SDS-PAGE. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to PVDF membranes. After being blocked with BSA, membranes were incubated overnight at 4°C with primary antibodies at 1:500 or 1:1,000 dilutions for α-, β-, or γ-ENaC subunits, 1:2,000 for NHE3, NKCC2, and 1:13,000 for NCC. Bound antibodies were detected using anti-rabbit IgG conjugated with alkaline phosphatase and detected with a chemiluminescence substrate (Western Breeze, Invitrogen) on autoradiography film (Denville Scientific, Metuchen, NJ). Films were scanned with Image Zone software (Hewlett-Packard, Palo Alto, CA) and band densities were quantitated using Photoshop (Adobe Systems, San Jose, CA).

**Analytical.** Urine was collected in metabolic cages for a period of 2–3 h in the morning. Na and K concentrations in urine and plasma were measured with a flame photometer (model 943; Instrumentation Laboratories, Lexington, MA). Creatinine in plasma and urine was measured with a flame photometer (model 943; Instrumentation Laboratories, Lexington, MA). Creatinine clearance, an index of glomerular filtration rate, was maintained at the expense of reduced levels of the ion. Creatinine clearance, an index of glomerular filtration rate, was similar in the two groups of animals. This indicates that the low rates of electrolyte excretion in the LS/LK rats were not due to decreased glomerular filtration rate (GFR).

To test whether Na channels were activated under these conditions, we measured amiloride-sensitive whole cell currents (I_{Na}) in principal cells of the isolated, split-open CCD, a model of the ASDN. Typical current-voltage relationships in the absence and presence of the blocker are shown for a LS/CK animal in Fig. 1A. Similar to previous results (6), I_{Na} was robust in nearly all cells under these conditions. In contrast, when the same protocol was carried out with the LS/LK rats, I_{Na} was virtually undetectable (Fig. 1B), similar to tubules from Na-replete animals (6, 9). Figure 1C shows mean values under the two conditions.

In K-replete animals, Na depletion alters the amount and character of ENaC proteins (4, 25). Immunoblots of total rat kidney proteins probed with antibodies against the three ENaC subunits are shown in Fig. 2. A prominent effect of the low-Na diet is the appearance of reduced molecular mass, proteolytically cleaved products of α- and γ-ENaC. As indicated in Fig. 2A, the abundance of the cleaved α-ENaC species (~30 kDa) was significantly reduced in the LS/LK relative to LS/CK kidneys. The content of the full-length α-ENaC band (~85 kDa) was not significantly different in the two groups. Intermediate bands (50–60 kDa) were also recognized by this antibody, but their relationship to ENaC is unclear; their abundance was not significantly altered by diet and they were not affected by treatment with the glycosidase PNGaseF (4). There was also a significant enhancement of the full-length γ-ENaC subunit (~80 kDa) and a decrease in the cleaved γ-ENaC subunit (~60–65 kDa) in the LS/LK relative to the LS/CK animals. In this series of animals, there was a broad distribution of cleaved protein suggesting multiple cleavage sites. The significance of this distribution is unknown. There was little difference in the overall amount of β-ENaC (Fig. 2B) in the two groups. However, the appearance of a higher molecular mass, maturely glycosylated form of the subunit, previously reported for Na-depleted animals (4), was much less in the K-depleted animals. Densitometric analysis of the areas of the blots immediately above the sharp band at ~90 kDa indicated a 2.9 ± 0.5-fold increase in the LS/CK vs. LS/LK groups. Thus, kidneys from animals ingesting a LS/LK diet had a lower abundance of fully processed ENaC subunits with mature glycosylation and proteolytic cleavage. These biochemical differences are consistent with the greatly reduced Na channel currents seen with the LS/LK rats. The lack of apical Na\(^{+}\) conductance will reduce the driving force for K\(^{+}\) secretion in these segments, lowering urinary K losses.

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**RESULTS**

Rats were fed with either a low-Na, control-K diet (LS/CK) or a low-Na, low-K diet (LS/LK) for 1 wk. During this time, the animals on LS/CK gained 9 ± 2 g (n = 6) of body weight while those on LS/LK gained 10 ± 2 g (n = 7). Both of these gains are smaller than expected for a normal diet but are not significantly different from each other. Steady-state data for plasma electrolytes, Na and K excretion, and creatinine clearance, measured in the morning of the last day, are given in Table 1. In both cases, the kidneys responded appropriately to the dietary manipulations, as the rates of excretion of Na (in the case of LS/CK) and both Na and K (in the case of LS/LK) were extremely low. For comparison, with a standard rat diet, rates of excretion were 1.55 ± 0.35 μmol/min (8), 50–100 times higher than those seen here on either of the sodium-deficient diets. In a few experiments, the rates of Na excretion were measured under transient conditions over three intervals within the 24 h of Na restriction (overnight, morning, and afternoon). The decline in the rates of Na excretion did not differ between the two groups (data not shown). Plasma K concentrations were reduced by 30% in the LS/LK group, indicating that K balance was maintained at the expense of reduced levels of the ion.

- **Table 1. Steady-state data for plasma electrolytes, Na and K excretion, and creatinine clearance**

<table>
<thead>
<tr>
<th></th>
<th>Plasma Na, mM</th>
<th>Plasma K, mM</th>
<th>Plasma Aldosterone, pg/ml</th>
<th>U_{Na}V, μmol/min</th>
<th>U_{K}V, μmol/min</th>
<th>U_{Na}/V_{Pcr}, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-Na/control-K</td>
<td>145 ± 2</td>
<td>3.8 ± 0.2</td>
<td>560 ± 250</td>
<td>0.032 ± 0.020</td>
<td>0.72 ± 0.08</td>
<td>0.77 ± 0.04</td>
</tr>
<tr>
<td>Low-Na/low-K</td>
<td>143 ± 1</td>
<td>2.7 ± 0.1</td>
<td>58 ± 9</td>
<td>0.012 ± 0.010</td>
<td>0.022 ± 0.007</td>
<td>0.87 ± 0.06</td>
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Data represent means ± SE for 6 different animals in each group, measured after 7 days on the altered diets. U_{Na}V, Na excretion rate; U_{K}V, K excretion rate; U_{Na}/V_{Pcr}, creatinine clearance. *Significant differences (P < 0.05) between low-Na/control-K and low-Na/low-K groups.
An alternative way to reduce K⁺ secretion would be to decrease the K⁺/H⁺ conductance of the apical membrane. To test this possibility, we measured whole cell currents attributable to ROMK (Kir1.1) channels \( (I_{SK}) \) using TPNQ to block outward currents. This peptide from honey bee venom blocks Kir1.1 and Kir3.1/3.4 channels with high affinity (18). It has been used previously to dissect ROMK currents from whole cell clamp measurements of principal cells in the rat CCD and CNT (11). In Na-replete, K-depleted animals, \( I_{SK} \) is reduced by \( \sim 50\% \) relative to controls (11). To see whether lowering dietary Na intake further reduced ROMK activity, we measured \( I_{SK} \) in CCDs from rats on LS/LK and on normal-sodium, low-K (CS/LK) diets. Typical measurements are shown in Fig. 3 and are similar in the two groups of animals. Mean values were slightly lower in the LS/LK group but this difference was not statistically significant (Fig. 3C). These results imply that the driving force, rather than the permeability, for K⁺ movement is the major factor in blunting K⁺ secretion under these conditions.

Although Na⁺ channel activity appears to be greatly reduced, if not absent, in the CCDs of the LS/LK rats, Na⁺ excretion is as low in these animals as in those receiving the LS/CK diet, in which channel activity is high (Table 1). This conundrum could be explained if Na⁺ transport through other mechanisms in other segments were increased. To pursue this possibility, we measured the expression of three additional apical transport proteins: the Na/H exchanger NHE3, the triple cotransporter NKCC2, and the Na-Cl cotransporter NCC. The results are illustrated in Fig. 4. The largest difference between the LS/LK and LS/CK animals was seen with NCC, the thiazide-sensitive cotransporter expressed in the distal convoluted tubule (12). Here, the protein levels were increased by \( \sim 70\% \). A smaller relative increase was observed for NHE3, which is found in both the proximal tubule and the thick ascending limb of Henle’s loop (TALH) (16); NHE3 abundance was \( \sim 30\% \) higher in the LS/LK-fed rats. A similar 35% increase in NHE3 was observed with K depletion in Na-replete animals (not shown). There was no significant difference in the levels of NKCC2, the major apical membrane Na transporter in

**Fig. 1.** Na⁺ currents in principal cells of cortical collecting ducts (CCDs) from Na-depleted rats are abolished by simultaneous K depletion. Currents were measured in Na-depleted (A) and Na- and K-depleted rats (B). Current-voltage relationships were obtained in the absence (■) and presence (□) of 10⁻⁵ M amiloride. C: average amiloride-sensitive currents \( (I_{Na}) \) measured at a voltage of \(-100 \text{ mV}\). Data represent means \( \pm SE \) for 18 cells from 3 animals [low-Na, control-K diet (LS/CK)] and 23 cells from 4 animals [low-Na, low-K diet (LS/LK)].
the TALH (12). The quantitative analysis of these proteins, as well as of the ENaC subunits, is presented in Fig. 5.

Normally a reduced Na intake will lead to extracellular volume depletion that will stimulate levels of renin, angiotensin II, and aldosterone (23). The appearance of Na\(^+/\)H\(^{-}\) channel activity in the CCD and CNT in response to Na depletion is mimicked by treatment with aldosterone (28), which interacts directly with principal cells in these segments (42). The lack of channel activity observed with simultaneous K depletion could reflect either decreased aldosterone production by the adrenals or reduced sensitivity of the renal cells to the hormone. Measurements of plasma aldosterone indicate much lower levels in the LS/LK compared with LS/CK animals (Table 1). This indicates that the differences in the regulation of Na\(^+/\)H\(^{-}\) channels under these two conditions reflect, at least in part, changes in adrenal function.

**DISCUSSION**

The most important, and for us unexpected, result of this study was that activation of epithelial Na\(^+/\) channels by Na depletion was strongly dependent on the K status of the animal. Under conditions where Na\(^+/\) channels would benefit Na balance and volume homeostasis by reabsorbing Na\(^+\), but would be detrimental to K balance by promoting K\(^+/\)H\(^{-}\) secretion in principal cells, channel activity was minimal. This suggests that at least in this scenario maintenance of K balance, rather than Na balance, had a higher priority in the CCD. We did not make electrophysiological measurements in the CNT, where Na and K transport is quantitatively more important than in the CCD (9), because of the difficulty of dissecting these segments from Na- and K-depleted animals. However, it is likely that Na\(^+/\) channel activity in this segment was also low, since immunoblots of total kidney tissue confirmed the lack of effect of Na depletion on ENaC. Here, the CNT will contribute most strongly to the signal, since Na\(^+/\) channels are most abundant in that part of the nephron (9). In addition, levels of aldosterone were greatly reduced, and the response of the CNT to Na depletion presumably involves this hormone. The inhibitory effect of K restriction on aldosterone levels during Na depletion confirms previous reports (21, 27).

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**Fig. 3.** K\(^+/\) currents in principal cells of CCDs from K-depleted rats are not decreased by simultaneous Na depletion. Outward currents were measured in K-depleted (A) and Na- and K-depleted rats (B). Membrane voltage was held at 0 mV. At the arrows, tertiapin-Q (TPNQ; 10\(^{-7}\) M) and Ba\(^{2+}\) (5 mM) were added to the bath. C: average TPNQ-sensitive currents (I\(\text{SK}\)). Data represent means \(\pm SE\) for 32 cells from 3 animals [normal-sodium, low-K diet (CS/LK)] and 28 cells from 3 animals (LS/LK).

**Fig. 4.** Na-Cl cotransporter (NCC) and Na-H exchanger type 3 (NHE3) protein are increased by simultaneous K depletion in Na-depleted rats. Total homogenates from LS/CK and LS/LK rat kidneys were separated by SDS-PAGE and probed with antibodies for NHE3 (A), Na-K-2Cl cotransporter type 2 (NKCC2) (B), and NCC (C). Each lane was loaded with 40 \(\mu\)g protein from a different animal. Quantification of band densities is shown in Fig. 5.
Despite the lack of Na⁺ channel activity, the Na⁺- and K⁺-depleted animals did not waste Na⁺ to a greater extent than the Na⁺-depleted, K⁺-replete rats. Since GFRs were similar in the two groups, this implies that other transport systems in other parts of the nephron compensate for the lack of Na channel activity. Two candidates for this role that emerge from our measurements are NCC, the thiazide-sensitive Na-Cl cotransporter in the distal convoluted tubule (DCT), and NHE3, the Na⁺-H⁺ exchanger expressed in the apical membranes of the proximal tubule and TALH. The abundance of both of these transporters was increased by low-KCl intake on a background of Na deficiency. Although the relative changes were not large, these transporters normally handle a large fraction of the filtered Na load, so that a small fractional increase in activity could result in a large increase in absolute rates of reabsorption.

The upregulation of NHE3 may be induced by the marked fall in renal intracellular pH during the negative K balance caused by K restriction (1). The acceleration of the rate of H⁺ ion secretion accounts for the increased bicarbonate reabsorption in proximal tubules that occurs in this condition (32). This is part of the mechanism of sustained metabolic alkalosis that accompanies hypokalemia (32).

Previous studies also suggested a role for the NCC in maintaining Na⁺ and K⁺ balance. Kim and colleagues (20) presented evidence that the cotransporter can be upregulated by aldosterone at least under some circumstances. Rozansky et al. (34) proposed that mineralocorticoids can activate NCC through an SGK1- and WNK4-dependent pathway. We (7) and others (41) found that NCC total protein levels, surface expression, and phosphorylation were negatively correlated with K intake. The importance of NCC in the conservation of K was underscored by studies of a mouse model of Gitelman’s syndrome that lacks the cotransporter (26). These mice have normal plasma K on a K⁺-replete diet. However, in response to modest K restriction for 1 wk, they develop hypokalemia due to increased urinary loss of K. Wild-type animals maintained plasma K under these conditions.

Since aldosterone secretion decreases with low-K intake, the increased abundance of NCC implies that the transporter can be regulated by aldosterone-independent factors. This was also suggested by measurements of NCC surface expression that showed increases with Na depletion that were not mimicked by aldosterone administration (10). One candidate for such a factor is angiotensin II. Plasma renin activity, which produces this hormone, is enhanced by both Na⁺ and K⁺ depletion, and these effects are synergistic (21). Acute upregulation of the activities (13, 17, 35, 44) as well as changes in subcellular localization (36, 49) of these transporters by angiotensin II have been described. However, effects of chronic manipulation of hormone levels on transporter protein content could not be documented (3, 39). Therefore, other factors are likely to be involved in the long-term responses.

A major advantage of upregulating transporters upstream of the ASDN would be that Na⁺ delivery to this part of the nephron would be reduced, decreasing K⁺ secretion rates. This is consonant with the general idea that the kidney can manage Na⁺ and K⁺ independently by shifting Na reabsorption between K⁺-secreting segments (e.g., the CNT and CCD) and NaCl-reabsorbing segments (e.g., the proximal convoluted tubule and DCT) (19, 22, 38, 43). We cannot rule out increased transport rates downstream of the CCD, such as in the inner medullary collecting duct. Some investigators suggested that this segment transports Na⁺ at high rates and determines the final rate of Na⁺ excretion (33, 37, 40). If this transport were uncoupled from K⁺ secretion, it could affect Na retention without a concomitant loss of K.

In principle, another way to dissociate Na⁺ and K⁺ transport in the ASDN would be to suppress the activity of apical K⁺ channels. Although studies of whole cell currents (11) and some single-channel measurements (2) demonstrated reduction of ROMK conductances with K depletion, the channel activity is not abolished under these conditions. In addition, with concurrent Na depletion, ROMK activity was not further reduced. This is consistent with the idea that K⁺ secretion is minimized under these conditions at least in part by reducing the driving force by decreasing electrogenic Na⁺ reabsorption through ENaC.

The adrenal represents an important site for the integration of the responses to changes in Na and K balances. In the K⁺-replete state, dietary Na restriction leads to large rates of aldosterone secretion by glomerulosa cells secondary to increased levels of circulating angiotensin II (23). Increases in plasma K⁺ can also elicit aldosterone secretion by the adrenals directly (31). However, increased circulating levels of the mineralocorticoid are not sufficient for the upregulation of ROMK channels observed with a high-K diet (11, 29, 45, 47), since activity was not increased by either aldosterone infusion or by a low-Na diet (29, 46). Our results indicate that these two responses of the adrenals to angiotensin II and K⁺ are interdependent. When plasma K levels are low, the aldosterone-secreting cells do not respond to Na depletion or, presumably, to ANG II. This modulation of the effects of the renin-angiotensin-aldosterone axis will prevent excess loss of K in the urine, but it will require that Na is conserved by aldosterone-independent mechanisms.

\begin{figure}
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\includegraphics[width=\textwidth]{fig5.png}
\caption{K depletion downregulates ENaC and upregulates NCC and NHE3 in kidneys of Na-depleted rats. Ratios of densities from LS/LK vs. LS/CK animals were computed from blots as shown in Figs. 3 and 4. For α-ENaC and γ-ENaC, ratios were obtained from both full-length and cleaved species. Data represent means ± SE for 4 animals in each group (ENaC subunits) or 6 animals in each group (Na⁺ transporters). *Significant difference (P < 0.05, unpaired t-test) between LS/LK and LS/CK groups.}
\end{figure}
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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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