Liddle’s Syndrome

In Liddle Syndrome, Epithelial Sodium Channel Is Hyperactive Mainly in the Early Part of the Aldosterone-Sensitive Distal Nephron

Viatcheslav Nesterov, Bettina Krueger, Marko Bertog, Anke Dahlmann, Ralf Palmisano, Christoph Korbmacher

Abstract—The epithelial sodium channel (ENaC) is rate limiting for Na⁺ absorption in the aldosterone-sensitive distal nephron comprising the late distal convoluted tubule (DCT2), the connecting tubule (CNT), and the entire collecting duct. Liddle syndrome (pseudohyperaldosteronism), a severe form of salt-sensitive hypertension, is caused by gain-of-function mutations of ENaC, but the precise tubular site of increased ENaC function is unknown. In the cortical collecting duct (CCD), ENaC is known to be regulated by aldosterone. In contrast, we recently reported aldosterone-independent ENaC regulation in the early part of the aldosterone-sensitive distal nephron. Here, we investigated ENaC function in the transition zone of DCT2/CNT or CNT/CCD microdissected from mice homozygous for Liddle syndrome mutation or from wild-type control mice. Whole-cell patch-clamp recordings were used to measure amiloride-sensitive ENaC currents in nephron fragments from mice maintained on different sodium diets to vary plasma aldosterone levels. Our data indicate that in mice with Liddle syndrome, the primary site of increased Na⁺ reabsorption is the DCT2/CNT. In addition, increased aldosterone responsiveness of ENaC in CNT/CCD may contribute to salt-sensitive hypertension in Liddle syndrome. Single channel properties of ENaC were similar in Liddle syndrome mutation and wild-type mice, but ENaC expression at the apical membrane was increased in Liddle syndrome mice when compared with wild-type mice, in particular, in animals maintained on a high salt diet. Our findings highlight the importance of ENaC function and regulation in the early part of the aldosterone-sensitive distal nephron for the maintenance of sodium balance and blood pressure control. (Hypertension. 2016;67:1256-1262. DOI: 10.1161/HYPERTENSIONAHA.115.07061.)

Key Words: aldosterone • ENaC • Liddle syndrome • micro dissected renal tubules • patch-clamp technique • pseudohyperaldosteronism • salt-sensitive hypertension

Liddle syndrome (pseudohyperaldosteronism) is a monogenic form of salt-sensitive hypertension associated with hypokalemia, metabolic alkalosis, suppressed plasma renin activity, and low plasma aldosterone levels.¹ It is caused by gain-of-function mutations in the epithelial sodium channel (ENaC).² ENaC constitutes the rate-limiting step for sodium absorption in the aldosterone-sensitive distal nephron (ASDN), which comprises the late distal convoluted tubule (DCT2), the connecting tubule (CNT), and the entire collecting duct. The appropriate regulation of ENaC is critically important for the fine tuning of renal sodium excretion and hence for the maintenance of body sodium homeostasis and the long-term control of arterial blood pressure.³ It is generally accepted that Liddle syndrome results primarily from an inappropriately high ENaC activity in the kidney,⁴ but the precise tubular site of this increased ENaC activity has not been established. In addition, ENaC localized in colon,⁵ lung,⁶ and brain⁷ has been reported to be hyperactive in mouse models of Liddle syndrome. ENaC activity depends on channel open probability and on the number of channels expressed at the cell surface. The latter is determined by the rate of channel insertion and channel retrieval. The majority of mutations causing Liddle syndrome disrupt PY motifs localized in the C termini of the β- or γ-subunit of ENaC.⁸ These PY motifs serve as binding site for the ubiquitin ligase NEDD4-2, a NEDD4 (neural precursor cell–expressed developmen tally down-regulated protein 4) family member.⁹ Binding at these sites facilitates NEDD4-2–mediated channel ubiquitination with subsequent channel retrieval and proteasomal degradation.¹⁰ Mutations in the PY motifs disrupt the physiological NEDD4-2/ENaC interaction. This results in an

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increase in the number of channels at the cell surface, which is thought to be the main mechanism by which Liddle syndrome mutations cause a gain-of-function effect.\textsuperscript{2,11–13} In addition, it has been reported that mutations causing Liddle syndrome increase channel open probability (\(P_o\)),\textsuperscript{13,14} enhance proteolytic channel cleavage,\textsuperscript{15} and reduce Na\(^+\) feedback inhibition.\textsuperscript{16}

Aldosterone stimulates the expression of the serum and glucocorticoid–regulated kinase 1 (SGK1), which phosphorylates NEDD4-2, and thereby reduces its affinity to ENaC. The resultinating inhibition of Nedd4-2–mediated channel retrieval is thought to play a key role in the stimulatory effect of aldosterone on ENaC function.\textsuperscript{17} This concept predicts that aldosterone should have a reduced stimulatory effect on ENaC with Liddle syndrome mutation because aldosterone and the mutation stimulate ENaC by the same mechanism. However, it has been shown that patients with Liddle syndrome readily reduce their urinary sodium excretion in response to aldosterone\textsuperscript{1} and that ENaC has an increased rather than decreased responsiveness to aldosterone in a mouse model of Liddle syndrome.\textsuperscript{5,12} and in cultured renal epithelial cells expressing ENaC with Liddle syndrome mutation.\textsuperscript{18} Moreover, in the cortical collecting duct (CCD)\textsuperscript{12} of mice homozygous for Liddle syndrome mutation (L/L), enhanced ENaC activity was prominent only when aldosterone levels were high, ie, when animals had a low dietary sodium intake or were treated with aldosterone. In contrast, patients with Liddle syndrome typically have low plasma aldosterone levels, and L/L mice develop significant arterial hypertension on high Na\(^+\) diet, when aldosterone levels are suppressed.\textsuperscript{19} How can this paradox be explained?

The absence of increased ENaC activity in the CCD of L/L mice on standard salt diet suggests that ENaC activity may be enhanced in more proximal parts of the ASDN, ie, in the DCT2 and CNT. These nephron segments have not yet been studied in Liddle mice but are thought to contribute substantially to ENaC-mediated sodium absorption in the kidney.\textsuperscript{20–22} Previously, we reported that ENaC activity in DCT2/CNT is constitutively high and independent of aldosterone. This is in contrast to the aldosterone dependence of ENaC activity in CNT/CCD.\textsuperscript{21} We hypothesized that mutations causing Liddle syndrome affect ENaC activity differently in the DCT2/CNT and CNT/CCT and that an increased ENaC activity in the DCT2/CNT may account for sodium retention and hypertension observed in patients with Liddle syndrome. Therefore, the aim of the present study was to investigate ENaC regulation in the DCT2/CNT and CNT/CCT in a mouse model of Liddle syndrome\textsuperscript{19} using different sodium diets to vary plasma aldosterone levels. The activity and biophysical properties of ENaC were investigated using the whole-cell and outside-out configuration of the patch-clamp technique. In addition, we studied the subcellular distribution of ENaC in isolated fragments of the ASDN by immunohistochemistry.

### Methods

#### Mouse Model

An established mouse model for Liddle syndrome\textsuperscript{12,19,24} was used, and animals were maintained as previously described.\textsuperscript{4} All animals used in the present study were from this mouse line and were either homozygous for the R566 Liddle syndrome mutation in \(\beta\)ENaC (L/L or Liddle mice) or homozygous for wild-type ENaC (WT mice).

Animals routinely were maintained on a standard salt diet (SD; 3.2 g Na\(^+\)/kg of food). Alternatively, mice were kept on a low-salt diet (LD; 0.13 g Na\(^+\)/kg) or high salt diet (HD; 16 g Na\(^+\)/kg and 0.9% saline as drinking water) for 2 weeks prior to the experiments. Food was obtained from Altromin (Lage, Germany).

#### Preparation of Renal Tubules and Electrophysiology

Tubules were prepared from mice aged 9 to 12 weeks, and patch-clamp experiments were performed essentially as previously described.\textsuperscript{21,25}

#### Immunohistochemical Analysis

To investigate the subcellular localization of ENaC, isolated tubules were stained using subunit specific antibodies against \(\beta\)- and \(\gamma\)ENaC.\textsuperscript{26} Specimens were examined by confocal laser scanning microscopy.

#### Statistics

Values are reported as mean±SEM. Statistical significance was evaluated by Student t test, and factorial ANOVA with post hoc Tukey HSD (honest significant difference) test, as appropriate; \(P<0.05\) was considered significant for the rejection of the null hypothesis. Experimental techniques are described in more detail in the online-only Data Supplement.

### Results

**In CNT/CCD, the Gain-Of-Function Effect of Liddle Syndrome Mutation on ENaC Is Most Evident in Mice Maintained on an HD**

Experiments were systematically performed in 2 distinguishable regions of the ASDN designated DCT2/CNT and CNT/CCD.\textsuperscript{23} After transfer of the tubular fragments to the perfusion chamber, experiments were routinely started in a bath solution containing 2 \(\mu\)mol/L amiloride to inhibit ENaC and reduce channel rundown.\textsuperscript{27} The appearance of an inward current component on washout of amiloride, as well as the rapid return of the whole-cell current toward its initial level on readdition of amiloride, indicated that the cell under investigation expressed active ENaC in its apical plasma membrane. Representative whole-cell current recordings obtained from the CNT/CCD of WT and Liddle mice are shown in Figure 1A and 1B, respectively. The middle traces were obtained from mice maintained on SD. As previously reported,\textsuperscript{23} under these conditions, the amiloride-sensitive ENaC whole-cell current (\(\Delta I_{\text{ami}}\)) in CNT/CCD from WT animals (Figure 1A, middle trace) was larger than that observed in L/L mice (Figure 1B, middle trace). However, the average \(\Delta I_{\text{ami}}\) was significantly higher in L/L mice (7.2±1.2 pA; \(n=10\); Figure 1A, middle trace). In L/L mice maintained on SD, the current response to amiloride removal and readdition was qualitatively similar to that observed in WT animals (Figure 1B, middle trace). However, the average \(\Delta I_{\text{ami}}\) was significantly higher in L/L mice (34±2.9 pA; \(P<0.05\); \(n=12\); Figure 1C) when compared with that observed in WT mice. This is consistent with a gain-of-function effect of Liddle syndrome mutation and the previously reported finding that isolated perfused CCD from L/L mice exhibit higher transepithelial potential differences than perfused CCD isolated from WT mice.\textsuperscript{23} Maintaining the animals on LD substantially increased \(\Delta I_{\text{ami}}\) in both, WT and Liddle mice (Figure 1A and 1B, right traces), with a nonsignificant trend for a larger average \(\Delta I_{\text{ami}}\) in L/L mice (343±60 pA; \(n=17\)) compared with that in WT mice (241±69 pA; \(n=11\); Figure 1C). HD largely suppressed the current response to amiloride washout and readdition in the CNT/CCD of WT mice (Figure 1A, left trace) and reduced average \(\Delta I_{\text{ami}}\) to 7.8±1.5 pA (\(n=12\); Figure 1C).
In contrast, in the CNT/CCD from L/L animals, HD failed to suppress ENaC activity, which was similar to that in L/L animals on SD (Figure 1B, left trace) with an average ΔI_ami of 71.7±10.8 pA (n=11). Thus, in the CNT/CCD, the gain-of-function effect of Liddle syndrome mutation on ENaC is most evident in animals maintained on HD, whereas the effect is less prominent in L/L and WT animals maintained on SD or LD (Figure 1C).

In DCT2/CNT, the Gain-of-Function Effect of Liddle Syndrome Mutation Is Largely Independent of Dietary Salt Intake

As illustrated by the representative current traces shown in Figure 2A, substantial ENaC currents were measured in the DCT2/CNT from WT mice with ΔI_ami averaging 187±67 pA (n=11), 295±70 pA (n=10), and 305±38 pA (n=13; Figure 2C) in animals maintained on HD, SD, and LD, respectively. Thus, under all dietary conditions, ENaC currents in the DCT2/CNT were of similar magnitude as ENaC currents observed in the CNT/CCD of WT mice maintained on LD. In the DCT2/CNT, LD did not significantly increase ΔI_ami and HD only partially reduced ΔI_ami, which is in contrast to the findings in the CNT/CCD. These results are in good agreement with our previous study in which we demonstrated aldosterone-dependent regulation of ENaC in the CNT/CCD and aldosterone-independent regulation in the DCT2/CNT. Importantly, in the DCT2/CNT of L/L mice, ENaC currents were much larger than those observed in WT animals under all dietary conditions used (Figure 2B). ΔI_ami averaged 718±160 pA (n=10), 1136±144 pA (n=12), and 991±116 pA (n=12) in DCT2/CNT of L/L mice maintained on HD, SD, or LD, respectively (Figure 2C). Thus, in DCT2/CNT, Liddle syndrome mutation stimulated ENaC currents ≈3-fold independently of the salt diet.

Single-Channel Properties of ENaC in L/L Mice Are Similar to Those in WT Animals

We compared single-channel current properties of ENaC recorded in L/L mice with those in WT animals. Representative single-channel current traces from a WT and L/L mouse are shown in Figure 3A and 3B, respectively. Measurements were started in the whole-cell configuration (Figure 3A and 3B, left), and then continued in the outside-out mode for the analysis of single-channel properties. In L/L mice, single-channel events could be resolved only in the CNT/CCD but not in the DCT2/CNT because of the high ENaC activity in this latter segment. However, we previously demonstrated that single-channel properties of ENaC in the DCT2/CNT are similar to those in the CNT/CCD. Single-channel current amplitudes and NP_o values were determined using binned amplitude histograms (Figure 3A and 3B). A binomial fit routine was used to estimate open probability (P_o) and number of channels in the patch (N) from NP_o and from the observed distribution of the probability of n channels being open simultaneously (Figure 3C and 3D; Methodological details are available in the online-only Data Supplement). Results from similar experiments as those shown in Figure 3 are listed individually in Table S1 in the online-only Data Supplement. On average, the single-channel current amplitude recorded in L/L mice at a holding potential (V_hold) of −60 mV was not significantly different from
That in WT (0.31±0.02 pA, n=8 versus 0.32±0.01 pA, n=5).
In both genotypes, the estimated \( P_0 \) values varied in a wide range (from <0.1 to 0.84 in WT and from <0.1 to 0.55 in L/L).
Importantly, the average \( P_0 \) value in L/L animals (0.31±0.09) was similar to that in WT mice (0.32±0.08), and there was no major effect of diet or segment on \( P_0 \), albeit in a small sample (Table S1). These findings indicate that the observed differences of ENaC whole-cell currents in WT and in L/L animals mainly reflect differences in the number of channels expressed at the cell surface, whereas the single-channel conductance and \( P_0 \) are similar in both genotypes.

The Liddle Syndrome Mutation Enhances the Apical Localization of ENaC in the ASDN in Particular in the DCT2/CNT
Using an antibody directed against \( \gamma \)-ENaC, typical ENaC staining\(^{28} \) was detected along the entire ASDN of WT and L/L animals under all dietary conditions used (Figures 4; Figure S1). Only a minority of ASDN cells, likely to be intercalated cells, did not show ENaC staining. Control experiments were performed with an antibody directed against \( \beta \)-ENaC to demonstrate the absence of ENaC staining in the ASDN of L/L mice that lack the C terminus of \( \beta \)-ENaC and therefore lack the epitope against which the antibody was raised (Figure S2).

In WT and L/L mice maintained on SD, prominent apical ENaC staining was limited to the DCT2/CNT in most tubular fragments investigated (Figures S1 and S3). Apical ENaC staining extended to the CNT/CCD region in WT and L/L mice maintained on LD (Figure 4; Figure S3), which is in good agreement with the observed increase in ENaC currents in the CNT/CCD under these conditions. In contrast, in mice maintained on HD, ENaC staining in the CNT/CCD of WT and L/L mice was mainly perinuclear (Figure 4; Figure S3) and was similar to the staining pattern observed in animals on SD. Importantly, in animals maintained on HD, prominent apical ENaC staining was clearly detectable in >70% of ENaC-positive cells in the DCT2/CNT of L/L mice but only in ≈10% of ENaC-positive cells in the DCT2/CNT of WT mice (Figure 4; Figure S3). Overall, these findings are consistent with our ENaC current measurements and indicate that Liddle syndrome mutation enhances the apical localization of ENaC in particular in the DCT2/CNT.

Discussion
Hyperactivity of ENaC in the DCT2/CNT Is Likely to Be the Primary Pathophysiological Mechanism of Liddle Syndrome
ENaC is expressed along the entire ASDN, but previous studies of ENaC regulation have mainly focused on the CCD. The key hormonal regulator of ENaC activity in the CCD is aldosterone. Indeed, ENaC activity is barely detectable in the CCD unless animals are kept on LD or treated with mineralocorticoids.\(^{12,29,30} \) Thus, ENaC-mediated sodium absorption in the CCD is probably essential in states of sodium depletion but plays a minor role when dietary sodium intake is normal or high. In contrast, ENaC activity in the DCT2/CNT is largely...
Hypertension independent and selective inactivation of ENaC in the CNT but not in the collecting duct severely compromises sodium balance in mice. In the present study, we demonstrate that in the DCT/CNT, the Liddle syndrome mutation has a profound gain-of-function effect on ENaC in mice maintained on LD, SD, or HD. Thus, our findings solve the conundrum that in the CCD, high ENaC activity was detectable only in L/L mice maintained on LD or treated with aldosterone, whereas aldosterone levels typically are suppressed in patients with Liddle syndrome. Our results establish the DCT/CNT as the main nephron site where ENaC is hyperactive in Liddle syndrome. This supports the emerging concept that appropriate regulation of ENaC activity in the DCT/CNT is critically important for renal sodium homeostasis and the long-term control of blood pressure, namely, under low plasma aldosterone conditions. The specific hormonal, local, and molecular factors involved in ENaC regulation in the DCT/CNT remain to be determined. The finding that ENaC activity in the DCT/CNT is preserved in aldosterone synthase–deficient animals suggests that glucocorticoids and angiotensin II may play a role because these hormones are upregulated in this mouse model. In addition, differences in subunit availability, channel trafficking, and channel activation by proteases may contribute to the differential regulation of ENaC in the DCT/CNT versus the CNT/CCD. A detailed analysis of these mechanisms will be necessary to understand why Liddle syndrome mutation has a predominant stimulatory effect in the DCT/CNT.

Increased Aldosterone Responsiveness of ENaC in CNT/CCD May Contribute to Salt-Sensitive Hypertension in Liddle Syndrome

Liddle mice become hypertensive on HD, and preliminary data from telemetric blood pressure measurements indicate that blood pressure values are significantly elevated in Liddle mice even under SD (Q. Wang and E. Hummler, personal communication, University of Lausanne). Moreover, plasma aldosterone is suppressed in Liddle mice on SD, suggesting chronic hypervolemia resulting from increased ENaC-mediated renal sodium absorption. As discussed above, our findings suggest that this is mainly because of an increased ENaC activity in the DCT/CNT. In Liddle syndrome, ENaC has been reported to have an increased responsiveness to aldosterone. Our present data confirm increased aldosterone responsiveness of ENaC localized in the CNT/CCD of Liddle mice. This becomes apparent when the data are interpreted in the context of previously reported plasma aldosterone values (P Ald) from mice of the same strain and maintained under essentially identical conditions. In animals maintained on SD, ENaC currents in the CNT/CCD of L/L mice are slightly higher than those in WT mice (Figure 1), despite a lower P Ald of ≈0.15 nmol/L in L/L mice than that of ≈0.55 nmol/L in WT mice. Interestingly, in L/L mice maintained on LD, P Ald was ≈0.54 nmol/L, which is similar to P Ald of WT mice maintained on SD. However, despite the similar P Ald, ENaC currents in L/L mice are much higher than those in WT mice (Figure 1).

Taken together, these findings support the concept that ENaC...
localized in the CNT/CCD has an increased responsiveness to aldosterone in Liddle syndrome. Moreover, our findings indicate that in L/L mice, ENaC activity in the CNT/CCD cannot be reduced sufficiently to compensate for the increased ENaC activity in the DCT2/CNT, which is present under all dietary conditions. This inability to compensate is most prominent when L/L mice are maintained on HD because their aldosterone levels are already suppressed to near-minimal values on SD. Because of the increased aldosterone sensitivity of ENaC in Liddle syndrome, some residual ENaC activity is maintained in the CNT/CCD even when aldosterone levels are maximally suppressed by HD. Thus, the failure of L/L mice to downregulate ENaC activity in the CNT/CCD when challenged with HD is likely to contribute to the salt-sensitive hypertension observed in Liddle syndrome. It is tempting to speculate that pathophysiologically increased ENaC activity in the DCT2/CNT and increased aldosterone sensitivity of ENaC in the CNT/CCD may contribute to other forms of salt-sensitive hypertension.

Increased ENaC Activity in Liddle Syndrome Is Mainly Caused by an Increased Expression of the Channel at the Apical Cell Surface in Particular in the DCT2/CNT

Immunocytochemical data reported in this study support the established view that the main cause of the gain-of-function effect of Liddle syndrome is an increased expression of the mutant channel at the cell surface. This does not exclude the possibility that an increased \( P_o \) of the mutant channel may contribute to the gain-of-function effect. However, in our outside-out patch-clamp recordings, we did not find any evidence for an increased \( P_o \) of ENaC in L/L mice. This is in agreement with the conclusion reached in a previous study using the same mouse model of Liddle syndrome and a noise analysis approach to estimate ENaC \( P_o \) in whole-cell recordings from isolated CNT. Our immunohistochemical data indicate that in L/L mice, ENaC expression is increased at the apical membrane, in particular, in the DCT2/CNT consistent with our ENaC current measurements.

In the kidney, long-term regulation of ENaC activity is mainly achieved by changes in the cell surface abundance of the channel. The number of ENaC channels in the luminal membrane is the net result of exocytotic delivery and the endocytotic retrieval of channels from the membrane. Liddle syndrome mutations have been shown to reduce the retrieval of ENaC from the membrane probably by interfering with Nedd4-2–dependent ubiquitylation and subsequent endocytosis. Under physiological conditions, Nedd4-2–mediated ENaC retrieval is thought to be inhibited by aldosterone via Nedd4-2 phosphorylation by SGK1. The resulting increase in ENaC expression at the cell surface is considered to be a major mechanism of ENaC stimulation by aldosterone. This concept predicts a reduced stimulatory effect of aldosterone on ENaC with Liddle syndrome mutation. In contrast, we demonstrate an increased aldosterone responsiveness of ENaC in L/L mice consistent with previous studies. Moreover, we demonstrate that the stimulatory effect of Liddle syndrome mutation is most prominent in the DCT2/CNT, where ENaC regulation is not dependent on aldosterone. Therefore, reduced Nedd4-2–mediated ENaC retrieval is probably not the only mechanism by which Liddle syndrome mutations increase the number of ENaC channels at the cell surface.

In conclusion, this study identifies the DCT2/CNT as the primary nephron site where the disease-causing gain-of-function effect of Liddle syndrome mutation occurs. This highlights the importance of ENaC regulation in the DCT2/CNT for long-term blood pressure control. The present study also confirms that in Liddle syndrome, the aldosterone responsiveness of ENaC is increased, rather than decreased. This may contribute to the development of salt-sensitive hypertension by impeding compensatory downregulation of ENaC activity in the CNT/CCD. Finally, our data confirm the concept that the gain-of-function effect of Liddle syndrome mutation is mainly the result of an increased channel expression at the cell surface, in particular, in the DCT2/CNT.

Perspectives

Increased ENaC activity may be an important contributing factor to the pathophysiology of salt-sensitive arterial hypertension not only in Liddle syndrome. Thus, the identification of the DCT2/CNT as the major nephron site for increased ENaC activity in Liddle syndrome may have implications for our understanding of the pathophysiology of other forms of salt-sensitive and low aldosterone hypertension. Moreover, our findings suggest that inhibiting ENaC activity in the DCT2/CNT may be a therapeutic strategy to treat patients with salt-sensitive hypertension. Finally, a better understanding of systemic and local mechanisms that contribute to ENaC regulation in the DCT2/CNT may reveal novel insights into the pathophysiology of salt-sensitive hypertension.

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Disclosures

None.

References


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IN LIDDLE'S SYNDROME ENaC IS HYPERACTIVE MAINLY IN THE EARLY PART OF THE ALDOSTERONE-SENSITIVE DISTAL NEPHRON

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Short title: Hyperactive ENaC in early ASDN in Liddle’s syndrome

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Supplementary Methods

Ethical approval for animal studies
For this study, renal tissue preparations were obtained from mice (Mus musculus) sacrificed by perfusion of the circulatory system with Leibovitz medium (LM) via the left ventricle after terminal anesthesia (thiopental, 50 mg/kg, i.p.). The animals were cared for and experiments were performed in accordance with the principles of German legislation. The study was approved by the animal welfare officer for the Friedrich-Alexander University Erlangen-Nürnberg (TS-1/03 ZellPhys) and by the state veterinary health inspectorate (License no. 621.2531.32-2/05). Housing and care of the animals was under the governance of the responsible regulatory authority in Erlangen, Germany.

Preparation of tubules
The circulatory system was perfused via the left ventricle with Leibovitz medium (LM), containing collagenase (1.33 mg/ml) and amiloride (2 μM). Kidneys were removed, cut into coronal slices, and incubated for 20-25 min at 37 °C in collagenase containing LM. Collagenase preparations may contain proteases capable of activating ENaC. However, it is unlikely that the collagenase or contaminating proteases reach the tubular lumen of the ASDN during perfusion or microdissection because of the glomerular filtration barrier and tubular collapse after cessation of circulation. Moreover, tubules were opened only after washout of the collagenase. Using a similar protocol, we previously demonstrated that trypsin can activate ENaC in microdissected mouse tubules1. The observed proteolytic stimulation was modest and variable which is consistent with findings in tubules microdissected without collagenase2 and in micropuncture studies in vivo3. Thus, renal perfusion with collagenase for tissue preparation does not seem to prevent proteolytic ENaC activation in the microdissected tubules and should not interfere with our measurements. Moreover, WT and Liddle animals were treated in the same way. Thus, any observed differences in ENaC activity is unlikely to be caused be the collagenase treatment.

Cortical tubules were separated manually using fine forceps. We identified and isolated tubular segments with characteristic branching indicative of the merging of CNT into CCD, and extended the dissection towards the DCT as described earlier4. We distinguished two regions of the ASDN: (i) DCT2 and initial CNT (DCT2/CNT), and (ii) late CNT and initial CCD (CNT/CCD). The isolated tubular segments were attached to glass coverslips coated with Cell-Tak (Collaborative Research, Bedford, MA, USA) and transferred to a temperature controlled perfusion chamber (37 °C) mounted on an inverted microscope (Leica DM IRB). To gain access with the patch pipette to the apical cell membrane, tubules were cut open with a broken glass pipette attached to a micromanipulator.

Experimental solutions and chemicals.
For patch clamp experiments, bath solution contained (in mM): 145 sodium-D-gluconate, 5 potassium-D-gluconate, 2 CaCl2, 5 barium acetate, 1 MgCl2, 5 HEPES and 3 glucose (pH adjusted to 7.4 with TRIS). Standard pipette solution contained (in mM): 85 potassium-D-gluconate, 5 sodium-D-gluconate, 2 Mg-ATP, 10 HEPES, 2 EGTA-Na, 2 MgCl2, 40 CsOH and 20 TEA-OH, (pH adjusted to 7.2 with gluconic acid). Amiloride hydrochloride was purchased from Sigma-Aldrich), collagenase (type: CLS III) from Biochrom (Berlin, Germany).

Electrophysiology.
A computer-controlled EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) was used to perform conventional whole-cell and outside-out patch clamp recordings from principal cells in microdissected tubules. Pipettes were made from borosilicate glass (Hildenberg, Masfeld, Germany) with a resistance of about 4-6 MΩ. Seals were formed at the apical cell surface by using
gentle suction. The whole-cell configuration was achieved by increasing the suction after the seal resistance had exceeded at least one GΩ. Only patches with seal resistances higher than 3 GΩ were used for outside-out recordings. Series resistance ranged between 10 and 15 MΩ, and was not compensated. For continuous whole-cell as well as outside-out current recordings, cells were voltage clamped using a pipette holding potential ($V_{\text{hold}}$) of -60 mV. Data were sampled at a rate of 2 kHz and initially filtered at 500 Hz. For further analysis, they were routinely re-filtered at 100 Hz. In the whole-cell mode, the amiloride-sensitive current ($\Delta I_{\text{Ami}}$) was determined by subtracting the whole-cell current measured in the presence of amiloride (2 µM) from the corresponding current measured in its absence. Amiloride in a concentration of 2 µM has previously been used to assess ENaC mediated currents in mouse ASDN and is expected to inhibit ENaC activity by about 95 % assuming an $IC_{50}$ value of about 0.1 µM. By including carboxyfluorescein in the pipette solution it has been demonstrated that rat CCD cells and mouse DCT2/CNT cells do not seem to be coupled by gap junctions in the whole-cell configuration of the patch clamp technique. Therefore, we can assume that currents measured in the whole-cell configuration reflect currents of a single cell. Thus, by determining $\Delta I_{\text{Ami}}$ overall ENaC activity in the apical membrane of a single cell can be assessed. In the outside-out mode, the current level at which all channels are closed (closed level) was determined in the presence of amiloride (2 µM). Current levels, corresponding to the closed level or to the opening of one or more channels were determined as maxima of binned current amplitude histograms and were numbered. Level zero corresponds to the all closed level (c). Levels 1, 2, 3 etc. correspond to simultaneous opening of 1, 2, 3 etc. channels (see fig. 3 of the main article). Single channel current amplitude was determined as the current interval between adjacent levels. Current values at each time point were attributed to the current level to which they were nearest. The probability of a current level to occur ($p_{\text{level}}$) was determined by dividing the number of data points attributed to this level by the total number of data points in an analysed recording. The channel activity was estimated as $NP_o$ which was determined as

$$NP_o = \sum_{\text{level}=1}^{\text{MaxLevels}} \text{level} \cdot p_{\text{level}}$$

where level is current level (0, 1, 2 ...); MaxLevels is the maximal number of observed levels, $p_{\text{level}}$ is the probability of each level. To estimate the number of channels in the patch, the observed probability for n channels being open simultaneously was fitted with a binomial distribution; the best fit was determined by maximal likelihood estimate. In addition, the weighted least squares method was used to verify the fit. In most cases both methods gave identical results and the difference in the estimate of N was never larger than 1. Apparent $P_o$ was estimated as a ratio of $NP_o$ to the best N estimate. A limitation of this approach is that it relies on the assumption that a patch contains a population of independent channels with identical properties. The good agreement between the observed and the fitted probability distribution of the current levels (see Fig. 3, C and D, main article) suggests that the assumption is valid for the purpose of this analysis. A specific software tool (Nest-o-Patch) was developed for the described analysis of patch-clamp data. It is available, including the source code, at [https://sourceforge.net/projects/nestopatch/](https://sourceforge.net/projects/nestopatch/).

**Immunohistochemistry and confocal microscopy.**

Tubules were isolated and attached to the glass slides covered with Cell-Taq using the same technique as for electrophysiology. Attached tubular segments were fixed with 4% paraformaldehyde in PBS for 30 minutes on ice. After fixation, tubular cells were permeabilized with...
0.1% Triton X-100 in PBS for 10 to 20 minutes on ice. Unspecific binding sites were blocked with 1x Roti-Immunoblock (Roth, Karlsruhe, Germany) for 10 minutes at room temperature. As primary antibodies against β- and γENaC we used rabbit polyclonal antibodies described previously\(^8\). These antibodies are directed against epitopes in the C-termini of the subunits. Therefore, the βENaC antibody should not recognize the C-terminally truncated β-subunit in the L/L animals. This prediction was confirmed as shown in figure S2. In contrast, the γENaC antibody can recognize the γ-subunit in WT and in L/L mice and therefore was used to compare the subcellular localization of ENaC in the ASDN of WT and L/L mice. The antibodies were used in a dilution of 1/2000. As a secondary antibody, a Cy3-conjugated goat anti-rabbit immunoglobulin G (H+L) from Dianova (Hamburg, Germany) was used. Tubules were incubated with the antibody in 0.5% BSA/ 0.04% sodium azide in PBS over night at 4°C. After washing, tubules were incubated with the secondary antibody for two hours at room temperature. Slides were covered with Fluoroshield including DAPI for nuclear counterstaining (Sigma, Taufkirchen, Germany). ENaC staining in microdissected tubules was evaluated by confocal microscopy using a Leica TCS 5 II Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) with LAS AF software version 2.6.0 build 7266. Images were taken using either HCX PL APO lambda blue 20.0 x 0.7 IMM UV or HCX PL APO CS 63.0 x 1.30 GLYC 21°C UV lenses.
References


Table S1. Single channel activity in the wild type and Liddle mice

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Genotype</th>
<th>Diet</th>
<th>Segment</th>
<th>$\Delta I_{\text{amil}}$(pA)</th>
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<th>NPo</th>
<th>N</th>
<th>Po</th>
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<tr>
<td>1</td>
<td>WW</td>
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<td>CNT/CCD</td>
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<tr>
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<td>3</td>
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<td>3</td>
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<td>0.34</td>
<td>2</td>
<td>0.21</td>
<td>n.d.</td>
</tr>
<tr>
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</tbody>
</table>

* Representative whole-cell and single channel current traces from these experiments are shown in Fig. 3 of the main paper.
† In these experiments the binomial distribution converges to a Poisson distribution; therefore, an accurate estimate of N could not be determined (n.d.); nevertheless, this implies that Po is less than 0.1, because this is the condition of convergence to a Poisson distribution.

HD, LD and SD indicate high, low and standard salt diet, respectively. $\Delta I_{\text{amil}}$(pA) - amiloride-sensitive whole-cell current; i – single-channel current amplitude; levels – number of observed channel open levels; N – best estimate of the number of channels in the patch according to the binomial fit analysis.
Figure S1: Subcellular distribution of γ-ENaC in microdissected ASDN of WT and Liddle mice maintained on standard salt diet. Red: γENaC staining, blue: DAPI staining. Open or filled arrow heads indicate cells with prominent apical ENaC staining or cells with predominant perinuclear ENaC localization, respectively. In the overview panels A and B the dotted rectangles delineate the portions of DCT2/CNT (C, D) and CNT/CCD (E, F) shown at higher magnification in the smaller panels labelled accordingly.
Using an antibody directed against an epitope in the C-terminus of β-ENaC the channel can be detected in the ASDN of wild-type but not of Liddle mice expressing a C-terminally truncated β-subunit. A. Isolated fragment of ASDN from WT mouse maintained on standard salt diet. Apical staining of β-ENaC is visible in DCT2/CNT and predominant perinuclear staining is observed in CNT/CCD. These results are consistent with those observed with the antibody against γ-ENaC shown in Fig. S1. B. Isolated fragment of ASDN from L/L mouse. As expected, no staining is visible, because the C-terminally truncated β-subunit lacks the epitope recognized by the antibody. These findings confirm that the ENaC antibodies used specifically recognize ENaC.
Figure S3: Ratio of cells with prominent apical ENaC staining to total number of ENaC-positive cells in DCT2/CNT and CNT/CCD segments from WT or L/L mice maintained on different salt diets. As illustrated in Fig. 4, from each microdissected tubular preparation one magnified portion of DCT2/CNT and of CNT/CCD was selected for further microscopic analysis. Cells with prominent apical ENaC staining and cells with predominant perinuclear ENaC staining were counted in every fragment. The total number of ENaC-positive cells per analyzed DCT2/CNT and CNT/CCD fragment averaged 16 ± 0.9 (n=50) and 22 ± 1.3 (n=54), respectively. For each analyzed fragment the ratio of cells with prominent apical ENaC staining to total number of ENaC-positive cells was calculated. This ratio is represented by a filled circle (L/L) or by an open circle (WT) for each individual fragment analysed. Average ratios with SEM values are indicated for each experimental group.