Dysfunction of the Epithelial Sodium Channel Expressed in the Kidney of a Mouse Model for Liddle Syndrome

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Abstract. The Liddle syndrome is a dominant form of salt-sensitive hypertension resulting from mutations in the β or γ subunit of ENaC. A previous study established a mouse model carrying a premature Stop codon corresponding to the R566stop mutation (L) found in the original pedigree that recapitulates to a large extent the human disease. This study investigated the renal Na⁺ transport in vivo, ex vivo (intact perfused tubules), and in vitro (primary cultured cortical collecting ducts [CCD]). In vivo, upon 6 to 12 h of salt repletion, after 1 week of low-salt diet, the L/L mice showed a delayed urinary sodium excretion, and the L/L mice exhibited higher I\text{eq} than their wild-type counterparts. The equivalent short-circuit current (Ieq) and the amiloride-sensitive Ieq was approximately twofold higher in cultured L/L CCD than in +/+ CCD. Aldosterone (5 × 10⁻⁷M for 3 h) further increased Ieq from cultured L/L CCD. Thus, this study brings three independent lines of evidence for the constitutive hyperactivity of ENaC in CCD from mice harboring the Liddle mutation.

The Liddle’s syndrome (or pseudoaldosteronism) is an autosomal dominant disorder that is characterized by early onset of salt-sensitive hypertension, hypokalemia, and metabolic alkalosis in the presence of low plasma renin and aldosterone levels (1–3). The disease has been genetically linked to mutations in either of two genes encoding the ENaC subunits, either ENaC β or γ subunits (4,5). ENaC is composed of three homologous subunits, α, β, and γ, with preferential assembly (6,7). This channel is mainly expressed in aldosterone-sensitive epithelia (late distal convoluted tubule [late DCT]), the connecting tubule (CNT), and the collecting ducts (CD) in the kidney; the surface epithelia of the distal colon; and the ducts of exocrine glands (8). In these epithelia, the apical entry of sodium into cells mediated by ENaC represents the rate-limiting step for the vectorial electrogenic movement of Na⁺ (9).

Each ENaC subunit consists of short intracellular NH2 and COOH termini, two transmembrane domains, and a large extracellular loop (10,11). The mutations identified to cause Liddle’s syndrome lead to truncation of the COOH terminus or affect a PPxY motif contained in the COOH terminus (4) of ENaC (9). The mutations identified to cause Liddle’s syndrome have been shown to be caused by both an increase in the number of channels at the plasma membrane (14,15) and an increased activity of single channels as a result of defective Na⁺-dependent feedback inhibition (16,17).

To generate a mouse model for the Liddle’s syndrome, we first introduced by homologous recombination in embryonic stem cells a stop codon (corresponding to residue R566 in human SCNN1B [BENaC] as found in the original pedigree described by Liddle) followed by a neomycin resistance (neo) gene in the mouse Scnn1b (BENaC) gene (18). The insertion of the selection marker resulted in destabilization and/or degradation of the βENaC mRNA transcripts, leading to a salt-losing phenotype (PHA-1) (18). To generate mice harboring an increase in ENaC activity, we therefore excised the neo gene in vivo, using the Cre/loxP-mediated recombination system (19).
The βENaC mutated allele carrying the R566 stop mutation but lacking the neo gene was named allele L. Under high salt intake, these mice develop all characteristics of the disease (high BP, metabolic alkalosis, hypokalemia, and low plasma aldosterone levels). In 3- to 6-month-old adult mice, however, under a standard diet (NaCl 3g/kg), we previously reported that the L/L or L/+ mice did not develop the characteristic features of the Liddle syndrome observed in human patients (hypertension, metabolic alkalosis and hypokalemia, suppressed aldosterone secretion). In our previous report (19), the only evidence for a hyperactive sodium channel was that animals that were fed a normal salt diet (L/+ and L/L animals) developed low plasma aldosterone, suggesting salt retention and hypervolemia. Salt retention could be, in part, attributed to an increased sodium reabsorption in the distal colon, as assessed by the in vivo measurement of rectal potential difference or, in part, in the kidney, but the renal phenotype was not investigated.

The present study was designed to assess the ENaC function in kidneys from mice before the development of the hypertensive disease. If dysfunction of ENaC in the kidney can be demonstrated in this “silent” phase, then it would favor the kidney hypothesis put forward by G. Liddle in 1963 (1). The aim of the study therefore was (1) to detect any dysfunction of ENAC in the kidneys of mice that were fed a standard salt diet or subjected to rapid changes from normal diet to a salt-free diet followed by rapid resalination—this protocol has been used in rats and mice to demonstrate the regulation of ENaC in the principal cell of the kidney (20–22)—and (2) to evaluate sodium/potassium balance to examine whether any disturbances could explain the suppression of aldosterone. In this study, we have not reevaluated the cardiovascular effects of the Liddle mutation, which was already assessed in our chronic (3 mo) experiments.

The activity of ENaC was estimated by measuring urinary electrolyte excretion of these animals that were fed a low- or high-sodium diet, and the γENaC protein localization along the renal collecting system was analyzed by immunofluorescence. We also used the technique of single isolated perfused tubule and primary cultures of isolated tubules to analyze the electrophysiologic parameters of microdissected cortical collecting ducts (CCD). The results indicate that the mutation of the βENaC subunit responsible for a Liddle phenotype in mice leads to a constitutive hyperactivation of ENaC in the kidney.

### Materials and Methods

#### Transgenic Mice

The generation of the Liddle mice has been described previously (18). For the present experiments, F2 mice were obtained and genotyped as described. For in vivo and ex vivo protocols, we first compared L/L with +/- animals. Because the differences observed were small (see Results), the evaluation of the L/+ phenotype would have required a very large number of animals to reach statistical significance and was therefore not performed. For in vitro experiments, L/L, L/+ and +/- littermates were used.

#### Salt Depletion/Repletion Studies

Adult mice (3 to 5 mo old) from the F2 generation were housed in individual metabolic cages (O’Har & Co., Tokyo, Japan) to separate the urine from the feces. Mice were housed in metabolic cages for 4 d before the beginning of the experiments. They were fed either a sodium diet containing 0.4% Na+ or a low-Na+ diet containing 0.01% Na+ (diet 212; UAR, Villemoisson-sur-Orge, France), with free access to tap water. Mice were fed a moderate Na+ diet for 4 consecutive days (days 1 to 4). Then, they were fed a low-Na+ diet for 7 d before being repleted in Na+ with the medium Na+ diet for 5 additional days. Mice were weighed, and food intake, water intake, and urine volume were measured every day. The body weight measured at day 4 was used as the reference weight. After salt repletion, urine was collected in four consecutive 6-h periods and in four consecutive 12-h periods. All experiments were performed in accordance with guidelines set out by the Swiss Veterinarian Office in conformity with legislation governing animal studies.

#### Plasma and Urine Analyses

Na+ and K+ contents were measured using flame photometry as described (18,19). Urinary and plasma aldosterone levels were determined by 125I RIA (Coat-A-Count Aldosterone kit; Diagnostic Products Corp.). Plasma and serum creatinine were determined, using an autoanalyzer (Ektachem DTSCII, Johnson and Johnson).

#### Immunohistochemical Studies

Kidneys of wild-type (wt) and L/L mice were fixed by intravenous perfusion with 3% paraformaldehyde and 0.05% picric acid, dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/kgH2O with sucrose), and 10% hydroxyethyl starch in saline (HAES-steril; Fresenius, Stans, Switzerland). Afterward, the kidneys were processed for immunohistochemistry, as described previously (21). Cryosections were incubated overnight at 4°C with newly generated rabbit antisera against rat γ-ENaC diluted 1:20000 in PBS/1% BSA, as described (21). After repeated rinsing with PBS, binding sites of the primary antibodies were revealed with Cy3-conjugated donkey-anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA), diluted 1:1000 in PBS/1% BSA. Finally, sections were washed with PBS and mounted with DAKO-Glycergel containing 2.5% 1,4 diazabicyclo (2.2.2)octane (Sigma, St. Louis, MO) to retard fading. Sections were studied by epifluorescence with a Polysar microscope (Reichert Jung, Vienna, Austria). Digital images were acquired with a charged-coupled-device camera (Visicam 1280; Visitron Systems, Puching, Germany) and processed by ImagePro and Photoshop software. In control experiments, the primary antibodies were replaced by a rabbit nonimmune serum. These sections were unstained.

#### Isolated Perfusion Tubule Studies

The methods used followed those first described by Grantham and Burg (23) and adapted in our laboratory (24). Quickly after death, both kidneys were removed from a mouse that was fed a regular salt diet (0.3% Na+; A03; UAR) and cut into 1-mm sagittal slices, which were immediately immersed into a chilled bathing solution (122 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 5 mM CH3COONa, 1.5 mM CaCl2, 2 mM Na2HPO4, 0.5 mM MgCl2, 6 mM urea, 8 mM d-glucose, 4 mM l-alanine, 1 g/100 ml BSA, buffered at pH 7.4 with 1 N HCl). The solution was saturated with oxycarbon (O2 95%, CO2 5%) and maintained at 4°C. CCD with branches (1 mm length) and thick ascending limb of Henle were dissected from bundles radiating from the papilla to the outer cortex. Tubules of similar length were
then transferred into a thermoregulated perfusion chamber containing approximately 1.2 ml bathing solution equilibrated with oxygen.

The perfusion technique used for CCD with branches was adapted from that previously described (24). In this “open tubule” configuration, only one end of the tubular segment was aspirated into a holding pipette, and the distal end was left floating in the bath. A concentric perfusion pipette was advanced into the lumen. The perfusing solution (88 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 2 mM NaHPO4, 0.5 mM MgCl2, 50 mM urea, buffered at pH 6.6 with 1 N HCl) was supplied with Trypan blue (0.05%) to check the viability of tubular cells. Perfusion was maintained by hydrostatic pressure of 5 to 40 cm H2O. Rapid perfusate changes could be accomplished by a remotely activated valve system that transferred the driving hydrostatic pressure from one side to the other. This system was used to switch, for a period of 0.5 to 3.0 min, from the normal perfusate to one containing 1 μM amiloride and back again.

The local transepithelial voltage (PD) was measured through the perfusion pipette via a salt bridge (1 M KCl in 3% agar) connected with Ag/AgCl wire to a high-input impedance dual-probe electrometer (1011 Ω, model KS-700; W-P Instruments). The voltage was referenced to the bathing medium, which was measured via a second salt bridge. The output was recorded on a strip chart recorder. All of the measurements were performed at room temperature.

Primary Cultures of CCD

Experiments were performed on confluent primary cultures of CCD microdissected from kidneys of βENaC +/+ , L+/+, and L/L mice. CCD fragments (0.5 to 1 mm long) from the kidneys of 5- to 8 wk-old mice were microdissected under sterile conditions in defined medium (25) (DM; DMEM:Ham’s F12 [1:1 vol/vol]; 60 mM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 2% charcoal-treated calf serum FCS, 20 mM HEPES, pH 7.4], supplemented with 0.1% (wt/vol) collagenase, as described (26). Isolated CCD were pooled (8 to 10 fragments), rinsed in fresh DM medium, seeded on Transwell filters (0.4 μm pore size, 0.33 cm2 diameter; Corning Costar Corp., Cambridge, MA) and grown in DM at 37°C in 5% CO2:95% air atmosphere. The medium was changed every 1 d after the fifth day of culture, and the experiments were carried out 1 wk after seeding on confluent cells having developed high transepithelial electrical resistance (>900 Ω · cm2).

Reverse Transcription–PCR

Total RNA was extracted from confluent primary cultures of CCD grown on 0.33 cm2 Transwell filters, using the RNA-PLUS extraction kit (Bioprobe Systems, Montreuil-sous-Bois, France). RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Eragny, France) at 42°C for 45 min, and cDNA was amplified for 28 cycles in 100 μl total volume containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 40 μM dNTP, 1.5 mM MgCl2, 1 μCi [α-32P]dCTP (NEN, Le Blanc Mesnil, France), and 1 unit Taq polymerase, and 15.7 pmol of βENaC primers. The βENaC primers were PLS (5’-CTTCCAAAGTCTCAACTCCG-3’) and P Las (5’-TCTACACGCTCCAGCACGGTG-3’). The αENaC and γENaC primers were the same as those described by Hummler et al. (27). The β-actin primers were sense 5’-GTTGGGCGCCCGCTAGGAGAAG-3’ and antisense 5’-TTGGGAGGTAGGTCTAGGGGG-3’. The thermal cycling programs were as follows: 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. Amplification products were run on 4% polyacrylamide gels and autoradiographed.

Electrophysiologic Studies in Primary Culture of CCD

CCD were grown on 0.33 cm2 Transwell filters in DM until confluence (day 12 after seeding) and then in hormone-free medium (HFM; DM without epidermal growth factor, hormones, and FCS), supplemented with 2% charcoal-treated steroid-free FCS for 24 h and then in HFM alone for a final 18 h. Transepithelial electrical resistance (R2) and voltage (V2) were measured, using dual silver/silver chloride (Ag/AgCl) electrodes connected to the Millicell Electrical Resistance Clamp apparatus (Precision Instrument Design, Tahoe City, CA), as described (25,26). Equivalent short-circuit current (Isc) was calculated with Ohm’s law from R2 and P2. Measurements under open-circuit conditions were chosen because current recordings by the classical short-circuit current (Isc) method still remained in our hands difficult to adapt to the small 0.33 cm2 Transwell filters, in particular for long-term Isc recordings needed to test the effects of aldosterone. In addition, isolated CCD failed to reach confluence on larger Transwell filters normally used for the Ussing chambers used in the laboratory. By convention, positive Isc corresponded to a flow of positive charges from the apical to the basal solution. For testing the effects of aldosterone (500 nM, supramaximal concentration), CCD were grown in DM, as described above, and then in HFM supplemented with 2% charcoal-treated steroid-free FCS for 24 h and then in HFM alone for a final 18 h.

Statistical Analyses

Results are given as means ± SEM from (n) experiments. Statistical significance was assessed by using unpaired t test or by one-way ANOVA using the Bonferroni t test or the Dunn’s t test for comparisons of two or more than two groups, respectively. P < 0.05 was considered significant.

Results

Effects of Salt Depletion and Salt Repletion on Body Weight, Urinary Electrolytes, and Aldosterone Excretion

To assess the role of the kidney for sodium retention in the Liddle mouse, we designed a protocol by looking at rapid changes in sodium intake to detect defects in achieving normal sodium balance. To assess the overall sodium balance, we followed short-term body weight changes, which have been found to be a sensitive index in humans and in mice (18). For instance, upon salt restriction, adult normal mice lose 6 to 7% of their body weight but then reach a new steady state unlike mice presenting a salt losing syndrome (i.e., PHA-1) (18). As shown in Figure 1, we were unable to detect any significant changes in body weight between the Liddle mice (L/L) and their littermate controls (+/+ or −/−) during salt depletion or salt loading; therefore, the L/J+ were not studied.

Next, Na+ and K+ concentrations were measured in the urine from Liddle and littermate control mice, using the same experimental protocol (Figure 2). Under a normal salt diet (0.4% Na+ in food), the urinary [Na+]/[K+] ratio from βENaC +/+ (1.27 ± 0.14; n = 18) and L/L mice (1.29 ± 0.13; n = 13) was not significantly different from that of wild-type mice (1.15 ± 0.11; n = 22). βENaC +/+ and βENaC L/L mice were then kept under a salt depletion diet (0.01% Na+ in food) for 7 d, then fed again with the 0.4% Na+ diet for an additional 5 d. The urine was collected and pooled over a 6-h period during the first 24 h of salt repletion (collection periods 1 to 4) and

Na+ Transport in CCD from Liddle Mice

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then pooled over a 12-h period for the next 24 h (collection periods 5 and 6). The urinary $[\text{Na}^+/\text{H}^+] / [\text{K}^+/\text{H}^+]$ ratio rapidly increased in $\beta\text{ENaC}^{+/+}$ mice to reach the steady-state level between 12 and 18 h after salt repletion (Figure 2A). In contrast, the urinary $[\text{Na}^+] / [\text{K}^+]$ ratio measured between 6 and 18 h after the salt repletion remained significantly lower ($P < 0.05$) in $\beta\text{ENaC} L/L$ mice than in $\beta\text{ENaC}^{+/+}$ mice. Plasma creatinine did not differ in $\beta\text{ENaC}^{+/+}$ and L/L mice (data not shown). The urinary aldosterone concentration was normalized to urinary creatinine concentration, and the aldosterone over creatinine ratio was followed during the phase of salt repletion. In both cases, this ratio rapidly decreased within 12 to 18 h after salt repletion (Figure 2B). However, the urinary aldosterone over creatinine ratio was lower for $\beta\text{ENaC} L/L$ than for $\beta\text{ENaC}^{+/+}$ mice, the differences reaching significance for the 12- to 18-h and 36- to 48-h collection periods ($P < 0.05$). During the same periods, renal sodium reabsorption was increased and prolonged over time in $\beta\text{ENaC} L/L$ mice as compared with the littermate controls ($+/+$) (Figure 2A), despite the two- to threefold lowered aldosterone urinary secretion.

**Subcellular Localization of $\gamma$ ENaC Protein in Kidneys from Wild-Type and Liddle Mice**

To determine whether Liddle mice may exhibit an increased apical abundance of ENaC in the renal collecting system (i.e., the CNT and the CD), we immunolocalized the $\gamma$ subunit of ENaC in kidneys of littermate ($+/+$) control and Liddle mice.

**Figure 1.** Influence of salt diet on total body weight. $\beta\text{ENaC}^{+/+}$ and $\beta\text{ENaC} L/L$ mice were kept under a medium-salt diet (0.4% Na$^+$ in food) for 4 d (days 0 to 4), then fed a low-salt diet (0.01% Na$^+$ in food) for 7 d (days 4 to 11) and fed again (day 11) with the medium-salt diet. Mice were weighed daily, and the results are expressed as percentage variations of body weights from day 4 (referred to as 100%). Values are means ± SEM from nine individual mice in each group.

**Figure 2.** Urinary electrolytes and aldosterone excretion. Mice kept under medium-salt diet (0.4% Na$^+$) were fed a low-salt diet (0.01% Na$^+$). At time 0, the low-salt diet was replaced by the medium-salt diet. Urine from $\beta\text{ENaC}^{+/+}$ and $\beta\text{ENaC} L/L$ mice was pooled in periods of 6 h during the first day after salt repletion and in period of 12 h during the next day. (A) Na/K represents the urinary $[\text{Na}^+] / [\text{K}^+]$ ratio measured in each group. Values are means ± SEM from seven to nine determinations in each group. *$P < 0.05$ versus $\beta\text{ENaC}^{+/+}$ urinary values. (B) Aldosterone/creatinine represents the urinary aldosterone concentration (mM) normalized to the urinary creatinine concentration in mM. Values are means ± SEM from seven to nine determinations in each group. *$P < 0.05$ versus $\beta\text{ENaC}^{+/+}$ values.

**Figure 3.** shows the immunofluorescent detection of $\gamma$ ENaC in representative CNT profiles from mice subjected to different dietary regimens. In wild-type and Liddle mice that were fed a standard sodium diet (0.4% Na), $\gamma$ ENaC was found diffusely distributed throughout the cytoplasm. Dietary sodium restriction (0.01% Na) caused a redistribution of $\gamma$ ENaC from the intracellular compartments toward the apical plasma membrane in the CNT from mice of both genotypes. The apical immunostaining was more pronounced in Liddle mice than in their control littermates. After dietary sodium repletion, $\gamma$ ENaC immunostaining vanished from the apical plasma membrane and became detectable in intracellular compartments in the CNT of wild-type mice, whereas in CNT of Liddle
mice, apical immunostaining for γENaC still prevailed. The apical immunostaining for γENaC, however, was slightly weaker than that seen under Na restriction. Qualitative similar changes in the subcellular localization of γENaC were found in the CD (data not shown). Apical localization of γENaC after dietary Na restriction was less prominent in CD than in CNT, consistent with the previously reported axial decrease of the apical abundance of ENaC along the renal collecting system. The sensitivity of the immunochemical localization of ENaC does not allow for the study of the L/+ phenotype in a meaningful way. The principal cell would be expected to express 50% of +/+ β channels, and our antibodies will not enable us to discriminate between wild-type and mutated β channel complexes.

**Transepithelial Potential Differences from Isolated Perfused βENaC +/+ and βENaC CCD**

To determine whether ENaC activity was increased in the kidneys of mice carrying the Liddle’s mutation, we measured the transepithelial potential difference (PDₜ) in isolated CCD microdissected from mice that were fed a normal salt diet (Figure 4A). In the “open tubule” configuration (see Materials and Methods), PDₜ from isolated perfused βENaC L/L CCD (−2.70 ± 0.88 mV) was significantly greater (P < 0.05) than that of perfused βENaC +/+ CCD (−0.30 ± 0.23 mV). In both cases, PDₜ were fully inhibited after addition of amiloride (10 μM) to the perfusion solution. To test the ability of CCD to recover their original PDₜ, the tubules were then perfused again with an amiloride-free solution. Isolated perfused CCD from βENaC L/L mice displayed 61% recovery of their initial PDₜ (−1.64 ± 0.64 mV versus −2.70 ± 0.88 mV; Figure 4A).

As negative controls, no significant differences were observed in the positive PDₜ values from isolated perfused thick ascending limb of Henle dissected from βENaC +/+ and L/L mice (Figure 4B). Plasma aldosterone levels from βENaC L/L mice from these experiments were lower than those from βENaC +/+ mice (L/L: 81 ± 26 pM versus +/+: 1058 ± 88 pM, n = 8; P < 0.001). Thus, under normal diet, βENaC L/L mice already exhibited lower plasma aldosterone levels and higher ENaC activity in CCD as compared with littermate controls.

**αβγENaC mRNA in Primary Cultures of βENaC +/+, L/+ and L/L CCD**

Primary cultures of CCD from βENaC +/+, +/+ and L/L mice were performed to analyze the consequence of the mutation on Na⁺ transport. First, reverse transcription–PCR was used to detect βENaC mRNA in confluent cultures of CCD. As shown in Figure 5, βENaC +/+ CCD cells exhibited one band of amplified products (260 bp) of the expected size, whereas CCD from heterozygous βENaC L/+ mice exhibited the expected additional band (370 bp) of amplified products generated from the mutated β allele (18). As expected, this band was the only one detected in primary cultures of βENaC L/L CCD cells (Figure 5A). Cultured +/+, L/+, and L/L CCD cells exhibited similar amounts of αENaC and γENaC mRNA transcripts as compared with the levels of actin transcripts, used as internal standard (Figure 5B and C). Thus, these results demonstrated that confluent cultured CCD have conserved qualitatively the expected mRNA expression of wild-type and mutated βENaC.
Na\textsuperscript{+} Transport by Primary Cultures of βENaC +/+ , L+/+ , and L/L CCD

The transport of Na\textsuperscript{+} was estimated by measuring the equivalent short-circuit current (I<sub:eq</sub>) on confluent cultures of CCD cells grown on 0.33 cm\textsuperscript{2} Transwell filters. Confluent cultures of control (+/+ ) CCD cells grown on permeable filters (day 14 after seeding) exhibited high R<sub>f</sub> (1937 ± 114 Ω · cm\textsuperscript{2}, n = 25) and negative P<sub>D</sub> (−34 ± 2 mV). In contrast, confluent (L/L) CCD cells had significantly (P < 0.001) lower R<sub>f</sub> (1220 ± 37 Ω · cm\textsuperscript{2}, n = 29) and greater negative P<sub>D</sub> (−50 ± 3 mV) than those of cultured βENaC +/+ CCD cells (Table 1). Confluent cultures of (L+/+) CCD cells exhibited intermediate R<sub>f</sub> (1426 ± 97 Ω · cm\textsuperscript{2}, n = 33) and P<sub>D</sub> (−43 ± 2 mV) values, of borderline significance with those of cultured βENaC L/L CCD cells. I<sub:eq</sub> was then measured on the three cell models before and after adding amiloride (10\textsuperscript{-6} M) to the apical side of the filters (Table 1). Under basal conditions, I<sub:eq</sub> was significantly greater (P < 0.001) in cultured L/L CCD cells (42.6 ± 3.1 μA/cm\textsuperscript{2}; n = 29) and L+/+ CCD cells (31.9 ± 1.8 μA/cm\textsuperscript{2}; n = 33) than in +/+ CCD cells (17.4 ± 0.5 μA/cm\textsuperscript{2}; n = 25).
Table 1. Electrophysiologic properties of primary cultured CCD

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<th>L/+ (33)</th>
<th>L/L (29)</th>
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<td>RT (Ω · cm²)</td>
<td>1937 ± 114</td>
<td>1426 ± 97a</td>
<td>1220 ± 37d</td>
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<tr>
<td>PD (mV)</td>
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<td>AmsIeq (µA · cm⁻²)</td>
<td>12.0 ± 0.4</td>
<td>16.5 ± 1.3b</td>
<td>23.0 ± 2.7d</td>
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*The transepithelial electrical resistance (Rt) and potential (Pd) were measured on confluent cultures (14 days after seeding) of CCD dissected from wt (+/+), L/+ and L/L mice, as described in Methods. Equivalent short-circuit current (Ieq) was calculated from Rt and Pd measured without or with 10⁻⁶ M amiloride added to the apical side of the filters. Values are means ± SE from (n) separate filters. AmrIeq, amiloride-resistant component of total Ieq; AmsIeq, amiloride-sensitive component of total Ieq.

b P < 0.05, c P < 0.01, d P < 0.001 versus untreated or amiloride-treated (+/+ cell values.

Adding amiloride to the apical side of the filters significantly (P < 0.001) decreased Ieq in all cultured CCD cells, but the fraction of amiloride-resistant Ieq was greater in cultured βENaC L/L and βENaC L/+ CCD cells than in cultured βENaC (+/+) CCD cells. As a consequence, the amiloride-sensitive component of Ieq (AmsIeq) was approximately twofold higher (P < 0.001) in cultured βENaC L/L CCD cells (23.0 ± 2.7 µA/cm²) than in βENaC (+/+) CCD cells (12.0 ± 0.2 µA/cm²). The AmsIeq from cultured βENaC L/+ CCD (16.5 ± 1.3 µA/cm²) was also significantly higher than the AmsIeq from βENaC (+/+ ) CCD cells. These results thus provided more evidence that mutation of the βENaC subunit leads to an increase in amiloride-sensitive Na⁺ absorption that is maintained in cells cultured for 2 wk. The amiloride-resistant fraction of total Ieq significantly increased in cultured L/+ and L/L CCD, as compared with that of wt (+/+ ) CCD. Because we used a concentration of amiloride (10⁻⁶ M) that did not fully block ENaC, it cannot be excluded that part of the amiloride-insensitive current corresponded to Na⁺ reabsorption mediated by ENaC that was not inhibited by amiloride. It also cannot be excluded that a significant portion of this amiloride-resistant Ieq also corresponded to electrogenic secretion of Cl⁻. Indeed, previous studies have shown that primary cultured or established cortical and inner medullary CD cell lines exhibit an apical electrogenic secretion of Cl⁻ that is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) (28,29). Thus, it is tempting to speculate that part of the amiloride-insensitive fraction of the Ieq measured in L/+ and L/L CCD reflects an apical secretion of Cl⁻, presumably through the CFTR Cl⁻ channel. Such a hypothesis deserves further studies to test whether the increase in Na⁺ reabsorption mediated by ENaC observed in the CCD from L/L mice is accompanied by an increase in secreted Cl⁻ mediated by CFTR. Although primary cultures of CCD seem to be a powerful system for ion transport analyses, it may not entirely reflect the in vivo situation, because the conditions of culture may induce rapid phenotypic changes of the cells. Nevertheless, under exactly the same experimental conditions of cultures, the results from the present study indicate clearly that cultured CCD isolated from L/+ and L/L mice exhibit a significant increase in Na⁺ reabsorption mediated by ENaC compared with that of cultured CCD isolated from wt (+/+ ) mice.

Effects of aldosterone on Na⁺ transport by primary cultures of βENaC (+/+), L/+ , and L/L CCD

The spontaneous increase in Na⁺ absorption measured in cultures of βENaC L/L CCD cells in the absence of any effectors raised the question as to whether hormones acting in the CCD could further stimulate Na⁺ absorption. We therefore analyzed the effects of aldosterone on confluent cultured βENaC (+/+), βENaC L/+ , and βENaC L/L CCD cells grown on filters. Aldosterone (5 × 10⁻⁷ M) added to the apical and basal sides of the filters for 3 h significantly increased by twofold the Ieq from cultured βENaC (+/+ ) CCD cells (Table 2). Aldosterone also significantly increased the Ieq from βENaC L/+ and βENaC L/L CCD, but the aldosterone-dependent increase in Ieq was less marked than in cultured βENaC (+/+ ) CCD cells. In all cases, apical addition of 10⁻⁶ M amiloride significantly reduced Ieq. The resulting aldosterone-stimulated AmsIeq was significantly higher (P < 0.05) in cultured βENaC L/L than in βENaC (+/+ ) and βENaC L/+ CCD cells. These latter results indicated that aldosterone was still able to increase the amiloride-sensitive Na⁺ absorption in cultured βENaC L/L CCD cells.

Discussion

Sodium Retention by the Kidney of L/L Mice Is Linked to a Transient Overexpression of ENaC at the Apical Membrane of Principal Cells

In their seminal 1963 report of a single patient, Liddle et al. (1) described a syndrome that mimicked primary aldosteronism characterized by severe hypertension and hypokalemia but with negligible aldosterone secretion. On the basis of simple metabolic studies and measurements of sodium balance under low- and high-salt diet and assessing the effects of mineralocorticoid antagonists (spironolactone and the potassium-sparing diuretic triamterene), the authors proposed that this syndrome was “a disorder in which the renal tubules transport ions with such abnormal facility that the end result stimulates that of a mineralocorticoid excess.” The data presented here provide direct experimental evidence for this brilliant speculation.

In the animal model (3- to 6-month-old adult), under a standard diet (NaCl 3g/kg), we previously reported that the L/L or L/+ mice (19) did not develop the characteristic feature of the Liddle syndrome observed in human patients (hyperten-
Table 2. Effect of aldosterone on electrophysiologic parameters of cultured CCD dissected from the kidneys of wild-type, L/+, and L/L mice

<table>
<thead>
<tr>
<th>CC (Ω · cm²)</th>
<th>−Aldosterone</th>
<th>+Aldosterone</th>
<th>Δleq</th>
<th>AmsIeq</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_T</td>
<td>2216 ± 96</td>
<td>1527 ± 160²</td>
<td>59.0 ± 2.6</td>
<td>30.4 ± 2.0</td>
</tr>
<tr>
<td>L/+</td>
<td>1528 ± 160</td>
<td>1230 ± 81</td>
<td>-30.0 ± 1.6</td>
<td>31.0 ± 4.8</td>
</tr>
<tr>
<td>L/L</td>
<td>1378 ± 117</td>
<td>1102 ± 72</td>
<td>-41.0 ± 2.0</td>
<td>43.3 ± 6.1³</td>
</tr>
<tr>
<td>P_D (mV)</td>
<td>−46.3 ± 3.3</td>
<td>−63.1 ± 3.7²</td>
<td>-18.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>L/+</td>
<td>−48.6 ± 4.6</td>
<td>−59.4 ± 6.4</td>
<td>-24.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>L/L</td>
<td>−64.5 ± 5.1</td>
<td>−67.6 ± 5.9</td>
<td>-29.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Ieq (μA/cm²)</td>
<td>20.6 ± 1.2</td>
<td>42.8 ± 3.1²</td>
<td>21.3 ± 2.6</td>
<td>30.4 ± 2.0</td>
</tr>
<tr>
<td>L/+</td>
<td>32.6 ± 2.9</td>
<td>50.8 ± 6.1²</td>
<td>18.2 ± 2.6</td>
<td>31.0 ± 4.8</td>
</tr>
<tr>
<td>L/L</td>
<td>43.6 ± 4.2</td>
<td>62.0 ± 6.2³</td>
<td>18.4 ± 3.9</td>
<td>43.3 ± 6.1³</td>
</tr>
</tbody>
</table>

*Values are the mean ± SEM from 16 to 19 individual filters. R_T, P_D, and Ieq were measured before (−Aldosterone) and after basal and apical additions of 5 × 10⁻⁷ M aldosterone (+Aldosterone) for 3 h at 37°C. Δleq, difference in Ieq measured before and after the 3-h incubation with aldosterone; AmsIeq, amiloride-sensitive component of Ieq from aldosterone-treated CCD.

² P < 0.05, ³ P < 0.01, ² P < 0.001 versus −Aldosterone values.

 supported by the present in vivo data. Isolated perfused tubules (Figure 4) showed a modest difference of amiloride-sensitive P_D between the L/L mice and the littermate controls. By contrast, when isolated CCD were put in primary culture, they developed an amiloride-sensitive sodium transport that is strongly correlated with the genotype (Figure 5), increasing progressively from +/+ to L/L animals (Table 1). These highly significant effects are in contrast with the small effect of the mutation observed in vivo or ex vivo. The discrepancy between sodium reabsorption measured in vivo or ex vivo and in vitro is not yet understood. It cannot be excluded, however, that the differences observed are due to the fact that primary cultured CCD are bathed with a Na⁺-rich medium containing serum and hormones, a culture condition that should favor the overexpression of ENaC. It is also possible that, in vivo, a number of regulatory mechanisms, triggered by increased salt reabsorption and hypervolemia, lead (by negative feedback) to the repression of the constitutively hyperactive ENaC. In vitro, these repressor mechanisms are lost and the phenotype corresponds to the genotype.

The present findings demonstrate clearly that the Liddle mutation does not completely block or inhibit the aldosterone responses in vitro (Table 2). Despite long-term repressions of aldosterone secretion and chronic low level of plasma aldosterone, the target cells remain sensitive to aldosterone, but the aldosterone responsiveness is inversely proportional to the genotype (or the baseline sodium transport) from high for +/+ to low for L/L animals. This finding in vitro contrasts with observations made recently ex vivo by Palmer et al., who found a remarkable high sensitivity to aldosterone administered in vivo before measuring ENaC activity ex vivo in the apical membrane of CCD principal cells (35). It is interesting that both of the effects of aldosterone and the Liddle mutation were
to increase the number of channels at the cell surface rather than to change its open probability.

In summary, we provide experimental evidence that dysfunction of ENaC expression in the kidney in vivo and in vitro is the first step in increased blood volume, which, in term, will lead to high BP. Our study cannot answer the question of what is the relative contribution of the dysfunction of ENaC in the colon versus the kidney, but the data from our previous reports, together with the present findings, suggest that the two organs can act synergistically in increasing abnormal sodium reabsorption in vivo. The possibility of selectively inactivating α ENaC in the CD (36,37) or in the colon (38) using Cre-lox technology may allow these questions to be addressed in the near future.

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