Peptide Inhibition of Constitutively Activated Epithelial Na\(^+\) Channels Expressed in Xenopus Oocytes*

Hong-Long Ji, Catherine M. Fuller, and Dale J. Benos‡

From the Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

The hypothesis that 30-amino acid peptides corresponding to the C-terminal portion of the β- and/or γ-subunits of epithelial sodium channel (rENaC) block constitutively activated ENaC was tested by examining the effects of these peptides on wild-type (wt) rENaC (αβγ-rENaC), truncated Liddle’s mutants (αβγ-, αβγγ-, and αβγγγ-rENaC), and point mutants (αβγγ-, αβγγγ-rENaC) expressed in Xenopus oocytes. The chord conductances of αβγγ-, αβγγγ-, and αβγγγ-rENaC were 2- or 3-fold greater than wt αβγ-rENaC. Introduction of peptides into oocytes expressing αβγγ-, αβγγγ-, and αβγγγ-rENaC produced a concentration-dependent inhibition of the amiloride-sensitive Na\(^+\) conductances, with apparent dissociation constants (K\(d\)) ranging from 1700 to 160 \(\mu\)M, depending upon whether individual peptides or their combination was used. Injection of peptides alone or in combination into oocytes expressing wt αβγ-rENaC or single-point mutants did not affect the amiloride-sensitive whole-cell currents. The single channel conductances of all the mutant ENaCs were the same as that of wild type (αβγγ). The single channel activities (N\(P\_o\)) of the mutants were 2.2-2.6-fold greater than wt αβγ-rENaC (1.08 ± 0.24, \(n = 7\)) and were reduced to 1.09 ± 0.17 by 100 \(\mu\)M peptide mixture (\(n = 9\)). The peptides were without effect on the single channel properties of either wt or single-point mutants of rENaC. Our data demonstrate that the C-terminal peptides blocked the Liddle’s truncation mutant (αβγγ) expressed in Xenopus oocytes but not the single-point mutants (αβγγ or αβγγγ). Moreover, the blocking effect of both peptides in combination on αβγγγ-rENaC was synergistic.

Hypertension is a common multifactorial disease imparting an increased risk of myocardial infarction, stroke, and end-stage renal disease. Epidemiological studies suggest that up to 30% of human hypertension may have a genetic basis (1). Epithelial sodium channels (ENaC)\(^1\) play a key role in regulating salt and water homeostasis by controlling sodium reabsorption in the distal nephron (22, 23). Similarly, stimulation of the ENaC subunits in families with a history of salt-sensitivity, low-renin, volume-expanded hypertension. These studies suggested that Liddle’s syndrome resulted from truncating or missense mutations deleting a PPPXY motif in the cytoplasmic C-terminus of either the β- (15–20) or γ-ENaC subunits (21). Systemic mutagenesis of the rat ENaC homolog, rENaC, suggested that normal ENaC activity could be modified by altering the consensus PPPXY sequence in an individual β- or γ-ENaC subunit (22). Each of these mutations resulted in increased channel activity when co-expressed with the other wt rENaC subunits in Xenopus oocytes, consistent with increased sodium reabsorption in the distal nephron (22, 23). Similarly, stimulated amiloride-sensitive Na\(^+\) currents in oocytes expressing the Liddle’s mutants of human ENaC were also observed (20, 24).

At present, two non-mutually exclusive mechanisms have been posited to account for the enhanced macroscopic Na\(^+\) channel activity resulting from Liddle’s mutations, namely an increased surface density of channel protein and/or increased channel open probability (P\(o\)) (24–26). While an increased residence time of ENaC at the cell surface has been attributed to defective internalization of the channel protein for ENaC constructs containing Liddle’s mutations, the mechanisms by which these mutations in the β- or γ-ENaC subunits lead to an increase in single channel P\(o\) are not well understood. Interestingly, the currents associated with Liddle’s mutants expressed in oocytes were not down-regulated by elevated intracellular Na\(^+\) concentration in contrast to wt ENaC currents (27). Furthermore, Dinudom et al. (28) suggested that elevated intracellular Na\(^+\) promotes the interaction of Nedd4 with the PY motif of β-ENaC. Hence, feedback inhibition of ENaC by intracellular Na\(^+\) would be lost with Liddle’s mutations. The differences between the regulation of wt ENaC and Liddle’s mutations have not been explored in detail. However, like other cation channels (29–31), the activity of truncated Liddle’s β-ENaC mutants immunopurified from human lymphocytes were also inhibited by synthesized peptides when reconstituted into planar lipid bilayers (32).

---

* This work was supported by NIDDKD Grant DK37206 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Physiology and Biophysics, University of Alabama at Birmingham, MCLM 704, Birmingham, AL 35294-0005. Tel.: 205-934-6200; Fax: 205-934-1445; E-mail: Benos@phybio.bsu.uch.edu

‡ The abbreviations used are: ENaC, epithelial sodium channels; rENaC, rat epithelial sodium channels; hENaC, human epithelial sodium channels; wt, wild type.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

The Journal of Biological Chemistry Vol. 274, No. 53, Issue of December 31, pp. 37693–37704, 1999

This paper is available on line at http://www.jbc.org

37693
We had previously demonstrated in planar lipid bilayer studies that truncation of the C termini of β- or γ-ENaC led to increased channel Pγ (32, 33). We wished to test further the hypothesis that the C-terminal peptides of these subunits could function as inhibitory peptides of rENaC expressed in Xenopus oocytes. In the present study, we investigated the effect of the 30-amino acid C-terminal peptides of β- or γ-ENaC subunits on the currents associated with the truncation and point mutants found in Liddle’s syndrome using whole-cell and single channel recording techniques. The results showed that both the individual peptides SPββ or SPγγ and a mixture of both peptides decreased the macroscopic currents of truncated Liddle’s mutants expressed in oocytes but had no effect on the currents associated with expression of the Liddle’s point mutants.

The single channel activity (NPFγ) of the truncated Liddle’s mutant was down-regulated by the peptides due to decreases in Pγ and the number of channels per patch. The conclusions of the present study were that the increased channel Pγ of the truncated Liddle’s mutants expressed in Xenopus oocytes could be reversed by the synthetic peptides, thus the C termini cytoplasmic tails of the β and γ subunits act in concert to form part of the normal gating mechanism of a functional ENaC.

MATERIALS AND METHODS

Peptide Synthesis and Purification—rENaC constructs, including wt αβγ-rENaC and β and γ subunits of Liddle’s mutations δβH514 (δβH514), δγT874 (δγT874), and γT874 (γT874) were kind gifts of Dr. Cecilia Caneessa (Yale University) and Bernard Rossier (Université de Lausanne). In vitro translation of each rENaC subunit and their mutations were as described previously (34).

Peptides, SPββ, SPγγ, and Pro-SPββ were synthesized by Research Genetics (Huntsville, AL). Following synthesis, the peptides were purified (to >90% purity) by reversed-phase high performance liquid chromatography. The amino acid sequence of each peptide was verified by mass spectroscopy. The primary amino acid sequences of the synthetic peptides were as follows: SPββ, 611PPGPNNYDSLRLQPLD-VIESDSEGDAL449; SPγγ, 620PQPGPKKNTLYRLAFSLTNLTDQTMYLDEL161; Pro-SPββ, 611PPGPNNYDSLRLQPLDPEGPESDSEGDAL449. Peptide concentration was calculated based upon the exact molecular weight of each peptide. The 1:1 peptide mixtures were equimolar, i.e., a 276 μM mixture was a 1:1 mix of two solutions of 276 μM, yielding a final concentration of each peptide of 138 μM.

Oocyte Expression of ENaC—The methods for isolation of oocytes and expression of rENaC cRNA were as described previously (34). Briefly, ovarian tissue was removed from frogs (Xenopus Express, Burley Hills, FL) under anesthesia through a small incision in the lower abdomen. Oocytes were digested with 2–4 mg/ml collagenase in Ca2+-free OR-2 solution for 2 h on a shaker. Following several washes in Ca2+-free OR-2 solution before patch clamping the cell.

For constructing current-voltage (I-V) curves and calculating the permeability ratio of Li+:K+, equimolar potassium was used to replace lithium. The oocytes were clamped at a holding potential of −100 mV. Data were filtered at 0.5–1 KHz, digitized, and stored on hardware for offline analysis. For ramp-I-V recordings, test voltages were elicited from a holding potential of −100 to −140 mV through +60 mV at a rate of 10 mV/25 ms for 500 ms. Currents at −100 mV were sampled at 20-s intervals. Capacitance neutralization was done for each recording using the circuit built into the TEA-200 voltage-clamp amplifier. In addition, the small volume of the holding chamber (<50 μl) also helped minimize the capacitance at the input of the head stage. The resting membrane potentials were read directly from the monitor window of the voltage clamp before and after application of amiloride.

Single Channel Recordings—The oocytes used for patch clamp experiments were first placed in a hypertonic solution prior to being used. The 1:1 peptide mixture from Research Biochemicals Int. (Natick, MA), and 5 mM stock (1:1 MeSO3H/O, v/v) was stored at 4 °C in a container wrapped with aluminum foil. The perfusate for two-electrode voltage clamp experiments was composed of (in mM) 116 NaCl, 2.0 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.0 Na-HEPES, pH 7.4.

For patch clamp experiments, we used a hypertonc solution for stripping the vitelline membrane from the oocytes composed of (in mM), 200 potassium aspartate, 20 KCl, 1 MgCl2, 10 EGTA, 10 NaHEPES, pH 7.4. The basal extracellular medium contained (in mM) 100 LiCl, 10 HEPES, pH 7.4. To obtain outside-out patches, 1.8 mM CaCl2 was included in the extracellular medium. In order to measure the permeability ratio of Li+:K+, equimolar potassium was used to replace lithium. The electrodes were dissolved in water to 100 mM and stored at 4 °C until used. All other reagents were obtained from Sigma.

Two-electrode Voltage Clamp—Whole-cell currents were measured in oocytes held at room temperature (22–25 °C) 24 h after rDNA injection. The currents were analyzed using pCLAMP software (Axon Instruments, Foster City, CA). Voltage clamp potentials were evoked using a TEA-200 voltage clamp (Dagan Corp., Minneapolis, MN) controlled by a personal computer connected via a TL-1 Interface (Axon Instruments). The injected oocyte was placed in a small holding chamber (<50 μl) and was initially superfused with the desired solution at a flow rate of 1.0 ml/min for at least 5 min before recording. Microelectrodes were filled with 3 M KCl and had a tip resistance of 0.5–2.0 MΩ. The bath electrodes consisted of two chloride-coated silver wires that made electrical contact with the bath through a 5% agar bridge (mM) CaCl2 (200 mM). For peptide injection, a third glass electrode (Drummond Scientific Co.) filled with a stock solution of peptide (usually 100 mM) was inserted into an oocyte subsequent to implantation with two recording electrodes. The desired concentration of peptides within an oocyte was defined by injecting a known volume of the peptide stock using an adjustable nanoliter injector (WPI, Sarasota, FL) and calculating the oocyte volume by assuming the oocyte was a prolate spheroid. The oocytes were clamped at a holding potential of −100 mV. Data were filtered at 0.5–1 KHz, digitized, and stored on off-line analysis. For ramp-I-V recordings, test voltages were elicited from a holding potential of −100 to −140 mV through +60 mV at a rate of 10 mV/25 ms for 500 ms. Currents at −100 mV were sampled at 20-s intervals. Capacitance neutralization was done for each recording using the circuit built into the TEA-200 voltage-clamp amplifier. In addition, the small volume of the holding chamber (<50 μl) also helped minimize the capacitance at the input of the head stage. The resting membrane potentials were read directly from the monitor window of the voltage clamp before and after application of amiloride.

Three all configurations (cell-attached, excised inside-out, and outside-out) were used. The reference electrode was an Ag/AgCl pellet bathed in the same solution as the bath. For construction current-voltage (I-V) curves and calculating Pγ, data obtained from cell-attached, inside-out, and outside-out patches were included. For plotting concentration dependence curves of amiloride inhibition, the outside-out patch configuration was employed, and a series (from 0 to 10 μM) of concentrations of amiloride was applied. Patch pipettes were made of borosilicate glass, pulled in two steps with a PP83 vertical puller (Narishige, Japan). The tip resistance of the electrodes was 1–10 MΩ. Single channel currents were recorded with an Axopatch 1-B current-voltage clamp amplifier (Axon Instruments, Inc., Burlingame, CA). The current traces were displayed both by CLAMPPEX version 7.0 associated Axoscope software on the monitor and on an oscilloscope and were stored on a hard drive connected with the working computer through the internet. By convention, for the outside-out configuration, the intracellular potential corresponds to the pipette potential (Vp), and negative (downward) single currents correspond to cation influx from the extracellular to the intracellular side of the membrane. For the cell-attached configuration, the membrane potential should be close to the actual potential across the membrane patch because the cell membrane potential is coupled to the transmembrane potential by two-electrode voltage clamp under our experimental conditions.

Current signals were filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized at 2 kHz using a Digidata 1200 interface and CLAMPPEX software (Axon Instruments, Inc., Foster City, CA). For display, currents were low-pass filtered at 100–200 Hz.
Data Analysis—Amiloride-sensitive macroscopic whole-cell currents were calculated by subtracting the currents in the presence of 10 μM amiloride from the currents in the absence of amiloride. Student’s t test was used to analyze the differences of amiloride-sensitive currents among each group. The results were presented as mean ± S.E., and the degree of significance was assessed using the Student’s t test. Data from rENaC expressing oocytes for quantifying the inhibitory effect of peptide were normalized to the maximum current in the absence of peptide. The apparent inhibition dissociation constants \( K_d \) were computed according to Equation 1,

\[
I / I_o = \frac{1}{1 + (\text{Pep}) / K_d}
\]  
(Eq. 1)

where \( K_d \) is the apparent dissociation constant and \( n \) is the Hill coefficient. \( I \) and \( I_o \) represent the amiloride-sensitive Na\(^+\) currents in the presence or absence of the C-terminal peptides, respectively. [Pep] indicates the concentration of individual peptide or the peptide mixture injected into oocytes. Thus, a plot of \( \log I/I_o - \log I \) versus \( \log [\text{Pep}] \) should yield a straight line with slope \( n \) (see Ref. 37 for discussion).

Analysis of single channel data was performed using FETCHAN and pSTAT program of software CLAMPEX version 7.0 (Axon Instruments). The holding potentials (or the polarity of the single channel currents) were corrected accordingly. Assuming the lowest level during more than 3 and up to 30 min of continuous recordings was the closed level, the single channel open probability \( (P_o) \) was calculated from using Equation 2,

\[
P_o = \frac{I}{N} \frac{1}{T} 
\]  
(Eq. 2)

where \( N \) is total number of channels, \( I \) is the mean current over the period of observations, and \( i \) is the main state unitary current determined from all points current amplitude histograms produced by FETCHAN. The mean current over the period of observation was calculated using the events list files generated by CLAMPEX version 7.0 software and Equation 3,

\[
\frac{I}{T} = \frac{1}{\sum \frac{t_m}{T}} \sum \frac{t_m}{N}
\]  
(Eq. 3)

where \( t_m \) is an event current (all level including the 0 level), \( t_m \) is an event dwell time, and \( M \) is the total number of events. The parameters calculated according to the above method may be underestimated due to the long open and closed transitions and multichannel nature of the patches of ENaC expressed in oocytes. Data are expressed as mean value ± S.E. for \( n \) patches. Because the pSTAT program could not be used to analyze the results of multichannel patches with more than five conductance levels, the program GAUSS (supplied to us by Dr. James Kenyon of the Department of Physiology, University of Nevada) was used to analyze channel data consisting of six or more discrete current levels (38).

The permeability ratios of wt αβγ-rENaC and Liddle’s mutants expressed in oocytes were computed from the measured reversal potentials using the Goldman-Hodgkin-Katz (GHK) equation.

RESULTS

Macroscopic Currents of Truncation Mutants Are Inhibited by Peptide Injection—In order to test the hypothesis that peptides corresponding to the C termini of the β- and/or γ-subunits (SF-30β and SF-30γ) inhibit Liddle’s mutations inserted into rENaC, the βγ-rENaC subunit truncated at amino acid position Arg-564 (βγ) and the γ-rENaC subunit truncated at amino acid position Arg-574 (γγ) were expressed in oocytes in combination with the wt α-rENaC subunit (αβγ-γ). Fig. 1 shows representative current traces in oocytes evoked by a voltage ramp from −140 mV to +60 mV. The chord conductance of αβγ-γ-rENaC-associated currents at hyperpolarizing potentials was ~3-fold greater than that of wt αβγ-rENaC. Injection of a mixture of β + γ C-terminal peptides (1:1, 138 μM each) into oocytes expressing the truncated ENaC constructs (αβγ-γ-rENaC) decreased the current by approximately 63% at hyperpolarizing potentials. In contrast, the current in oocytes expressing wt αβγ-rENaC was not affected by the same concentration of peptide mixture. The αβγ-γ-rENaC-associated current was not decreased by water injection or by injection of either of two control peptides, one comprised of the last 13 amino acids of the C terminus of CFTR (KEETEEYQDTRL, final concentration = 1.7 μM; \( n = 4 \)) or a 30-mer peptide identical in all respects to SP-30β except for substitution of 3 prolines for the single valine and two isoleucines (Pro-SP30β). Fig. 2 shows the time course of peptide inhibition of inward Na\(^+\) current in αβγ-γ-rENaC-expressing oocytes (Fig. 2A) and the lack of effect of the control Pro-SP30β peptide (Fig. 2B). Although Pro-SP30β and the CFTR peptides carried the same or a greater total number of negative charges as the C-terminal peptides SP-30β and SP-30γ, neither the CFTR nor Pro-SP30β peptides had any effect on the whole-cell Na\(^+\) currents induced by αβγ-γ-rENaC expression. Similarly, in parallel patch clamp experiments the addition of the C-terminal CFTR peptide to
Peptide Inhibition of Liddle’s Mutants

A $\alpha\beta_{\gamma\gamma}-rENaC+$ peptide mixture

| Current @ -100 mV (nA) |
|-------------------------|
| 0                      |
| -100                    |
| -200                    |

5 min

B $\alpha\beta_{\gamma\gamma}-rENaC+$Pro-SP$_{30\beta}$

| Current @ -100 mV (nA) |
|-------------------------|
| 0                      |
| -100                    |
| -200                    |

5 min

C $\alpha\beta_{\gamma\gamma}-rENaC$

D + 1.7 mM Pro-SP$_{30\beta}$

FIG. 2. Effect of 30-mer peptide mixture on macroscopic currents in $\alpha\beta_{\gamma\gamma}$-rENaC expressing oocytes. A, continuous recording of inward current at -100 mV from an $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocyte, showing the effects of both amiloride (10 $\mu$M) and 100 $\mu$M SP$_{30\beta}$ at 1.7 mM. B, continuous current recording from an $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocyte showing the lack of inhibitory effect of a control peptide Pro-SP$_{30\beta}$ at 1.7 mM. C, typical ramp current-voltage curves for $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocytes without injection of Pro-SP$_{30\beta}$. D, typical ramp current-voltage relation for $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocytes previously injected with 1.7 mM Pro-SP$_{30\beta}$.

The data are averaged amiloride-sensitive Na$^+$ rENaC conductance in oocytes. (data not shown).

Summarized data showing inhibition of wt and $\alpha\beta_{\gamma\gamma}$-rENaC-induced macroscopic Na$^+$ currents both for the individual peptides and a 1:1 mixture of SP$_{30\beta}$ and SP$_{30\gamma}$ peptides (138 $\mu$M each) are plotted in Fig. 3. The mean chord conductance of $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocytes was 123.7 ± 12.6 $\mu$S (n = 11). This value was more than twice that of wt $\alpha\beta_{\gamma\gamma}$-rENaC-expressing oocytes (51.9 ± 50 $\mu$S, n = 5; p < 0.001). Injection into oocytes of the mixture of SP$_{30\beta}$ plus SP$_{30\gamma}$ peptides decreased the $\alpha\beta_{\gamma\gamma}$-rENaC-associated Na$^+$ conductance to 37.6 ± 10.7 $\mu$S (n = 11), a value statistically indistinguishable from wild-type (p > 0.4). The individual peptides, at the same final concentration as the mixture, i.e. 276 $\mu$M, also inhibited the macroscopic inward Na$^+$ currents but were not nearly as effective as the peptide mixture. In contrast, neither the individual peptides nor the peptide mixture inhibited wt $\alpha\beta_{\gamma\gamma}$-rENaC-associated Na$^+$ conductance (Fig. 3).

To study the inhibitory kinetics of the individual peptides and their mixture, increasing concentrations of the SP$_{30\beta}$ and/or SP$_{30\gamma}$, varying from 92 to 1732 $\mu$M, were injected into the cytoplasm of oocytes expressing $\alpha\beta_{\gamma\gamma}$, $\alpha\beta_{\gamma\gamma}$, and $\alpha\beta_{\gamma\gamma}$-rENaC. The concentration-response curves of peptide(s) on amiloride-sensitive Na$^+$ currents in oocytes injected with cRNA of rENaC constructs associated with Liddle’s syndrome (Fig. 4A) were well described by the Hill equation (Fig. 4B).

The $K_I$ values and the corresponding Hill coefficient are summarized in Table I. The Hill coefficients for the individual peptides and the peptide mixture range from 0.7 to 1. These results suggested that the individual C-terminal peptides and their mixture interact with one rENaC subunit or with a single

FIG. 3. Summarized data showing the effect of the individual peptides or the peptide mixture injection on wt $\alpha\beta_{\gamma\gamma}$ and $\alpha\beta_{\gamma\gamma}$-rENaC conductance in oocytes. The data are averaged amiloride-sensitive Na$^+$ chord conductances determined as the slope of the current-voltage relation between -140 and 0 mV. Each oocyte was injected with a total of 276 $\mu$M peptide (individual or in combination) or an equivalent volume of water. n stands for the number of eggs recorded, and the standard errors (S.E.) are shown. Without injection of the C-terminal peptides, the amiloride-sensitive Na$^+$ conductances for $\alpha\beta_{\gamma\gamma}$ and $\alpha\beta_{\gamma\gamma}$-rENaC were 52.0 ± 5.0 $\mu$S (n = 6) and 123.7 ± 12.6 $\mu$S (n = 11), respectively. Injection of the individual peptides SP$_{30\beta}$, SP$_{30\gamma}$, or the peptide mixture decreased the conductances of $\alpha\beta_{\gamma\gamma}$-rENaC by 103.4 ± 3.7 $\mu$S (n = 5), 79.8 ± 5.7 $\mu$S (n = 4), and 37.6 ± 10.7 $\mu$S (n = 11), respectively. In contrast, the amiloride-sensitive Na$^+$ conductance of $\alpha\beta_{\gamma\gamma}$-rENaC did not decline after injection of either the peptide mixture (53.0 ± 4.3 $\mu$S, n = 6) or injection of the individual peptides SP$_{30\beta}$ (51.8 ± 5.4 $\mu$S, n = 6) and SP$_{30\gamma}$ (48.1 ± 6.4 $\mu$S, n = 5).
compared with the wild-type construct when expressed in ab. 

abg rENaC expressed in oocytes, the question arose as to whether this SP30 single (lines) the two complementary subunits of wt rENaC, were expressed m concentrations injected into oocytes (from 0 to 1732 ab from the same frog were injected on the same day. The amiloride (SS) or open squares U-trENaC, was 15.7 S M). The individual SP30 (b 1) (11) 163.2 (6) 6-rENaC was 15.7 S M by repeated injections into the same oocyte. The dose-response curves were fit with the Hill equation: \( I_c = I_{c0} + \left( \frac{[Pep]}{K_d} \right)^n \). The two far right dashed lines are the double truncation mutant (abg-rENaC) injected with SP30 (closed squares) and SP30 (closed circles). The remaining lines (open symbols) represent single truncation mutant abg-rENaC injected with SP30 (b peptide (open squares) and abg-rENaC injected with peptide SP30 (open circles), respectively. The open triangles represent data obtained from abg-rENaC-expressing oocytes injected with a 1:1 combination of SP30 and SP30 (peptides). The apparent dissociation constants (Ka) and the corresponding Hill coefficient constants determined from the associated Hill plots (B) are listed in Table I.

### Table I

|        | Ka (μM) | Hill coefficient constant |
|--------|---------|---------------------------|
| abβ2γ + SP30 β (5) | 108.3 ± 22.4 | 0.9 ± 0.2 |
| abβ2γ + SP30 γ (4) | 81.4 ± 14.6 | 0.7 ± 0.2 |
| abβ2γ + SP30 γ (5) | 1734.7 ± 0.6 | 1.0 ± 0.1 |
| abβ2γ + SP30 (4) | 1053.3 ± 0.7 | 0.9 ± 0.1 |
| abβ2γ + SP30 (b + γ) (11) | 163.2 ± 19.5 | 0.8 ± 0.1 |

binding site.

**Macromolecular Currents of Point Mutants Are Not Inhibited by Peptides**—Among the Liddle’s ENaCs, both β and γ-Terminus truncated (nonsense) mutants and the single amino acid point mutants resulted in greater amiloride-sensitive Na+ currents compared with the wild-type construct when expressed in *Xenopus* oocytes (22). Because the C-terminal peptides inhibited the currents associated with abβ2γ - rENaC, and abβ2γ - rENaC expressed in oocytes, the question arose as to whether this specific effect was based on the ability of the peptides to restore the wild-type nature of these truncation mutants through the specific interaction with the truncated channel or by involvement of intracellular secondary components. To test this hypothesis, subunits containing point mutations associated with Liddle’s syndrome, βENaC (βL) or γENaC (γL), combined with the two complementary subunits of wt rENaC, were expressed in oocytes, and the effect of the peptides was examined.

In oocytes injected with wt abβγ-rENaC and point mutant constructs, abβγ and abβγ, three parallel groups of oocytes from the same frog were injected on the same day. The amiloride-sensitive conductance in oocytes injected with abβγ-rENaC and abβγ-rENaC were 45.77 ± 3.8 μS (n = 6) and 43.5 ± 3.9 μS (n = 7), respectively. In comparison, the chord conductance of wt abβγ-rENaC was 15.7 ± 4.0 μS (n = 6, p < 0.001). The reversal potentials among each group did not show a statistically significant difference, indicating that the cationic selectivity was not modified by point mutagenesis of the tyrosines in the β- (in position 618) or γ- (in position 628) rENaC subunit. The reason why the absolute value of Na+ conductance in this batch of oocytes was lower than that measured in the experiments presented in Fig. 3 was because less ENaC cRNA was injected (7.5 versus 25 ng/oocyte).

As shown in Fig. 5, following an injection of either 276 μM SP30β or SP30γ into oocytes expressing abβγ-rENaC, the current amplitudes were slightly increased to 108.9 ± 2.5 and 114.07 ± 1.83%, respectively. For oocytes expressing abβγ-rENaC, the Na+ currents were decreased slightly to 85.31 ± 2.4 and 91.53 ± 2.4%, respectively, following β- or γ-peptide injection. No significant inhibitory effects of the individual peptides were found (variation less than 15%) on abβγ-rENaC-associated amiloride-sensitive Na+ currents in oocytes (Fig. 5). Similarly, injection of the peptide mixture into oocytes expressing either abβγ or abβγ-rENaC did not significantly affect the current amplitudes (Fig. 5). The above experiments were repeated using the peptides at a concentration of 1732 μM; comparable results, i.e. no significant current inhibition (within 15% of the preinjection control), were observed (n = 3 each; data not shown).

**Amiloride Blockade of rENaCs Expressed in Oocytes**—One of the defining features of ENaC is its sensitivity to the potassium sparing diuretic amiloride, which reversibly blocks the channel at nanomolar to micromolar concentrations (for review see Ref. 7). To identify that the currents were carried by exogenous rENaC expression, inhibition of current by amiloride was studied in rENaC-injected oocytes in H2O-injected oocytes as a control. Under voltage clamp conditions, amiloride-blockable Na+ currents could not be detected in H2O-injected oocytes (I = 10 μA). In comparison, small currents observed in un.injected or water-injected oocytes, large amiloride-sensitive inward Na+ currents of 5–10 μA in oocytes injected with cRNA of wt rENaC were seen, indicative of the fact that the currents recorded were indeed produced by exogenous rENaC expression (data not shown).

In order to verify that the currents we observed were produced by exogenous cRNAs of rENaC rather than by activation of an endogenous cation channel, amiloride was applied to outside-out patches. Outside-out patches from oocytes expressing rENaC (wt or mutant varieties) invariably contained multiple channels. Similar continuous recordings were made on oocytes expressing all of the other Liddle’s truncation constructs used in these experiments (Fig. 6). When perfused with 100 nM amiloride, the multichannel currents of rENaC invariably contained multiple channels. Similar continuous recordings were made on oocytes expressing all of the other Liddle’s truncation constructs used in these experiments (Fig. 6). When perfused with 100 nM amiloride, the multichannel currents of rENaC and the Liddle’s mutants became flaccid. After washing out amiloride, the original multichannel traces were recovered, indicating that the inhibitory effect of amiloride was reversible. In contrast, no amiloride-sensitive single channel currents could be seen in 10 outside-out patches of H2O-injected oocytes.

In order to determine whether the efficacy of amiloride was changed by any of these truncation mutations, we constructed dose-response curves for amiloride inhibition of channel activity (Np) in these outside-out patches (Fig. 6B). Kd for wt abβγ - γENaC, abβγ - γENaC, and abβγ - γENaC at a holding potential of -20 mV were 55.4, 71.8, 76.4, and 96.7 nM, respectively (p > 0.1, analysis of variance). These results indicate that there was no difference in amiloride sensitivity between wt abβγ-rENaC and the Liddle’s variants. Furthermore, amiloride inhibited single channel activity (Np) by acting as an open channel blocker (39).

**Effect of a 1:1 Peptide Mixture on Single Channel Currents of Truncated Mutants**—We also used single channel analysis to
assay the inhibitory effect of the C-terminal peptides on wt abg-rENaC and the Liddle’s constructs when expressed in oocytes. Fig. 7 shows the single channel current traces for abg (top) and abgT-rENaC (middle) and their respective I-V curves (bottom) in inside-out patches of oocytes membrane under bi-ionic conditions. The unitary conductances of both wt abg (n = 9) and abgT-rENaC (n = 6) averaged around 7 pS.

The reversal potentials under bi-ionic conditions averaged 97 mV, indicating a PLi/PK of 42.

The effects of 1000 μM peptide mixture on the kinetics of the double-truncated Liddle’s mutant (abgT) are shown in Fig. 8. It can be seen that the peptide mixture decreased NPo, but not the conductance of abgT-rENaC, and was completely reversible (Fig. 8C). The inhibition produced by the peptides on abgT-rENaC was very different than that seen for amiloride (cf. Fig. 6A), i.e. the peptides did not appear to act as open channel blockers.

If the C-terminal regions of the β- and γ-ENaC subunits act as intrinsic gating particles, exogenously added β and γ C-terminal peptides should not block wt abg-rENaC because of the presence of intact β- and γ-ENaC C termini. The effect of the peptide mixture on wt abg-rENaC was thus explored following application of peptides to inside-out patches (Fig. 8A).

In contrast to abgT-rENaC, the C-terminal peptides had no effect on the gating of wt abg-rENaC currents nor did 1000 μM Pro-SP30b peptide produce any inhibition of abgTγ-rENaC (Fig. 8B), consistent with the macroscopic current recordings (Fig. 1). The single channel activity (NPo) and Po of each group are summarized in Table II. NPo of abg and abgY were 2.65- and 2.18-fold over that of wt abg-rENaC, respectively, at a holding potential of 240 mV. The corresponding Po of abg and abgY increased only slightly, namely by 6.8 and 4.5%, respectively (Table II), values not significantly different than that for the wild-type channel. The number of channels per patch and the
Peptide Inhibition of Liddle’s Mutants

The number of channels per patch, the single channel activity, and the \( P_o \) were not affected by application of the peptide mixture to the cytosolic side of the patch from the oocytes expressing \( \alpha \beta \gamma \) or \( \alpha \beta \gamma \) rENaC (Fig. 12 and Table II). Taken together, these data indicated that the C-terminal peptides inhibited the amiloride-sensitive macroscopic Na\(^+\) currents and the single channel activity of \( \alpha \beta \gamma \) rENaC but not those of \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC, consistent with a role for the C terminus acting as an intrinsic inhibitory particle of ENaC.

**DISCUSSION**

The hypothesis that the cytosolic tails of \( \beta \)- or \( \gamma \)-ENaC subunits may function as inherent inhibitory particles involved in the normal gating behavior of epithelial Na\(^+\) channels arose from our previous observations made in planar lipid bilayers that synthetic peptides made up of the last 10 or the last 30 amino acids of either \( \beta \)- or \( \gamma \)-ENaC could inhibit Na\(^+\) channels whose \( P_o \) was increased because they possessed Liddle’s truncation mutations (40, 41). In the present study, we extended these initial observations to the oocyte expression system, using a combination of two-electrode voltage clamping and excised patch clamping, to confirm the inhibitory actions of these peptides on ENaCs containing truncations in their \( \beta \) and/or \( \gamma \) subunits when expressed in a more native environment.

**Expression of Liddle’s Mutants**—Two non-mutually exclusive mechanisms have been proposed to account for the basal-activated, macroscopic amiloride-sensitive Na\(^+\) currents induced by expression of the Liddle’s mutants: the retrieval theory (i.e. decreased channel internalization) and/or enhanced single channel activity (42). Identification of specific Nedd4 and spectrin binding domains in the cytosolic region of the ENaC subunits (43, 44) and the interactions of ENaC with actin and other cytoskeletal elements (45) supported the idea that the fundamental problem in Liddle’s disease could be inefficient internalization of the channel from the cell surface. Furthermore, observations by Snyder et al. (24) that suggested that >90% of the basal-activated Na\(^+\) currents are correlated to an increase in ENaC expression at the cell surface. This finding was verified in oocytes expressing a rENaC construct containing a truncated version of the \( \beta \) subunit (R564X). Cell surface expression of \( \alpha \beta \gamma \) rENaC was increased over wt rENaC expression, corresponding to a 5.6-fold increase in ENaC currents (25). However, we previously reported that a 35% increase in the fluorescence intensity in oocytes injected with \( \alpha \beta \gamma \) rENaC was accompanied by a 4.4-fold increase in amiloride-sensitive Na\(^+\) current (26). Thus, a smaller increase in cell surface channel density was associated with a large increase in current, suggesting that the retrieval theory alone did not adequately explain the large macroscopic Na\(^+\) current seen in Liddle’s mutants (46). It is possible that the differences between these studies could be due to different responses produced by rENaC and hENaC (24).

The idea that ~90% of the ENaC channels at the cell surface are inactive and only 10% are active (27) underscores the methodological limitations in quantitatively determining not only active ENaC channel number at the cell surface but also single channel \( P_o \). Electrophysiological recordings, including two-electrode voltage and patch clamping, report only electrically active ENaC channels at the cell surface and cannot reveal the existence of quiescent channels within the plasma membrane. The assessment of single channel activity, specifically open probability, can only be done if the total number of channels (active plus inactive) per patch is known. The relationship between macroscopic currents and single channel activity is given by \( I = i \cdot N \cdot P_o \), where \( I \) is the mean current amplitude; \( i \) indicates the amplitude of the unitary current; \( N \) represents the number of active channels at the cell surface,

**single channel activity of \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC on the other hand were significantly increased compared with wt rENaC, \( p < 0.01 \).**

The unitary conductances of \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC were 6.61 ± 0.01 (n = 5), and 6.68 ± 0.02 (n = 10), respectively (Fig. 10), indicating that these single-point mutations in the C terminus did not influence single channel conductance. The cation permeability ratios (\( P_o \)/\( P_c \)) for \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC were similar to that of wt \( \alpha \beta \gamma \) rENaC when expressed in Xenopus oocytes, i.e. around 40 (Fig. 10, bottom).

The effect of peptides on \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC was also assessed using the inside-out patch configuration. As shown in Fig. 11, no change in channel activity was recorded for both \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \) rENaC. Unlike the truncated Liddle’s mutant,
and $P_o$ is the open probability of the channel. Therefore, in a case where the majority of channels are quiescent, the true $P_o$ will be very low. Thus, a significant component of the basal-activated ENaC currents that is not due to increased ENaC channel expression at the cell surface can result from altered single channel behavior. Such alterations may include an increase in $P_o$ or an increase in single channel unitary conductance.

The findings of the present study showed that the unitary conductances for both the truncation mutants ($\alpha\beta\gamma$-rENaC) and the missense Liddle's mutants ($\alpha\beta\gamma$- and $\alpha\beta\gamma$-rENaC) did not differ from that of wt $\alpha\beta\gamma$-rENaC, in accordance with the observations of others (23, 24). However, the electrically active ENaC number at the cell surface of Liddle's mutants described as the number per patch were 1.8–2.5-fold over that of wt $\alpha\beta\gamma$-rENaC. Significantly, in our experiments, $P_o$ for $\alpha\beta\gamma$-rENaC was 40% higher as compared with wt $\alpha\beta\gamma$-rENaC (Table II). The single channel activity ($N_{ch}$) for all the mutant channels showed a ~3-fold increase, paralleling the macroscopic ENaC currents in oocytes expressing ENaC-containing Liddle's mutations. Moreover, the $P_{ch}/P_R$ permeability ratios for all of the mutant channels were comparable to those of wt $\alpha\beta\gamma$-rENaC expressed in oocytes or of the native ENaC in epithelia (47–49), as was the amiloride sensitivity. Thus, like Firsov et al. (25) and Ismailov et al. (32), we propose the open probability of Liddle's mutations producing truncations of the $\beta$ or $\gamma$ subunit carboxyl tails is also an important parameter contributing to enhanced macroscopic ENaC currents seen in this disease.

It is important to reconcile our findings with those of other laboratories claiming that single channel $P_o$ does not change when a $\beta$ or $\gamma$ subunit truncating Liddle's mutation is present in ENaC (24, 50). As indicated above, analyses of $P_o$ from single channel data recorded by patch clamp are difficult because the true number of channels in the patch must be known. Moreover, appropriate observation times must also be attained. Fyfe and Canessa (50) concluded that subunit composition of ENaC primarily determines channel kinetics, yet only two, not three, subunit channels were used in their studies. As they and others (50–53) indicate, the $P_o$ of ENaC is quite variable, ranging from 0.01 to 0.9. This variability in basal $P_o$ reflects uncertainties in channel number within any given patch. If the channel number is underestimated, for example, due to the presence of quiescent channels, then higher values of $P_o$ will be calculated. More reliable estimates of $P_o$ for wild-type ENaC would be obtained because there would be on average fewer quiescent channels present. In our experiments, wide variability in $P_o$ was not observed from patch-to-patch. Although our experiments also suffer from lack of certainty of the absolute number of channels per patch, we only performed our analyses on multichannel patches (2–7 channels), not “single” channel patches, so that a more representative (and potentially more reliable) determination of channel number could be made.

Peptide Inhibition of Liddle's Mutants—By using the whole-cell configuration of the patch clamp technique, the ability of a 10-amino acid polypeptide (200 $\mu$m) identical to either the cytosolic tail of $\beta$-rENaC or $\beta$-hENaC to inhibit amiloride-sensitive inward Na$^+$ currents in lymphocytes of Liddle's disease was found (41). This finding was confirmed in planar lipid bilayers reconstituted with the Na$^+$ channel complex immunopurified from lymphocytes derived from patients affected with Liddle's disease (32). Unresolved questions concern the molecular mechanism underlying peptide inhibition of ENaC and whether peptides comprising the missing segments of the intracellular C-terminal tails of the $\beta$ or $\gamma$ subunit can inhibit basal-activated Na$^+$ currents caused by both single and double truncation mutations ($\alpha\beta\gamma$-rENaC, $\alpha\beta\gamma$-rENaC, and $\alpha\beta\gamma$-rENaC).
rENaC) and missense mutations (αβγ-rENaC) identified in Liddle’s syndrome cases. In the present study, a combination of the two peptides markedly inhibited αβγ-rENaC-associated amiloride-sensitive Na\(^{+}\) currents in a dose-dependent fashion. Whereas the individual peptides were also inhibitory, their efficacy was much reduced (Fig. 4). Expression of only one truncation mutant of either the β subunit (βT) or the γ subunit (γT) combined with complementary wt ENaC sub-units also induced a 3-fold increase in macroscopic amiloride-sensitive Na\(^{+}\) currents (Refs. 23, 24, and 27 and present study). The significantly lower inhibitory effect of the individual peptides compared with the peptide mixture on αβγ-rENaC suggests that both the β and γ subunit C termini are required for efficient inhibition of an activated ENaC. This idea is supported by our data showing that the efficacy of either SP30β or SP30γ used individually was enhanced in the single subunit truncation mutant (αβγT) as compared with the double truncation constructs (αβγT). Furthermore, the fact that these peptides did not block the point mutant channels (αβγT) and the fact that these channels did not have an increased P\(_o\) is consistent with the argument that the cytoplasmic C-terminal tails of β and γ-ENaC are involved in the normal gating of ENaC. We hypothesize that there is β-sheet formation between the C-terminal tails of β and γ that, in essence, forces the tails to act as a single binding peptide. Evidence supporting such an interaction comes from intrinsic fluorescence and circular dichroism studies of these synthetic peptides in solution (33). Measured Hill coefficients of 1 for the peptide mixture are also consistent with this hypothesis.

The specificity of the peptide inhibition on Liddle’s ENaC (truncation mutants) was verified in experiments using non-ENaC peptides (e.g., a 14-amino acid-mer of CFTR or Pro-SP30β). Cytoplasmic injection or perfusion of either of these peptides at the same concentrations used for the peptide mixture did not cause any decrease of αβγ-rENaC-associated amiloride-sensitive Na\(^{+}\) currents. Because rENaC activity is down-regulated by hypo-osmotic stress (14) and peptide injection may have increased the oocyte volume (the cell volume increased ~13% assuming the mean liquid volume of oocyte is 500 nl), it is also possible that the decreased channel activity could be due to oocyte swelling. However, injection of the same volume of distilled water had no effect on the current amplitude (Fig. 1). The observations of Kellenberger and colleagues (27) excluded the possibility that the inhibitory effects of the C-terminal peptides of rENaC on the basal-activated currents of Liddle’s mutations result from rundown. Their examination of the rundown phenomenon suggested that the wt rENaC-associated, rather than the Liddle’s mutant-associated, Na\(^{+}\) cur-

**Fig. 8.** Single channel inhibition of αβγ-rENaC by the peptide mixture. Continuous single channel records (inside-out patches) of wt αβγ-rENaC (A) or αβγ-rENaC (B and C) expressed in oocytes are shown. Excised inside-out patches were prepared in order to apply the C-terminal peptides to the cytosolic side of the rENaC channels. The desired concentration of peptide was added directly to a small (0.3 ml) chamber. The effect of SP30β + SP30γ on the wt (A) and αβγ and channel (C) and the lack of effect of 200 μM Pro-SP30β peptide on αβγ-rENaC (B) are depicted. The holding potential was ~40 mV. Each condition was replicated six times.
Peptide Inhibition of Liddle’s Mutants

The peptide mixture.

The macroscopic currents depicted in Figs. 1 and 2 before the addition of the peptide mixture containing active ENaC.

of channel per patch, single channel activity, and open probability of αβγ-λɛNaC. The summarized data on single channel activity (normalized N·P₀ of wt αβγ-λɛNaC as 1.0, middle), the number of channel per patch (N, bottom), and open probability (P₀, top) for wt αβγ- and αβγ-λɛNaC are averaged from the results of five experiments. The peptide mixture of SP30β and SP30γ (1:1, 100 μm) was applied to the isolated inside-out patches from oocytes expressing wt αβγ- and αβγ-λɛNaC. All three investigated parameters for αβγ-λɛNaC exceeded those for wt αβγ-λɛNaC by the order of 2- or 3-fold, in accordance with the macroscopic currents depicted in Figs. 1 and 2 before the addition of the peptide mixture.

rent shows a time-dependent decrease (by 27% in 25 min). In the present study, the C-terminal peptides did not exert any effect on wt λɛNaC or the point mutant constructs (αβγ-λɛNaC), indicating that they did not have untoward nonspecific effects in this expression system. The amiloride-sensitive Na⁺ currents generally achieved stabilization after ~10 min of perfusion under our experimental conditions, and the rundown of wt λɛNaC currents was correlated to the accumulation of intracellular Na⁺ (27). Comparable experiments were performed on excised inside-out patches of oocyte membranes containing active ENaC.

Our data show that the αβγ-λɛNaC number per patch, P₀, and thus overall channel activity (N·P₀) were inhibited to wild-type levels by the peptide mixture, similar to that seen in the macroscopic experiments. The inhibition of the channel by peptide occurs only from the cytoplasmic side and is dissimilar to that produced by amiloride, in that a “flickering”-type block was not evident. These results are essentially identical to those seen in the bilayer, in that increasing concentrations of the peptides increase a second slow component of channel closed time distribution, leaving the open time distribution unaffected (33), again consistent with the reconstituted planar lipid bilayer system (33).

Deletion of the C termini of the β- and γ-ENaC subunits leads to loss of the PPPXY conserved sequence. The PPPXY motifs located within the C termini have been found to play an
addition is due to a decrease in the fraction of the electrically detected rENaC per unit of cell surface, simply by a reduction in $P_o$. Because the C-terminal domains of the $\beta$- and $\gamma$-ENaC subunits contain phosphorylation sites for protein kinase A and protein kinase C (8), in vivo the C-terminal regions may interact with some target for protein kinase A- or protein kinase C-mediated phosphorylation on rENaC (subunits or associated cytoskeletal elements) and therefore regulate the activity of rENaC by altering its phosphorylation state (32, 46). The enhanced sensitivity to the activating effects (via protein kinase A) of normal endogenous cAMP levels in affected lymphocytes of Liddle’s disease (41) is consistent with this possibility. Also, based on the finding that the channel activity of truncated Liddle’s mutants is no longer down-regulated in a feedback manner by the increasing intracellular Na$^+$ concentration and that $<10\%$ of ENaC population located at the cell surface is in the active channel pool, it has been proposed that a greater fraction of “silent” rENaCs has escaped from the inactive pool due to the rise in cytosolic Na$^+$ concentration and thus has contributed to the increased electrically detected number of channels per patch (27).

In summary, our results demonstrate that both a greater ENaC number of channels at the cell surface and an increased $P_o$ contribute to the increased basal ENaC currents associated with expression of ENaC truncation mutants identified in Liddle’s disease. In addition, C-terminal peptides structurally identical to the cytoplasmic tails of individual ENaC subunits afforded us an opportunity to examine the regulatory function imposed by the C-terminal tails of rENaC and thus further elucidate the pathogenesis of Liddle’s disease. The results in the present study confirmed that the constitutively activated Na$^+$ absorption through truncation mutants of the ENaC channel located on the apical membrane of kidney collecting duct correlates to the loss of the inhibitory tails of the $\beta$ and/or $\gamma$ subunits. Physical addition of the missing regions for both subunits of the truncation Liddle’s mutations could achieve successful correction of currents in vitro, both at the macroscopic and single channel levels. Combined with similar observations obtained from patch clamping on human B lymphocytes (41), dual-electrode voltage clamping on oocytes expressing ENaC (26), and planar lipid bilayer incorporation experiments (32, 33), our results provide an experimental basis for understanding inherent ENaC function. Our results also support the idea that different Liddle’s mutations, e.g. truncation versus point mutations, produce enhanced macroscopic Na$^+$ currents through different mechanisms.

Acknowledgment—We thank Eddie J. Walthall (Department of Physiology and Biophysics, University of Alabama, Birmingham) for the superb technical assistance in the construction of the patch clamp setup. We thank Drs. C. Canessa (Department of Physiology, Yale University) and L. Schild (Institute of Pharmacology and Toxicology, Lausanne, Switzerland) for their kind gifts of ENaC, and Drs. S. Kellenberger (Lausanne, Switzerland) and Dr. Y. L. Zhu (Physiology, State University of New York, Buffalo) for their helpful discussion. We thank C. Guy for excellent secretarial service.

REFERENCES

1. Lifton, R. P., and Jeunemaitre, X. (1993) J. Hypertens. 11, 231–236
2. Canessa, C. M., Schild, L., Buell, G., Thorenz, B., Gantschl, I., Horisberger, J.-D., and Rossier, B. C. (1994) Am. J. Physiol. 267, C1682–C1690
3. Lingueglia, E., Renard, N., Waldmann, R., Vollely, N., Champigny, G., Plass, H., Lazauski, M., and Banchy, P. (1994) J. Biol. Chem. 269, 13736–13739
4. Canessa, C. M., Merillat, A. M., and Rossier, B. C. (1994) Am. J. Physiol. 267, C1682–C1690
5. Sariban-Sohraby, S., Sorscher, E. J., Brenner, B. M., and Benos, D. J. (1988) J. Biol. Chem. 263, 13875–13879
6. Benos, D. J., Awa CL, M. S., Ismailov, I. I., and Johnson, J. P. (1995) J. Membr. Biol. 143, 1–14
7. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
8. Shimkets, R. A., Lifton, R., and Canessa, C. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3301–3305
9. Chraibi, A., Vallet, V., Firsov, D., Hess, S. K., and Horisberger, J. D. (1998)
Peptide Inhibition of Liddle’s Mutants

J. Gen. Physiol. 111, 127–138
10. Palmer, L. G. (1982) J. Membr. Biol. 83, 57–69
11. Ismaiel, I. I., Berdiev, B. K., and Benos, D. J. (1995) J. Gen. Physiol. 106, 445–466
12. Ishikawa, T., Marunaka, Y., and Rotin, D. (1998) J. Gen. Physiol. 111, 825–846
13. Smith, P. R., Saccomani, G., Joe, E. H., Angelides, K. J., and Benos, D. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6971–6975
14. Ji, H. L., Fuller, C. M., and Benos, D. J. (1998) Am. J. Physiol. 275, C1182–C1190
15. Shimkets, R. A., Warnock, D. G., Bositis, C. M., Nelson-Williams, C., Hansson, J. H., Schambelan, M., Gill, J. R., Ulick, S., Milora, R. V., and Findling, J. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 825–846
16. Ji, H. L., Fuller, C. M., and Benos, D. J. (1998) Cell 79, 457–414
17. Hansson, J. H., Schild, L., Lu, Y., Wilson, T. A., Gautschi, I., Shimkets, R., Nelson-Williams, C., Rossier, B. C., and Lifton, R. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11485–11499
18. Tong, H., Schild, L., Enomoto, N., Matsui, N., Marumo, F., Rossier, B. C., and Sasaki, S. (1996) J. Clin. Invest. 97, 1780–1784
19. Baker, E. H., Dong, Y. B., Sagnella, G. A., Rothwell, M., Onipinlu, A. K., Markandu, N. D., Cappuccio, F. P., Cook, D. G., Persu, A., Corvol, P., Jeunemaitre, X., Carter, N. D., and MacGregor, G. A. (1998) Lancet 351, 1388–1392
20. Jackson, S. N., Williams, B., Houtman, P., and Trembath, R. C. (1998) J. Med. Genet. 35, 510–512
21. Hansson, J. H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C., Iwasaki, T., Rossier, B. C., and Lifton, R. P. (1995) Nature Genet. 11, 76–82
22. Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., Lifton, R. P., and Rossier, B. C. (1996) EMBO J. 15, 2381–2387
23. Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P., and Rossier, B. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5099–5103
24. Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, A. M., Ertl, W., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
25. Araya, M. S., Touson A., and Benos, D. J. (1997) Am. J. Physiol. 273, C1889–C1899
26. Kellenberger, S., Gautschi, I., Rossier, B. C., and Schild, L. (1998) J. Clin. Invest. 101, 2741–2750
27. Dinudom, A., Haran, J. K., Komwata, P., Young, J. A., Kumar, S., and Cook, D. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7169–7173
28. Vassiley, P. M., Scheuer, T., and Catterall, W. A. (1988) Science 241, 1668–1661
29. Ehelting, G., Zagotta, W. N., and Catterall, W. A. (1998) J. Gen. Physiol. 111, 75–82
30. French, R. J., Prusak-Sochaczewski, E., Zamponi, G. W., Becker, S., Kularatna, A. S., and Horn, R. (1996) Neuron 16, 407–413
31. Ismaiel, I. I., Berdiev, B. K., Fuller, C. M., Bradford, A. L., Lifton, R. P., Warnock, D. G., Bubien, J. K., and Benos, D. J. (1996) Am. J. Physiol. 270, C214–C223
32. Ismaiel, I. I., Sbyhonsky, V. G., Serpersu, E. H., Fuller, C. M., Cheung, H. C., Muccio, D., Berdiev, B. K., and Benos, D. J. (1999) Biochemistry 38, 354–363
33. Ji, H. L., DuVall, M. D., Patton, H. K., Satterfield, C. L., Fuller, C. M., and Benos, D. J. (1998) Am. J. Physiol. 274, C455-C464
34. Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S., and Sakmann, B. (1988) Pflügers Arch. 407, 577–588
35. Tamura, H., Schild, L., Enomoto, N., Matsui, N., Marumo, F., Rossier, B. C., and Sasaki, S. (1996) J. Clin. Invest. 97, 1780–1784
36. Baker, E. H., Dong, Y. B., Sagnella, G. A., Rothwell, M., Onipinlu, A. K., Markandu, N. D., Cappuccio, F. P., Cook, D. G., Persu, A., Corvol, P., Jeunemaitre, X., Carter, N. D., and MacGregor, G. A. (1998) Lancet 351, 1388–1392
37. Jackson, S. N., Williams, B., Houtman, P., and Trembath, R. C. (1998) J. Med. Genet. 35, 510–512
38. Hansson, J. H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C., Iwasaki, T., Rossier, B. C., and Lifton, R. P. (1995) Nature Genet. 11, 76–82
39. Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., Lifton, R. P., and Rossier, B. C. (1996) EMBO J. 15, 2381–2387
40. Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P., and Rossier, B. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5099–5103
41. Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, A. M., Ertl, W., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
42. Araya, M. S., Touson A., and Benos, D. J. (1997) Am. J. Physiol. 273, C1889–C1899
43. Kellenberger, S., Gautschi, I., Rossier, B. C., and Schild, L. (1998) J. Clin. Invest. 101, 2741–2750
44. Dinudom, A., Haran, J. K., Komwata, P., Young, J. A., Kumar, S., and Cook, D. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7169–7173