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A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system

(amiloride/human hypertension/kidney/genetic disease)

LAURENT SCHILD†, CECILIA M. CANESSA†‡, RICHARD A. SHIMKETS§, IVAN GAUTSCHI†, RICHARD P. LIFTON§, AND BERNARD C. ROSSIER†

†Institut de Pharmacologie et de Toxicologie de l'Université, rue du Bugnon 27, CH-1005 Lausanne, Switzerland; and §Howard Hughes Medical Institute, Departments of Medicine and Genetics, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT 06510

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ABSTRACT **We have studied the functional consequences of a mutation in the epithelial Na^+ channel that causes a heritable form of salt-sensitive hypertension, Liddle disease. This mutation, identified in the original kindred described by Liddle, introduces a premature stop codon in the channel β subunit, resulting in a deletion of almost all of the C terminus of the encoded protein. Coexpression of the mutant β subunit with wild-type α and γ subunits in *Xenopus laevis* oocytes resulted in an ≈3-fold increase in the macroscopic amiloride-sensitive Na^+ current (I_{Na}) compared with the wild-type channel. This change in I_{Na} reflected an increase in the overall channel activity characterized by a higher number of active channels in membrane patches. The truncation mutation in the β subunit of epithelial Na^+ channel did not alter the biophysical and pharmacological properties of the channel—including unitary conductance, ion selectivity, or sensitivity to amiloride block. These results provide direct physiological evidence that Liddle disease is related to constitutive channel hyperactivity in the cell membrane. Deletions of the C-terminal end of the β and γ subunits of rat epithelial Na^+ channel were functionally equivalent in increasing I_{Na}, suggesting that the cytoplasmic domain of the γ subunit might be another molecular target for mutations responsible for salt-sensitive forms of hypertension.**

The amiloride-sensitive epithelial Na^+ channel (ENaC) constitutes the rate-limiting step for Na^+ reabsorption in epithelial cells lining the distal nephron (1–3). Proper regulation of the activity of this channel is crucial for maintenance of Na^+ balance, blood volume, and control of blood pressure. In 1963, Liddle *et al.* (4) described a disorder affecting a 16-year-old female and some of her siblings characterized by severe hypertension, hypokalemia, and low urinary levels of aldosterone. Pharmacological evaluation of these patients showed no effects of spironolactone, a mineralocorticoid antagonist, but administration of triamterene, a specific blocker of the ENaC during salt restriction, normalized blood pressure and hypokalemia. The pedigree of the original kindred described by Liddle has been extended (5), clearly demonstrating autosomal dominant inheritance of this form of familial hypertension. The constellation of these findings was consistent with a mutation resulting in a constitutive activation of the ENaC and suggested the subunits of the ENaC as candidate genes for hypertension.

The primary structure of the subunits of the amiloride-sensitive ENaC of the rat colon has recently been identified (6, 7). The functional channel is a heterooligomeric protein composed of at least three homologous α, β, and γ subunits that share ≈35% identity in amino acid sequence. The ENaC is highly conserved, functionally and structurally, throughout evolution (8–11, 13). Each subunit has two transmembrane domains, with short cytoplasmic N and C termini and a large ectocytoplasmic loop (14–16). The biophysical properties of the ENaC include a low channel conductance (4–5 pS), ionic selectivity for $Li^+ > Na^+ >> K^+$, and a high affinity for amiloride (K_i = 0.1 μM) (7, 13).

Recently, complete linkage of Liddle syndrome with the locus encoding the β subunit has been established (17). Analysis of this gene in five Liddle syndrome kindreds has revealed mutations that, in each case, introduce premature stop codons or frameshift mutations in the cytoplasmic C terminus of the β subunit. The mutation identified in the affected patients of the original Liddle kindred is a C → T substitution that results in the introduction of a stop codon at Arg-564 (β564). This mutation leaves the second transmembrane domain intact but removes the major part of the downstream cytoplasmic portion of the protein.

The nature of the mutation and the pathophysiology of Liddle syndrome suggested that these mutations result in constitutive activation of the ENaC. The purpose of this study was to define the functional consequences of the β564 truncation of the C terminus of the β subunit of ENaC that causes Liddle disease. We chose this mutation because it represents the most proximal deletion identified to date that causes this disorder (17). We have used a well-characterized system for expressing the α, β, and γ subunits of the rat ENaC (rENaC) in *Xenopus laevis* oocytes, for at least two reasons. (*i*) The rat and human homologues of ENaC share a high degree of sequence homology—including 81% identity in the second transmembrane domain and the C-terminal segment of the β subunit (17), the most relevant functional domains of the protein. (*ii*) The biophysical properties of the human channel have not yet been established, whereas the properties of the channel of the rat cortical collecting duct are well documented (18). The present study demonstrates that deletion of the C terminus of the rat β subunit resulted in an increase in channel activity when expressed in *X. laevis* oocytes. In addition, the corresponding deletion in the γ subunit, but not in the α subunit, resulted in an equivalent abnormal increase in channel activity. These findings establish the molecular basis of a form of human hypertension and have implications for the mechanism of regulation of the ENaC.

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Abbreviations: ENaC, epithelial Na^+ channel; rENaC, rat ENaC; $_T$, C-terminus truncated subunit; I_{Na}, Na^+ current; WT, wild type; PKC, protein kinase C.

‡Present address: Department of Cellular and Molecular Physiology, Yale University, New Haven, CT 06520-8026.

MATERIALS AND METHODS

Truncation mutants of the rat α, β, and γ subunits were obtained by introduction of a premature stop codon in the C-terminal end of the cDNA, using a one-step PCR amplification with two oligonucleotide primers: a 5′ specific primer upstream to a unique restriction site of the rENaC cDNA and a 3′ primer with the stop codon followed by another unique restriction site for subsequent ligation into the expression vector. In the α subunit a stop codon was introduced instead of Pro-646 codon, in the β subunit it replaced the Arg-564 codon, and in the γ subunit the stop codon was introduced at the Lys-571 position. The sequence of each mutation was confirmed by DNA sequencing. Complementary RNAs (cRNAs) of each α, β, and γ subunits were synthesized *in vitro*, and an equal quantity of each subunit cRNA (5–8 ng of total cRNA) was injected into stage-V to -VI oocytes of *X. laevis*.

Electrophysiological measurements were taken 24–36 hr after injection. Whole-oocyte macroscopic currents were measured by using the two-electrode voltage clamp in 115 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes·NaOH, pH 7.2. The amiloride-sensitive current was measured in oocytes clamped at −100 mV as the difference between the current measured without and with 5 μM amiloride in the bath.

Single-channel recordings were done by using the patch-clamp technique in the cell-attached configuration at 20–22°C (7). The pipette solution contained 110 mM NaCl or 110 mM LiCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes, pH 7.2. Bath solution was identical to pipette solution, except when KCl replaced NaCl to clamp the oocyte at 0 mV. Data were sampled at 2 kHz and filtered at 100 Hz for analysis. Construction of current–voltage curves for the open state was done with computer-aided measurements of peak current from amplitude histograms containing at least 50 current transitions. The open-state probability (P_o) was determined from 5- to 60-min segments of digitized single-channel data, using a 50% threshold criterion to distinguish open from closed states. Channel kinetics of amiloride block was analyzed from dwell-time histograms compiled from 2- to 50-min channel records.

Results are reported as mean values ± SEMs and represent the mean of n independent experiments in which average Na^+ current (I_{Na}) was obtained from 8–10 oocytes originating from the same frog. Comparisons of the effects of C-terminal truncations in α, β, or γ subunits (α_T, β_T, γ_T) were obtained from two different sets of experiments: in one protocol, oocytes within the same batch (same frog) were injected with either wild-type (WT) $\alpha\beta\gamma$, $\alpha_T\beta\gamma$, $\alpha\beta_T\gamma$, or $\alpha_T\beta_T\gamma$, and average I_{Na} was measured from 8–10 oocytes in each of the different conditions. In the second protocol, average I_{Na} was measured in 8–10 oocytes injected with (WT) $\alpha\beta\gamma$, $\alpha\beta\gamma_T$, $\alpha\beta_T\gamma$, or $\alpha\beta_T\gamma_T$ cRNA. Statistical significance was determined by use of a paired t test.

RESULTS

We have investigated the functional consequences of a mutation causing Liddle disease that introduces a premature stop codon at the Arg-564 codon of the β subunit of the rENaC (rat homologue). Arg-564 is located 10 residues after the putative second transmembrane α-helix (M2 segment), and the stop-564 mutation results in a deletion of 75 amino acids from the C terminus of the β subunit.

Effects of the β564 Deletion in β Subunit on Whole-Oocyte Macroscopic I_{Na}. The WT rENaC and β564-truncation mutant were expressed in *X. laevis* oocytes by injection of increased amounts of cRNA encoding the three α, β, and γ subunits. Fig. 1*A* shows that I_{Na} measured in oocytes expressing the WT channel was not dependent on total cRNA injected for concentrations from 5 to 25 ng, consistent with saturation concentrations of cRNA for channel expression. For each condition tested, the β564 mutant induced a significantly higher level of I_{Na}. To verify that the β564 mutant protein was made correctly and in a quantity similar to that of the WT protein, we metabolically ([^{35}S]methionine) labeled oocytes injected with either $\alpha\beta\gamma$ cRNA or $\alpha\beta_T\gamma$ cRNA, according to reported procedures (14). Oocyte membranes were immunoprecipitated (14) by a rabbit polyclonal antibody directed against the ectodomain of the rat β subunit. SDS/PAGE analysis revealed that the WT β subunit migrated as a single band with an apparent molecular mass of ≈92 kDa, whereas the β564 mutant protein migrated as a single band with an apparent molecular mass of ≈85 kDa, as expected from the truncation of the last 73 amino acids (data not shown). There was no change in the total protein of either WT or mutant subunits. Fig. 1*B* shows amiloride-sensitive currents (I_{Na}) in oocytes expressing either WT or mutant heterotrimeric $\alpha\beta\gamma$ or heterodimeric $\alpha\beta$ channels. For both $\alpha\beta\gamma$ and $\alpha\beta$ channels, the β564 truncation resulted in a highly significant increase in I_{Na}. As compared with channels constituted by the three WT subunits, the mean increase in I_{Na} due to the β564 truncation was 2.96 ± 0.7-fold. In oocytes expressing the $\alpha\beta$ subunits, the level of amiloride-sensitive current was 20-fold lower compared with that observed by coexpression of $\alpha\beta\gamma$ as shown previously (7), but the β564 truncation in the β subunit caused an average increase in I_{Na} of 4.51 ± 0.73-fold. The channel composed only of β subunit was nonfunctional, and the β564 mutant did not induce any detectable amiloride-sensitive current. These experiments show that the mutation causing Liddle disease consistently increased Na^+ channel activity.

The C terminus of the β subunit appears to be an important structural determinant regulating Na^+ channel activity. We

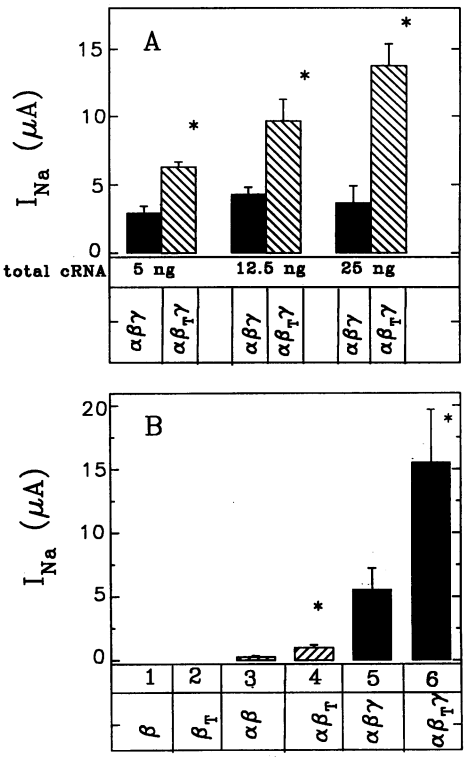

FIG. 1. Effects of C-terminus truncation (β564 mutant) of the β subunit of rENaC on macroscopic amiloride-sensitive I_{Na} expressed in *X. laevis* oocytes. (*A*) I_{Na} measured in oocytes injected with 5–25 ng of total cRNA ($\alpha\beta\gamma$ subunits) of WT rENaC or β564 truncation mutant ($\alpha_T\beta\gamma$). *, $P < 0.01$ for mutant over control WT values (n = 4–6 experiments). (*B*) Mean I_{Na} obtained with injection of WT β (bar 1) or β564 truncation (β_T) subunits alone (bar 2) (n = 2 experiments), together with α subunit (bar 4) (n = 4 experiments) or $\alpha\gamma$ subunits (bar 6) (n = 9 experiments). WT $\alpha\beta$ (bar 3) and $\alpha\beta\gamma$ (bar 5) are shown as controls. *, $P < 0.01$ for bar 3 vs. 4 and bar 5 vs. 6.

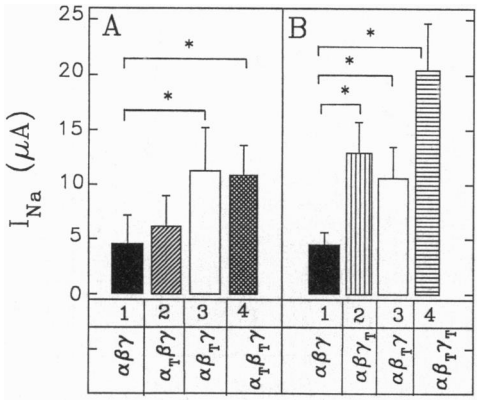

FIG. 2. Comparative effects of C-terminus truncations in $\alpha\beta\gamma$ subunits of rENaC on the whole-oocyte I_{Na}. One set of experiments (*A*) compares mean I_{Na} (n = 4 experiments) expressed by four different channels: WT $\alpha\beta\gamma$ (bar 1), single-truncation mutants $\alpha_T\beta\gamma$ (bar 2) and $\alpha\beta_T\gamma$ (bar 3), the double-truncation mutant $\alpha_T\beta_T\gamma$ (bar 4). The other set of experiments (*B*) compares mean I_{Na} in nine experiments from oocytes injected with WT $\alpha\beta\gamma$ (bar 1), single-truncation mutants $\alpha\beta_T\gamma$ (bar 2) and $\alpha\beta\gamma_T$ (bar 3), the double-mutant $\alpha\beta_T\gamma_T$ (bar 4). *, Statistical significance ($P < 0.01$) over values obtained with WT $\alpha\beta\gamma$ using a paired t test.

have investigated whether the regulatory function of the C terminus is specific for the β subunit by measuring the effects of corresponding truncations of the α and γ subunits on I_{Na}. These C-terminal truncations at Pro-646 in the α subunit, 36 residues after the M2 segment, and at Lys-571 in the γ subunit, located seven residues downstream from the putative M2 segment, delete the most conserved sequences of the C termini. Fig. 2 illustrates the comparative effects of the α646, β564, and γ571 truncations on the macroscopic I_{Na}. In Fig. 2*A*, the effect of the α646 truncation was compared with WT channel and to the truncation mutant of the β subunit of the heterotrimeric $\alpha\beta\gamma$ rENaC. The α-subunit truncation (bar 2) resulted in a slight (but significant) increase in expressed I_{Na} over control (bar 1) (mutant versus WT: 1.6 ± 0.2-fold increase; $P < 0.05$). The corresponding mutation in the β subunit (bar 3) resulted in a larger and highly significant increase in current over both WT (3.1 ± 0.7-fold increase; $P < 0.01$) and $\alpha_T\beta\gamma$ (bar 2) (2.1 ± 0.2-fold increase; $P < 0.02$). I_{Na} of the double α646/β564 mutant (bar 4) and the single β564 truncation (bar 3) were identical. These experiments indicate that the truncations of the α and β subunits are not functionally equivalent with respect to the magnitude of current expressed. The second set of experiments shown in Fig. 2*B* illustrates the effects of the C-terminal truncations in β and γ of the heterotrimeric rENaC. In contrast to results obtained with the α subunit, the γ571 (bar 2) and the β564 (bar 3) truncations resulted in quantitatively similar increases over WT (bar 1) in I_{Na} (2.4 ± 0.6- and 2.5 ± 0.4-fold increase; $P <$ 0.01, respectively). Furthermore, the effects of the double γ571/β564 (bar 4) mutations were additive and resulted in a further increase in I_{Na} compared with the β564 truncation alone (bar 3) (2.1 ± 0.4-fold increase; $P < 0.01$) or to the γ571 truncation alone (bar 2) (1.8 ± 0.4-fold increase; $P < 0.01$). These experiments show that the C termini of the $\alpha\beta\gamma$ subunits are not essential for channel function because the truncations always resulted in expressed functional channels. However, the C terminus in the β and γ subunits plays an important function in regulating channel activity and differs from the α subunit in this respect.

Single-Channel Analysis of Liddle Mutation. The total macroscopic amiloride-sensitive I_{Na} expressed in *X. laevis* oocytes depends on the number of active channels expressed (N), the channel unitary conductance, and the channel open probability (P_o). We have analyzed at the single-channel level the effects of β564 truncation on each parameter that determines I_{Na}.

Fig. 3 shows unitary current tracings obtained in the cell-attached mode in oocytes expressing the rENaC $\alpha\beta\gamma$ WT complex and the $\alpha\beta_T\gamma$ mutant. Both recordings show current fluctuations between multiple current levels, three for the WT and seven for the $\alpha\beta_T\gamma$ mutant, indicative of the presence of several channels in the patch. The dwell times in the different current levels are on the order of seconds in duration. The single-channel conductance was determined from plots of single-channel current versus transmembrane potential in the presence of Na^+ or Li^+ in the pipette. For both the WT channel (Fig. 4*A*) and the $\alpha\beta_T\gamma$ mutant (Fig. 4*B*), the single-channel current was higher with Li^+ as the charge carrier. The slope conductance determined for membrane potentials between −50 and −110 mV was 4.6 ± 0.4 pS and 5.2 ± 0.6 pS with Na^+ and 7.7 ± 0.5 pS and 8.7 ± 0.5 pS with Li^+ for the WT and the $\alpha\beta_T\gamma$ mutant, respectively. The *I–V* data points could be well fitted with a Goldman–Hodgkin–Katz equation; the extrapolated reversal potential greater than +50 mV indicates a high Na^+/K^+ selectivity for both channel complexes. Thus neither the single-channel conductance nor the channel selectivity $Li^+ > Na^+ >> K^+$ was affected by the C-terminal truncation of the β subunit.

Because the single-channel conductance is not altered by the β564 truncation, the density of active channels at the membrane surface (N) and/or the open probability of the channel (P_o) are the main parameters that could be affected by truncation of the β subunit in Liddle disease. When membrane patches show channel activity, they usually contain >1 channel per patch (see Fig. 3), and the product $N*P_o$ represents the mean number of channels open in the patch; it is a convenient way of representing the overall Na^+ channel activity in the membrane patch. The number of channels per patch (N) is estimated by the number of current levels detected during the recording, but clearly N may be underestimated because more channels could be present in the patch than detected by the number of discrete current levels. Fig. 5 compares the mean apparent number of channels open in patches performed on

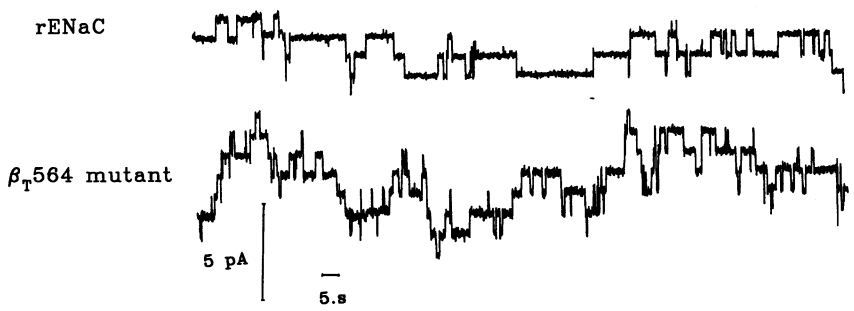

FIG. 3. Current tracings in cell-attached patches on oocytes expressing WT $\alpha\beta\gamma$ subunits of rENaC (upper tracing) and the rENaC mutant with a premature stop codon at amino acid 564 in the C terminus of the β subunit (lower tracing). Upward deflections indicate channel openings. Unitary currents were recorded at −100 mV.

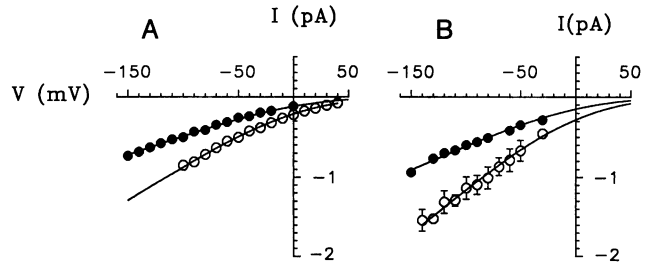

FIG. 4. Current–voltage (*I–V*) relationship of unitary currents recorded from cell-attached patches in *X. laevis* oocytes expressing WT rENaC and the β564 truncation mutant. (*A*) *I–V* curves for WT rENaC in the presence of Na^+ (100 mM, ●) or Li^+ (100 mM, ○) in the pipette. Solid lines represents best fits to the constant field equation. Single-channel conductance calculated from regression analysis between −50 and −110 mV was 4.6 ± 0.4 pS for Na^+ (n = 8) and 7.7 ± 0.5 pS for Li^+ (n = 6). (*B*) *I–V* curves for the β-truncation mutant show single-channel conductance of 5.2 ± 0.6 pS with Na^+ (n = 6) and 8.7 ± 0.5 pS with Li^+ (n = 7). Data points were fitted as for *A*.

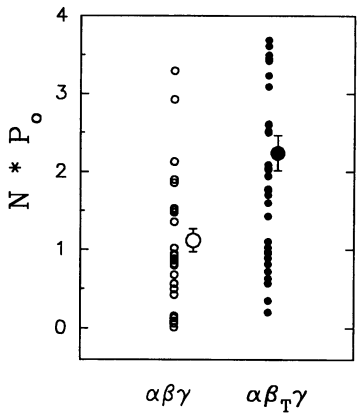

FIG. 5. Comparison of the mean number of channels open ($N*P_o$) in cell-attached patches done on oocytes expressing wild-type αβγ rENaC (○) and the $\alpha\beta_T\gamma$ rENaC channel with the Arg-564 truncation in the β subunit (●). Mean $N*P_o$ was 1.12 ± 0.15 for αβγ and 2.24 ± 0.22 for the $\alpha\beta_T\gamma$ truncation mutant (P = 0.002). The number of channels in the patch (N) was 2.22 ± 0.29 and 3.97 ± 0.43 for the WT and the β-truncation mutant, respectively (P = 0.002). The mean open probability per individual channel P_o ($P_o = N*P_o/N$) was 0.483 ± 0.054 and 0.590 ± 0.053, respectively ($P > 0.05$).

oocytes expressing either WT rENaC or the $\alpha\beta_T\gamma$ mutant. In recordings lasting >5 min (to reduce the probability of underestimating N), $N*P_o$ was 2-fold higher in patches containing the β564 mutant (2.24 ± 0.22) than the WT rENaC (1.12 ± 0.15). The higher number of active channels ($N*P_o$) found in patches with the β564 truncation mutant is consistent with the increased magnitude of the macroscopic current seen in oocytes. Assuming that the channels open independently of one another, the open probability P_o of a single channel can be calculated by dividing $N*P_o$ by N, the number of channels in the patch. Higher, but not statistically significant, P_o values were observed for individual mutant channels (P_o = 0.482 ± 0.054 for the WT and 0.590 ± 0.053 for β564 mutants; $P >$ 0.05), implying that a higher number of channels per patch, as determined by the number of current levels, is mainly responsible for the increase in $N*P_o$ measured for the β564 truncation mutant. The β564 mutant shows an ≈2-fold higher value for N (3.97 ± 0.42) compared with the WT (2.22 ± 0.29, $P <$ 0.005).

Finally, we investigated the effects of C-terminal truncations on the effects of external channel block by amiloride at the single-channel level. Channel kinetics were analyzed at +100 mV (pipette potential) with 400 nM amiloride in the pipette. With amiloride, the overall channel activity was higher for the β564 truncation mutant ($N*P_o$ = 1.17 ± 0.23; N = 6 ± 1, n = 3) than for the WT channel complex ($N*P_o$ = 0.24 ± 0.11; N = 2.3 ± 0.8, n = 3), consistent with our previous observation of a higher number of active channels per patch for the β564-truncation mutant. From the mean open- and blocked-time duration with amiloride, we estimated ON rate constants (k_{on}) of 35.3 ± 3 and 61 ± 9 $s^{-1}\cdot\mu M^{-1}$ and OFF rate constants (k_{off}) of 1.28 ± 0.3 and 2.5 ± 0.4 s^{-1} for the WT and the β564 truncation mutant, respectively. These values indicate that the dissociation constant ($K_d = k_{off}/k_{on}$) for amiloride of both the WT and the β564-truncation mutant is the same as the value reported for the native channel (Table 1; refs. 1–3 and 21) and that the C-terminus deletion did not affect channel affinity for amiloride.

DISCUSSION

Pseudoaldosteronism and Liddle Disease. We have analyzed the functional consequences of a truncation mutant of the β subunit of ENaC, which causes heritable hypertension in the original kindred described by Liddle *et al.* (4, 17). Our study shows that the β564 mutation causing Liddle disease in this family results in an increased Na^+ channel activity when the mutated gene product is expressed in *X. laevis* oocytes, as evidenced by an increased macroscopic I_{Na} (Figs. 1 and 2) and a higher number of open channels per patch (Fig. 3 and Table 1). The β564 truncation mutant deletes the major part of the cytoplasmic end of the β subunit but does not affect the biophysical or the pharmacological properties of the channel (Table 1), indicating that this region is not critically involved in the pore-forming region of the channel molecule and is not structurally related to the amiloride-binding domain of the channel. Thus, our study provides direct physiological evidence that abnormal regulation of ENaC activity represents the underlying mechanism for a form of genetically determined salt-sensitive hypertension and supports the early postulate made by Liddle that the syndrome was due to a "disorder in which renal tubules reabsorb ions with an abnormal facility despite suppression of aldosterone secretion" (4). This physiologic correlate of the β564 truncation can explain the observed pathophysiology in the original kindred described by Liddle *et al.* (4, 5); this form of pseudoaldosteronism can now be referred to as Liddle disease.

Possible Molecular Mechanisms Determining Channel Hyperactivity. With only the very recent identification of the primary structure of the subunits of the ENaC, little is known about the important structural domains that govern overall channel function. This study of the functional consequences of Liddle's mutation enabled us to define the C terminus of the β subunit as an important cytoplasmic structure that regulates

Table 1. Single-channel characteristics of native, cloned, and mutant ENaCs

ENaC	g, pS	P_{Na}/P_{Li}	K_d (amiloride), nM	P_o	N	$N*P_o$
Rat CCD*	4.9 ± 0.2	0.6	70	0.47 ± 0.03	2.3 ± 0.2	1.08 ± 0.15
αβγ WT	4.6 ± 0.4	0.6	36 ± 8	0.48 ± 0.05	2.22 ± 0.29	1.12 ± 0.15
β564 mutant	5.2 ± 0.6	0.6	42 ± 6	0.59 ± 0.05	3.97 ± 0.42	2.24 ± 0.22

The data from cortical collecting ducts (CCD) are from low-salt diet-fed rats—i.e., aldosterone-stimulated. No channel activity is seen in rats on a normal diet. P_{Na}/P_{Li}, ionic permeability ratio.
*CCD data were from Palmer and Frindt (18) and from Table 1 in Pacha *et al.* (19).

channel activity. The molecular mechanism(s) underlying the regulatory function of the C terminus of the β subunit cannot be established from our experimental data. Two hypotheses can be proposed. (*i*) This structural domain could be involved in regulating the expression of channel complexes at the membrane surface, by affecting their distribution between an intracytoplasmic pool and a membrane pool in favor of the plasma membrane. (*ii*) Alternatively, the density of channels at the membrane surface may remain unchanged, but the mutant channel may be lacking an inactivation mechanism that normally would result in long-term deactivation of the channel. Clearly, the elucidation of the molecular mechanisms involved in channel regulation may take on a critical importance in our understanding of the pathogenesis of more common forms of salt-sensitive hypertension.

Several mutations have been identified in Liddle syndrome that introduce stop codons or frameshifts at different positions in the C terminus of the β subunit. The molecular target for the abnormal channel regulation in Liddle syndrome has not yet been identified, but all mutations linked to this syndrome result in the deletion of potentially important structural domains, such as a conserved proline-rich sequence, and a protein kinase C (PKC) phosphorylation site that is common to the human and the rENaC homologues. Proline-rich sequences are found in α, β, and γ subunits of ENaC and resemble the Src homology 3 binding motifs found in protein involved in cell growth signal transduction (20). Rotin *et al.* (20) have recently shown that this Src homology 3 region of α rENaC binds to α-spectrin *in vitro* and that this cytoskeletal interaction may stabilize the channel protein within the membrane. Deletion of a Src homology 3 binding motif in the β subunit could be involved in the increased channel activity found in the oocyte plasma membrane for the β564 mutant. However, the small effect of C-terminal truncation of the α subunit compared with β subunit suggests that an interaction with α-spectrin is not related to the abnormal Na^+ channel regulation that has been linked to truncations of the β subunit. Alternatively, the proline-rich region of the β subunit (or the γ subunit) may be involved in binding interactions with other unknown proteins that modulate Na^+ channel activity.

In the close vicinity of the proline-rich region, the β and γ subunits share a conserved PKC phosphorylation site, which is absent in the α subunit. We showed that C-terminal truncations of the β and γ subunits were functionally equivalent and resulted in a quantitatively similar increase in Na^+ channel activity and that a double truncation of β and γ subunits was even more efficient in enhancing Na^+ channel activity than either truncation alone. The ENaC activity is regulated by hormones such as aldosterone or vasopressin. Interestingly, the β-mutation of Liddle disease mimics the physiological effect of aldosterone *in vivo* in the rat cortical collecting duct (19). Transduction of hormonal signaling involves intracellular second-messenger cascades such as protein kinase A/cAMP, G proteins, or calcium (for review see ref. 21). By contrast, prostaglandin E_2 applied at the basolateral membrane of cortical collecting ducts decreases apical Na^+ channel activity. Evidence that Ca^{2+}-dependent PKC mediates the prostaglandin E_2 regulation of ENaC derives from the observations that PKC antagonists inhibit prostaglandin E_2 effects and that PKC agonists such as phorbol 12-myristate 13-acetate or 1-oleoyl-2-acetylglycerol inhibit ENaC activity (12). Deletion of the putative PKC phosphorylation sites in the C terminus of β and γ subunits could constitutively activate ENaC and would explain the additive effects of the double truncation of β and γ subunits on Na^+ channel activity. Further studies using site-specific mutations will be needed to determine whether these PKC phosphorylation sites represent the molecular target for the abnormal Na^+ channel regulation observed in Liddle disease.

Possible Implications for the Genesis of Salt-Sensitive Forms of Hypertension. Because the corresponding truncation of the γ subunit is functionally equivalent to the β564 truncation in increasing Na^+ channel activity, it would not be surprising if mutations in the C terminus of the γ subunit may also be linked to other genetically determined forms of salt-sensitive hypertension. Combining genetic approaches to identify such mutations contributing to development of hypertension with functional investigations of these mutant channels in the *X. laevis* expression system will provide detailed information regarding the structure–function relationships of ENaC and will help define some of the basic molecular features underlying various forms of salt-sensitive hypertension.

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