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Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome

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Liddle syndrome is an autosomal dominant form of hypertension, resulting from mutations in the cytoplasmic C-terminus of either the β or γ subunits of the amiloride-sensitive epithelial Na channel (ENaC) which lead to constitutively increased channel activity. Most mutations reported to date result in the elimination of 45–75 normal amino acids from these segments, leaving open the question of the identity of the precise amino acids in which mutation can lead to an enhanced channel activity. To address this question, we have performed a systematic mutagenesis study of the C-termini of the α, β and γ ENaC subunits of the rat channel and have analyzed their function by expression in *Xenopus* oocytes. The results demonstrate that a short proline-rich segment present in the cytoplasmic C-terminus of each subunit is required for the normal regulation of channel activity. Missense mutations altering a consensus PPPXY sequence of the α, β or γ subunits reproduced the increase in channel activity found in mutants in which the entire cytoplasmic C-termini are deleted. This proline-rich sequence, referred to as the PY motif, is known to be a site of binding by proteins bearing a WW domain. These findings show that the three PY motifs in the C-termini of ENaC are involved in the regulation of channel activity, probably via protein–protein interactions. This new regulatory mechanism of channel function is critical for the maintenance of normal Na reabsorption in the kidney and of Na^+ balance and blood pressure.

Keywords: amiloride/ENaC/hypertension/proline-rich domain/protein–protein interactions

Introduction

The amiloride-sensitive epithelial Na channel (ENaC) is a highly selective Na channel found in the apical membrane of epithelial cells from different tissues including the distal nephron, the colon, exocrine gland ducts, the lung and the skin. In these polarized epithelial cells, sodium entry into the cell from the apical surface via ENaCs represents the rate-limiting step for the vectorial electrogenic movement of sodium. In the distal nephron, ENaC activity is regulated by aldosterone and vasopressin, serving to maintain sodium balance, extracellular volume and blood pressure (Rossier *et al.*, 1994).

The primary structure of the rat ENaC has been elucidated by cloning of the channel subunits by functional expression (Canessa *et al.*, 1994b). The functional channel is a hetero-oligomeric protein composed of at least three subunits, α, β and γ, which are of similar structure, sharing 35% identity in their amino acid sequences. The biophysical properties and pharmacological profile of ENaC expressed in *Xenopus* oocytes are similar to those of the native channel in the distal nephron (Palmer and Frindt, 1986; Canessa *et al.*, 1994b).

A role for ENaC in the maintenance of blood pressure in humans has been demonstrated by elucidation of the pathogenesis of Liddle syndrome, an inherited form of salt-sensitive hypertension. Mutations that lead to a loss of the last 45–75 amino acids of the C-terminus of the β subunit of ENaC cause this disease (Shimkets *et al.*, 1994). Coexpression in *Xenopus* oocytes of a truncated β subunit with the normal α and γ subunits of the rat ENaC gene resulted in a marked increase in the amiloride-sensitive current compared with the current resulting from expression of wild-type ENaC subunits, consistent with a gain-of-function mutation causing increased renal sodium reabsorption *in vivo* (Schild *et al.*, 1995). These expression studies also provided evidence that deletion at the corresponding position in the C-terminus of γENaC induced a similar increase in channel activity, while truncated αENaC had only a small effect on channel activity. The subsequent investigation of an additional Liddle syndrome kindred identified a mutation in γENaC which introduced a premature stop codon that deleted the last 76 amino acids of the C-terminus of this protein (Hansson *et al.*, 1995a), further confirming the relevance of the *Xenopus* expression system to *in vivo* ENaC function.

These truncating mutations leave open the question of the identity of the critical amino acid sequences in the C-termini of ENaC subunits in which mutations result in channel activation. Potential targets for these mutations include proline-rich regions or protein kinase C (PKC) phosphorylation sites. To address this question, we systematically mutagenized the C-terminus of the β subunit of rat ENaC and measured the effect of these mutations on channel activity. The results demonstrate that mutations in a short proline-rich segment PPPXY (called the PY motif) induce an increase in channel activity. This PY motif is conserved among the three ENaC subunits, and mutations within this sequence in αENaC and γENaC result in a similar gain of function. These results provide evidence that the PY motif in the three ENaC subunits is the target sequence for mutations causing Liddle syndrome, and provide a new understanding of the possible mechanisms regulating ENaC channel activity.

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            610                                                  650
α-rENaC:..MLLRRFRSRYWSPGRGARGAREVASTPASSFPSRFCPHPTSPPP
            555       564                              590
β-rENaC:..ASCKGLRRRRPQRPYTGPPPTVAELVEAHTNFGFQPDTTSCRPN
            565       574                              600
γ-rENaC:..RQWHKAKDCWARRQTPPSTETPSSRQGQDNPALDTDDDLPTFTS


            654                  671                          739
α-rENaC:..SLPQQGMTPPLALTAPPPAYATLGPSAPPLDSAAPDCSACALAAL
            599         610      616                  638
β-rENaC:..AEVYPDQQTLPIPGTPPPNYDSLRLQPLDTMESDSEVEAI
            609         620      626                  650
γ-rENaC:..AMRLPPAPGSTVPGTPPPRYNTLRLDRAFSSQLTDTQLTNEL
WBP-1   :..............PGTPPPPYTVGIG.............
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Fig. 1. Primary sequence of the C-terminus of α, β and γ subunits of rENaC, and sequence comparison between the distal proline-rich region of the α, β and γ subunits of rENaC (P2 domain) and the WBP-1 protein (Chen and Sudol, 1995). Underlined residues indicate nonsense mutations reported to cause Liddle syndrome (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a).

Results

The Xenopus oocyte expression system as a model to study gain-of-function mutations

The amino acid sequences of the cytoplasmic C-termini of α, β and γ ENaC subunits are shown in Figure 1. When expressed in *Xenopus laevis* oocytes, C-terminus deletion mutants of either β or γ ENaC subunits result in a marked increase in the macroscopic amiloride-sensitive Na^+ current (Schild *et al.*, 1995). This effect is illustrated in Figure 2, where the amiloride-sensitive current in oocytes expressing either ENaC wild type (Figure 2A) or a ENaC mutant with a C-terminus deletion at codon R564 (Figure 2B) of the β subunit is shown. Comparison of these current–voltage relationships shows that at every membrane potential tested, the amiloride-sensitive Na^+ and Li^+ currents were larger in oocytes expressing βR564-stop mutant than the rENaC wild type. A unique functional characteristic of ENaC is its higher permeability for Li^+ compared with Na^+. The Li^+:Na^+ permeability ratio of the macroscopic amiloride-sensitive current is >1, and remains unchanged in oocytes expressing wild-type ENaC and βR564 mutant (Figure 2A and B), indicating that the amiloride-sensitive current reflects ENaC channel activity at the membrane surface. The higher Na^+ and Li^+ currents expressed by the βR564-stop mutant are a good indication of an increase in channel activity at the plasma membrane. The macroscopic current–voltage behavior of the wild type and βR564-stop mutant is consistent with the single-channel recordings reported previously, showing that the βR564-stop deletion changes neither the biophysical properties of the channel, e.g. channel conductance or ionic selectivity, nor the affinity for amiloride; rather it increases the number of active channels in the plasma membrane and the channel's open probability (Schild *et al.*, 1995). Thus the macroscopic amiloride-sensitive current represents a reliable functional assay for the identification of sequences within the C-termini of ENaC subunits that are involved in the regulation of channel activity.

The larger amiloride-sensitive current expressed by the βR564-stop mutant is consistently associated with a significant depolarization of the resting membrane potential. Oocytes expressing wild-type ENaC or βR546-stop mutant show significant differences in their resting membrane potential, –0.70 ± 1.50 (n = 54) and +9.43 ± 1.10 mV (n = 54; mean ± SE) for the wild-type ENaC and βR564-stop mutant respectively ($P < 0.001$). This 10 mV difference in membrane potential indicates that the βR564 deletion mutation, which induces channel gain of function, leads to an influx of Na^+ into the cell that is large enough to make the intracellular potential positive. Because of this Na^+ load, the oocytes expressing the mutant channel gene are unable to extrude enough Na^+ via the Na-K-ATPase, and the electrochemical gradient for Na^+ between the intracellular and extracellular milieu tends to dissipate. This can be observed in Figure 2 by the depolarizing shift in the reversal potential of the amiloride-sensitive Na current in oocytes expressing the βR564 deletion mutant.

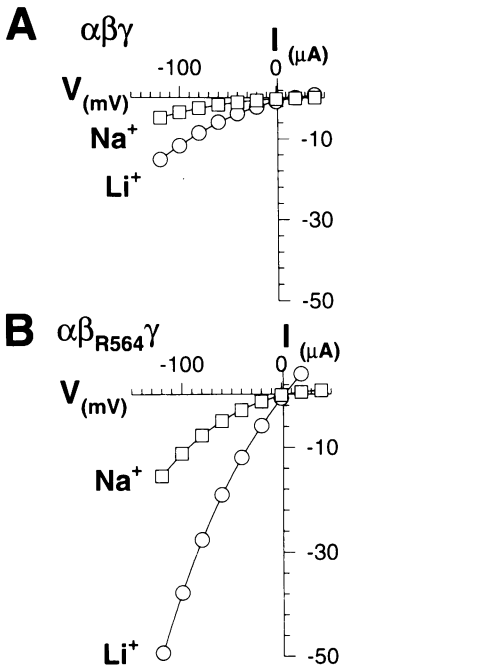

Fig. 2. Representative example of the current–voltage relationship of the amiloride-sensitive Na^+ and Li^+ currents expressed by oocytes injected with (**A**) wild-type rENaC and (**B**) the rENaC βR564 deletion mutant. Fits to the experimental data were obtained using a second-order regression analysis. Predicted reversal potentials were: $V_{rev}Na^+$ = +21 mV, $V_{rev}Li^+$ = +24 mV for wild-type rENaC (A); and $V_{rev}Na^+$ = +4 mV, $V_{rev}Li^+$ = +10 mV for the rENaC βR564 mutant (B).

Thus the oocyte represents a useful one-cell model for the investigation of the pathophysiological basis of hypertension in Liddle syndrome. We have used this cell model to identify the target sequence in the C-terminus of ENaC subunits which when mutated is responsible for the constitutive activation of the channel observed in patients affected by Liddle syndrome.

Effects of C-terminus deletions in ENaC subunits on channel activity

Important functional domains for the regulation of channel activity were first identified by sequential deletions along the C-termini of β and γ subunits and measurements of the expressed amiloride-sensitive current at a holding potential of –100 mV. Figure 3A shows the effects on channel activity of deletions in the β subunit which were performed downstream of the R564 codon. Sequential

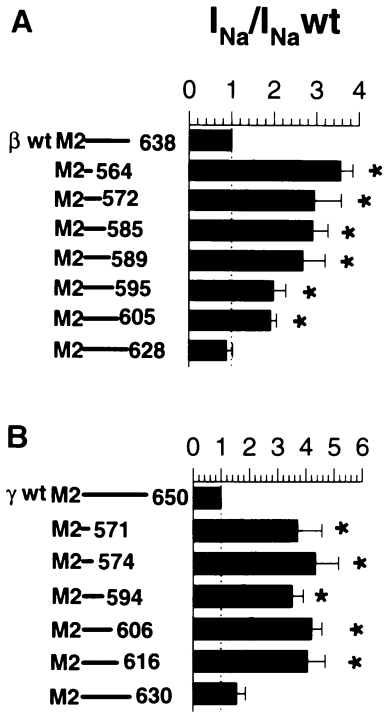

Fig. 3. Effects of C-terminal deletions in the βENaC and γENaC subunits on amiloride-sensitive Na^+ current (I_{Na}). (**A**) RNAs encoding wild-type βENaC and mutant subunits were coinjected with wild-type αENaC and wild-type γENaC. Schematic localizations of the deletion mutations in the C-terminus with respect to the second transmembrane domain M2 are represented on the left-hand side of the figure. Numbers define the codon at which the encoded protein is truncated. The corresponding bars indicate the I_{Na} normalized for wild-type ENaC values; I_{Na} was measured at the holding potential −100 mV; macroscopic I_{Na} for wild-type ENaC was 3.15 ± 0.55 μA (n = 8). (*) Statistical significance $P < 0.05$. (**B**) Wild-type γENaC and mutants were coinjected with wild-type αENaC and wild-type βENaC. The left-hand side of the figure represents the position of the deletion with respect to the M2 transmembrane segment, and the codon number in the γENaC sequence. Corresponding bars show I_{Na} normalized for values of wild-type ENaC. I_{Na} was recorded at −100 mV, and macroscopic I_{Na} for wild-type ENaC was 4.08 ± 0.42 μA (n = 8). (*) Statistical significance $P < 0.05$.

deletions down to the Q605 codon of the β subunit resulted in a significant 2.0- to 3.5-fold increase in the amiloride-sensitive Na^+ current, whereas deletion of the last 10 residues starting at the T628 codon did not induce a significant increase in channel activity. A gradual attenuation of the stimulatory effect on channel activity was observed as the deletions were performed along the C-terminal end, and deletion at codon Q605 reproduced only 35% of the increase in I_{Na} induced by the most proximal deletion at R564. This relationship between the size of the deleted fragment and the increase in I_{Na} suggests that the length of the C-terminus is a factor in determining the importance of the functional alteration of the channel activity. In addition, the significant gain of function obtained with the Q605 but not the T628 deletion suggests the presence of a functional domain located between these two residues which regulates channel activity.

As for the β subunit, deletions in the C-terminus of the γ subunit have been shown to induce significant channel activation (Schild *et al.*, 1995). More recently, such a deletion mutation has been identified in a kindred affected by Liddle syndrome (Hansson *et al.*, 1995a). We have investigated how sequential deletions in the C-terminus of the γ subunit compare with those data obtained in the β subunit. The functional consequences of deletion mutations performed along the C-terminus of the γ subunit are shown in Figure 3B. Changes in normalized I_{Na} induced by these deletions indicate that channel activation is consistently reproduced by deletions of the C-terminus from codons K571 to P616. Deletion of the last 21 residues, including a putative PKC phosphorylation site (see Figure 1), did not induce a significant increase in I_{Na}. These experiments clearly identify a target site for the stimulation of channel activity located between amino acids P616 and T630 in the γ subunit. This functional domain includes a proline-rich region in γENaC, a sequence that is conserved not only between the β and γ subunits but also in the α subunit (Figure 1).

Determination of target sequences by triple-alanine scanning or single-alanine substitutions of the β subunit

The above deletion experiments are consistent with a target between amino acids P616 and T630 in the γ subunit. However, it is not clear whether there is one or more targets in the β subunit. Deletions in this latter subunit suggest a significant effect on the deletion of amino acids Q605–T628, and perhaps further increases in activity with deletions from amino acids Q589–C595. To further characterize sequences in the C-terminus of the β subunit responsible for the activation of channel activity, we substituted three alanine residues for groups of three normal amino acids from these potential target regions and measured the resulting current in oocytes expressing ENaC and containing these mutant subunits. The effects of these mutations, expressed as the ratio of mutant to wild-type amiloride-sensitive Na^+ current (I_{Na}mutant/I_{Na}wt), are shown in Figure 4A, with the βR564 deletion mutant used as a positive control for activated channel activity. This alanine scanning of the C-terminus of the β subunit shows that only two such constructs have significant stimulatory effects on ENaC activity, i.e. those that substitute alanine for normal amino acids in the interval from codons 613 to 619 (this segment contains the proline-rich motif PPPXY which is conserved among all three ENaC subunits). None of the more proximal substitutions had significant stimulatory effects on I_{Na}, suggesting that the only target for mutation causing Liddle syndrome is the short segment from amino acid 613 to 626 of the C-terminus of the β subunit. The increase in I_{Na} caused by Ala substitutions at positions 613 and 617 was equivalent in magnitude to the effect of the βR564-stop mutation linked to Liddle syndrome, consistent with the notion that the entire effect produced by this proximal deletion can be accounted for by substitutions in this single target. Significantly, the critical segment for activation of the γ subunit identified by deletion experiments includes the PPPXY segment of this subunit.

With evidence pointing to this segment as the target for Liddle syndrome mutations, we next substituted single alanine residues for each of the 11 amino acids from codons I610 to S620. The amiloride-sensitive currents I_{Na} expressed by the mutants were compared with those from wild-type ENaC, with the mutant D591A as a negative

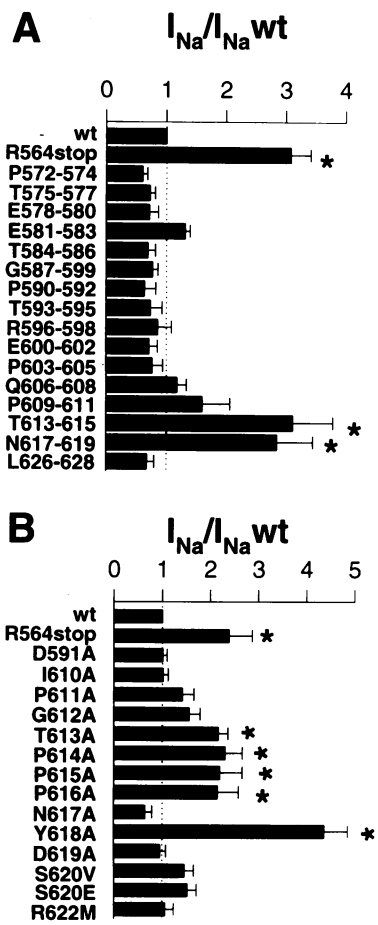

Fig. 4. Effects of amino acid substitutions in the C-terminus of βENaC on the amiloride-sensitive Na^+ current. (**A**) Comparative effects of AAA substitutions in the C-terminal sequence of the β subunit starting at codon 572 on the amiloride-sensitive Na^+ current. Mutants were coinjected with wild-type αENaC and wild-type γENaC. The I_{Na} value for wild-type ENaC was 4.15 ± 0.83 μA (n = 6). (*) Statistical significance $P < 0.05$. (**B**) Comparative effects of single-point mutations in the proline-rich sequence of βENaC. Bold residues denote substitution mutations. I_{Na} measured at −100 mV was normalized for values obtained for wild-type ENaC which averaged 3.50 ± 0.48 μA (n = 6). (*) Statistical significance $P < 0.05$.

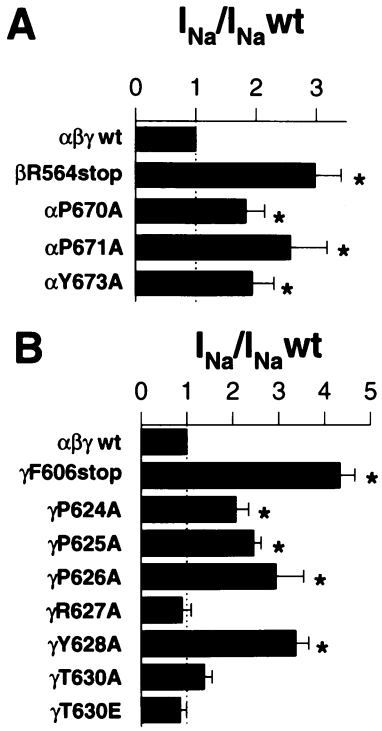

Fig. 5. Effects of mutations within the PY domains of the αENaC (**A**) and γENaC (**B**) subunits. I_{Na} was measured at −100 mM. I_{Na} values for wild-type ENaC were 4.58 ± 0.93 μA (n = 6) in (A) and 4.54 ± 1.13 μA (n = 6) in (B). (*) Statistical significance $P < 0.05$.

control (see data in Figure 4B) and the R564 deletion mutant as a positive control. Five of these mutations, T613A, P614A, P615A, P616A and Y618A, showed a significant increase in the amiloride-sensitive I_{Na}, consistent with increased channel activity; the magnitudes of these increases were comparable with that found for the R564 deletion. Importantly, the mutations N617A and D619A adjacent to Y618 did not affect channel activity, and mutations in the putative PKC phosphorylation site S620V or S620E and R622M had no significant effect on channel activity. Thus this series of missense mutations identifies the amino acid sequence TPPPXY as a site required in some fashion for the negative regulation of channel activity, because a mutation at any of these residues reproduces the ENaC channel activation found with C-terminal deletions.

Evidence for a similar functional PY motif in α and γ subunits of ENaC

The PPPXY sequence (PY motif) identified in the β subunit of ENaC is conserved in the α and γ subunits. This observation motivates an examination of the effects of alanine substitutions at corresponding positions in the α and γ subunits on the activity of ENaC. Alanine substitutions of any of the conserved PPXY residues in the α subunit led to a significant increase in I_{Na}, consistent with channel activation (Figure 5A). Similarly, the substitution of alanine for the conserved P624–P626 or Y628 in the γ subunit also significantly increased I_{Na} (Figure 5B). Some of these latter missense mutations may be less efficient than the deletion of this proline-rich domain in stimulating channel activity. As for the β subunit, missense mutations of R627 located between P626 and Y628, as well as mutations within the adjacent putative PKC site, did not modify channel activity. In summary, these findings indicate that the PPPXY domains in all three subunits are involved in the regulation of channel activity.

We reported previously that the truncation in the αENaC C-terminus (αP646-stop) resulted in a small but significant increase in the expressed I_{Na} over the control (I_{Na} α646/I_{Na} wt = 1.6 ± 0.2-fold, $P < 0.05$; Schild *et al.*, 1995). However, this effect was not additive to the effect of the deletion mutation in βENaC (βR564-stop), in contrast to the potentiation observed when the βR564 mutant was coexpressed with the γENaC deletion mutant at codon K571. At that time we concluded that the C-terminus deletion of αENaC had a minimal effect on the channel activity.

We have re-examined this question here. As shown in Figure 6, we again find a small but significant channel activation when the αP646 deletion mutant is expressed (2.1 ± 0.3-fold increase in I_{Na} over control, $P < 0.05$), which was significantly lower than the increase in I_{Na} obtained with the βR564-stop deletion mutant (3.4 ± 0.2-

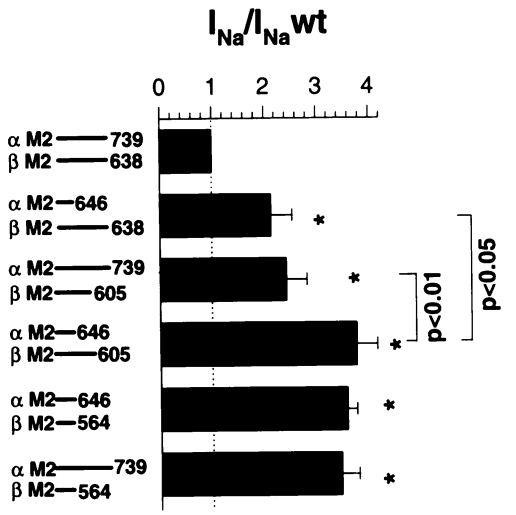

Fig. 6. Effects of combined deletions in the C-termini of the αENaC and βENaC subunits on the amiloride-sensitive Na current. Combinations of wild-type αENaC and βENaC or mutant subunits shown on the left-hand side of the figure were coinjected with wild-type γENaC. Schematic representations of the deletions are shown with their codon number where mutations were performed. I_{Na} was measured at −100 mV and the I_{Na} value for wild-type ENaC was 2.72 ± 0.60 μA. (*) Statistical significance $P < 0.05$.

fold increase in I_{Na} over control, $P < 0.01$). The data obtained in a large number of experiments reveal the following order of efficiency of C-terminal deletions on channel activation: γENaC (5.1 ± 0.6-fold increase in I_{Na}, $n = 29$) > βENaC (3.8 ± 0.3-fold increase in I_{Na}, $n = 52$) > αENaC (2.0 ± 0.8-fold increase in I_{Na}, $n = 12$).

Interestingly, the αP646-stop mutant increased the channel activity to the same level as the more distal βENaC deletion at codon Q605 (2.4 ± 0.4 increase in I_{Na} over control). When the βQ605-stop and αP646-stop mutants were coexpressed, a significant synergism was observed on the amiloride-sensitive current. In contrast, there was no synergism with the coexpression of the αP646 and βR564 deletion mutants, consistent with our previous report (Schild *et al.*, 1995). These data indicate that the C-terminus of αENaC participates in channel regulation, and that the critical domain for this effect comprises the PY motif in the distal end the C-terminus. In addition, the deletion studies are consistent with the notion that the length of the C-terminus of βENaC contributes to the functional integrity of the PY motifs in αENaC.

Discussion

αENaC: a novel candidate gene involved in the genesis of salt-sensitive hypertension

We have shown that the conserved PPPXY motifs in the C-termini of the α, β and γ subunits of the ENaC are required for the normal regulation of channel activity. Mutations altering this sequence in any of these subunits result in a constitutive increase in channel activity, indicating that these domains are involved in the repression of channel activity. The functional relevance of this regulatory domain *in vivo* is indicated by the finding that all patients thus far reported with Liddle syndrome have mutations which either delete this segment from the β or γ subunit (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a) or introduce missense mutations, thus altering this domain (Hansson *et al.*, 1995b; Tamura *et al.*, 1995). Evidence that the PPPXY motif represents the target for mutations causing Liddle syndrome is also provided by the observation that the P616L missense mutation linked to the disease induces channel activation (Hansson *et al.*, 1995b). Mutations in αENaC causing Liddle syndrome have not yet been reported, but it appears from our study that the α subunit of ENaC represents another potential target gene. An analysis of the sequential deletions as well as the alanine substitutions across the C-terminus of the β subunit does not suggest the presence of additional sites that, when mutated, can by themselves lead to an increased channel activity.

The PY motif is critical for channel repression

The ENaC protein is composed of at least three subunits that share ~35% identity among them (Canessa *et al.*, 1994b). Each subunit spans the membrane twice. All three subunits are glycosylated, and the six potential N-linked glycosylation sites, located in the large hydrophilic loop between the two transmembrane domains, are used in intact cells (Canessa *et al.*, 1994a; Renard *et al.*, 1994; Snyder *et al.*, 1994). This glycosylation pattern is consistent with a topological model leaving the N- and C-termini in the cytoplasm. Comparison of the cytoplasmic C-tail of the ENaC subunits shows a low level of identity, with virtually the only conserved portion being the PPPXY motif (Figure 1).

In the *Xenopus* oocyte expression system, we have no evidence that the PKC sites of the β and γ subunits regulate ENaC channel activity. Deletion of this domain cannot account for the effect of Liddle mutations on the channel activity. However, this does not exclude the possibility that in epithelial cells the phosphorylation of PKC plays a role in channel regulation. Patch–clamp studies have shown that PKC agonists inhibit channel activity in a culture cell line and that PKC antagonists inhibit the down-regulation of ENaC induced by prostaglandin E_2 (Ling *et al.*, 1992). These observations suggest that in epithelial cells, in contrast to the oocyte, a putative PKC site could directly or indirectly act as a repressor of channel activity. This potential phosphorylation site has not yet been identified in ENaC subunits.

Proline-rich sequences are often involved in protein–protein interactions, in particular with signal transduction or cytoskeletal proteins containing SH3 domains (Mayer and Baltimore, 1993; Feng *et al.*, 1994). Recently, an *in vitro* binding study has demonstrated that the distal proline-rich domain named P2 in the C-terminus of αENaC is involved in binding to the SH3 domain of α-spectrin. The functional significance of this interaction remains to be determined, in particular because microinjection of the C-terminus of αENaC localized the protein to the apical membrane of polarized epithelial cells (Rotin *et al.*, 1994).

A sequence comparison of the more distal proline-rich regions (P2) of the α, β and γ C-terminal ends is shown on Figure 1 and reveals those motifs potentially involved in channel function. The β and γ subunits share in common a consensus sequence for SH3 binding characterized by the PXXP motif (Feng *et al.*, 1994). A number of signaling proteins contain SH3 domains (reviewed in Cohen *et al.*,

1995). However, a recent study has failed to demonstrate the binding of SH3 domains to βENaC or γENaC (Staub *et al.*, 1996). Moreover, the involvement of the PXXP motifs of β and γ subunits in regulating the channel activity is unlikely because alanine substitution of the first proline residue (P611) of the PXXP motif in the β subunit has no effect on the expressed channel current.

Recently, Bork and Sudol (1995) and André and Springael (1994) have identified a novel domain called the WW (or WWP) domain in the yes-associated protein YAP65, as well as in several unrelated proteins, including dystrophin, FE65, Nedd4 and others. The WW domain contains two strictly conserved tryptophan residues in a 38 amino acid segment, and recent binding studies have shown that the WW domain of YAP binds to the PY motif of two proteins, WBP-1 and WBP-2 (Chen and Sudol, 1995). This PY motif has a consensus binding sequence defined by the site-directed mutagenesis of PPXY. This PY segment matches the sequence identified here as the critical sequence for the regulation of ENaC activity, leading to the inference that the normal negative regulation of ENaC channel activity requires the binding of a WW domain-containing protein to these PPXY segments in the ENaC subunits.

Nedd4 as a candidate for the negative regulation of channel activity

In a companion paper, Staub *et al.* (1996) have identified in a two-hybrid screen of rat lung library the rat homolog of Nedd4 as the binding partner to the PY motifs of ENaC. Rat Nedd4 contains three WW domains, and binding to the channel subunits was mediated by these WW domains (Staub *et al.*, 1996). The ability of α, β and γ ENaC C-termini to bind the Nedd4-WW domain, and the weaker binding interaction for αENaC compared with β and γ (Staub *et al.*, 1996), correlate nicely with the respective efficiencies of deletions in α, β and γ ENaC to increase channel activity. In addition, two single-point mutations in the PY motif of βENaC linked to Liddle syndrome, which abolish Nedd4-WW binding in the two-hybrid binding assay (Staub *et al.*, 1996), are shown here to increase the channel activity of ENaC. Together, these data raise the possibility that Nedd4 is the protein that binds to the ENaC subunit PPXY sequences *in vivo*, and that the three WW domains of Nedd4 may interact with the PY domain of each channel subunit. The ability of mutations that abolish this binding (by either deleting this binding site or mutating residues critical for binding) to cause the activation of channel activity implicates Nedd4 or a related interacting protein in the normal negative regulation of channel activity in the distal nephron and oocytes. Indeed, Staub *et al.* (1996) have demonstrated recently the endogenous expression of Nedd4 in *Xenopus* oocytes.

As discussed by Staub *et al.* (1996), all Nedd4 proteins from yeast to rat contain a C-terminal ubiquitin ligase homology (Hect) domain (Kumar *et al.*, 1992). Ubiquitination regulates protein degradation by proteosomes, and ubiquitin ligase mediates the final step of this degradation pathway (Ciechanover, 1994). The protein–protein interaction of ENaC with Nedd4 may be involved in tagging the active channel in the apical membrane for degradation by proteosomes. These observations provide new insights into the molecular mechanisms underlying the increased channel activity caused by mutations of ENaC in patients with Liddle syndrome.

How the binding of Nedd4 to PY elements in ENaC may mediate the negative regulation of channel activity remains uncertain. Patch–clamp experiments have shown that deletions of the β subunit causing Liddle syndrome result in an increase in the overall channel activity without altering the channel conductance, selectivity or pharmacological properties (Schild *et al.*, 1995). These observations would be consistent with a model in which Nedd4 binding is involved in promoting either the removal or degradation of ENaC from the membrane surface. According to this scenario, Liddle syndrome would be associated with an increased number of active-channel molecules at the membrane. Further work will be required to investigate the mechanism by which Nedd4 or related proteins negatively regulate ENaC activity.

Materials and methods

Preparation of the ENaC mutants

ENaC mutants were produced by two-step PCR mutagenesis. The first PCR step employed a 5′ mutagenic primer and a normal 3′ primer, using cloned rat ENaC cDNA as a template. The product of this PCR was then used as the 3′ primer in conjunction with another 5′ primer to again direct PCR using the ENaC cDNA as template. This PCR product contained the desired mutation flanked by a normal cDNA sequence; restriction endonucleases cutting on either side of the engineered mutation were used to cleave the product. In parallel, the corresponding segment of rat ENaC cDNA in vector PSD5 was removed by digestion with these same endonucleases, and the mutant PCR product was then ligated into the ENaC cDNA and transformed into *Escherichia coli* strain DH5α. The DNA sequence of the resulting clones confirmed the introduction of the expected mutations.

Expression of ENaC channels in X.laevis oocytes

Complementary RNAs of each α, β and γ subunits were synthetized *in vitro*. Equal amounts of each subunit cRNA at saturating concentrations for maximal channel expression (5 ng total cRNA) were injected into stage V oocytes of *X.laevis*. Electrophysiological measurements were performed 24 h after injection using the two-electrode voltage–clamp technique. A bathing solution contained 115 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES–NaOH. The amiloride-sensitive current I_{Na} was determined at −100 mV as the difference between the whole-oocyte current measured in the presence of 5 μM amiloride in the bath and after washout of the drug.

Results are reported as means ± SEM and represent the mean of n independent experiments in which the average amiloride-sensitive current I_{Na} was measured for eight to 10 individual oocytes originating from the same frog. Experimental protocols compared the I_{Na} expressed by ENaC deletion mutants, or amino acid substitution mutants with I_{Na} obtained for the ENaC wild type as control. Measurements were performed blinded to the nature of the construct being analyzed. Data on graphs represent the fractional changes in I_{Na} for the mutants over the value measured for wild-type ENaC (I_{Na}/I_{Na} wt). Statistical significance was determined using the unpaired t-test.

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