Roles of the C Termini of α-, β-, and γ-Subunits of Epithelial Na\(^{+}\) Channels (ENaC) in Regulating ENaC and Mediating Its Inhibition by Cytosolic Na\(^{+}\)**

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The amiloride-sensitive epithelial Na\(^{+}\) channels (ENaC) in the intralobular duct cells of mouse mandibular glands are inhibited by the ubiquitin-protein ligase, Nedd4, which is activated by increased intracellular Na\(^{+}\). In this study we have used whole-cell patch clamp methods in mouse mandibular duct cells to investigate the role of the C terminus of the α-, β-, and γ-subunits of ENaC in mediating this inhibition. We found that peptides corresponding to the C terminus of the β- and γ-subunits, but not the α-subunit, inhibited the activity of the Na\(^{+}\) channels. This mechanism did not involve Nedd4 and probably resulted from the exogenous C termini interfering competitively with the protein-protein interactions that keep the channels active. In the case of the C terminus of mouse β-ENaC, the interacting motif included βSer\(^{631}\), βAsp\(^{652}\), and βSer\(^{653}\). In the C terminus of mouse γ-ENaC, it included γSer\(^{460}\). Once these motifs were deleted, we were able to use the C termini of β- and γ-ENaC to prevent Nedd4-mediated down-regulation of Na\(^{+}\) channel activity. The C terminus of the α-subunit, on the contrary, did not prevent Nedd4-mediated inhibition of the Na\(^{+}\) channels. We conclude that mouse Nedd4 interacts with the β- and γ-subunits of ENaC.

The epithelial Na\(^{+}\) channel (ENaC)\(^{1}\) (1) is a major protein supporting the transepithelial transport of Na\(^{+}\) in many epithelia, including the renal collecting duct, the descending colon, the salivary ducts, and the respiratory epithelium (2, 3). It plays an important role in the regulation of blood pressure (4) and of fluid balance across the respiratory tract (5). Activating mutations in these channels, such as those found in the autosomal dominant form of hereditary hypertension known as Liddle’s syndrome, produce hypertension both in humans (6) and in mouse models (7). Conversely, inactivating mutations produce salt-sensitive hypertension and fluid accumulation in the respiratory tract (8–10).

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The abbreviations used are: ENaC, epithelial Na\(^{+}\) channels; PY motif, PPXY sequence; GST, glutathione S-transferase; NMDG, N-methyl-D-glucamine.

The C termini of each of the three subunits of ENaC contain PY motifs that bind the ubiquitin-protein ligase, Nedd4 (11–13). Nedd4 then ubiquitinates the Na\(^{+}\) channel (14–16) leading to its endocytosis (17) and degradation (16). Thus, mutation or deletion of the PY motifs leads to increased surface expression of Na\(^{+}\) channels in the Xenopus oocyte expression system (15, 18–20) and in renal epithelial cells (21). Furthermore, over-expression of wild-type Nedd4 reduces Na\(^{+}\) channel activity in Xenopus oocytes (15, 22), whereas over-expression of a Nedd4 mutant that is inactive as a ubiquitin ligase leads to increased Na\(^{+}\) channel activity (15, 22). The binding of Nedd4 to the PY motifs in the Na\(^{+}\) channel has also been proposed as a prerequisite for the insertion of the channels into the cell membrane in response to increased intracellular cyclic AMP (23).

The interaction between Nedd4 and Na\(^{+}\) channels appears to be regulated. Increased intracellular Na\(^{+}\) triggers the inactivation of Na\(^{+}\) channels by Nedd4 in Xenopus oocytes (20, 24–26) and in mouse salivary duct cells (14, 27–29). The importance of this regulation is evident from the finding that Liddle’s syndrome is induced by the deletion or mutation of the PY motifs in the C termini of the β- or γ-subunits (6, 30–33). Yet little is known of the mechanism by which Nedd4 binds to ENaC. WW1, one of the three WW domains present in mouse Nedd4, does not bind any of the subunits of the epithelial Na\(^{+}\) channel (29), and the same observation has been made for the WW1 domain of human Nedd4 (11). Given that all three subunits of the Na\(^{+}\) channel, α, β, and γ, contain PY motifs in their C termini, this finding raised the question of whether the remaining two WW domains in mouse Nedd4 bind all three subunits of the Na\(^{+}\) channel indiscriminately or whether they bind specifically to only two of them. The available information that might have resolved this question is contradictory. Thus, although no cases of Liddle’s syndrome have been reported to be caused by the deletion or mutation of the PY motif in the α-subunit (4), deletion of the C terminus of the α-subunit does lead to increased ENaC activity in Xenopus oocytes (33).

We investigated this question in mouse mandibular duct cells which, like the cells of other aldosterone-sensitive epithelia (34, 35), express all three subunits of ENaC (3, 29) and contain an amiloride-sensitive Na\(^{+}\) conductance that is activated by aldosterone (35). This amiloride-sensitive Na\(^{+}\) conductance is inhibited by increased intracellular Na\(^{+}\) acting via a Nedd4-mediated mechanism (14, 29). In the present study we used whole-cell patch clamp methods to examine whether the inclusion in the pipette solution of fusion proteins and synthetic peptides corresponding to the C termini of the α-, β-, and γ-subunits of the epithelial Na\(^{+}\) channel could prevent down-regulation of Na\(^{+}\) channel activity in mouse mandibular duct cells. We found that the C termini of β- and γ-ENaC, but not of...
Materials and Methods

Expression Plasmids—The regions encoding the C termini of human and mouse α-, β-, and γ-ENaC subunits were polymerase chain reaction-amplified and cloned into either BamHI or BamHI/EcoRI sites of pGEX-2TK. Mouse C terminus β-ENaC-10, mouse C terminus γ-ENaC-10, and mouse C terminus γ-ENaC-19 were polymerase chain reaction-amplified and cloned into the BamHI/EcoRI sites of pGEX-2TK. Human β-ENaC PPNA, human γ-ENaC S637G, and mouse γ-ENaC PPRA and S643G mutants were generated by quick-change mutagenesis (Stratagene). Mouse α-, β-, and γ-ENaC subunit cDNAs were cloned from mouse mandibular duct cells as previously described (29) and found to be identical with the published sequences (36). Human α-, β-, and γ-ENaC DNA clones were the gift of Dr. M. Welsh (University of Iowa).

Production of GST Fusion Proteins—Overnight cultures of Escherichia coli DH5α harboring the appropriate GST expression plasmid were diluted 1/50, incubated for 2 h at 37 °C, induced with 1 mM isopropyl β-thiogalactoside, and incubated for an additional 5 h at 37 °C. Bacterial cell pellets were resuspended in phosphate-buffered saline, lyzed by sonication, and clarified by centrifugation at 10,000×g for 10 min. Glutathione-Sepharose (Amersham Pharmacia Biotech) was incubated with the cleared lysate for 60 min at room temperature, after which the beads were washed three times with phosphate-buffered saline. Fusion protein was eluted with glutathione buffer according to the manufacturer’s protocol. Protein concentration was measured using a BCA kit (Pierce). Fusion proteins were routinely checked by SDS-PAGE and immunoblotting.

Fusion proteins (Fig. 1) on Na⁺ feedback inhibition of the Na⁺ channel, we checked whether they affected Na⁺ channel activity when the Na⁺ feedback system is inactive. We did so by including 300 μg of each GST fusion protein ml in the zero Na⁺ pipette solution and then observing the effect of the fusion protein on the amiloride-sensitive Na⁺ current. To our surprise, we found that both the fusion protein containing the C terminus of human β-ENaC and that containing the C terminus of mouse γ-ENaC inhibited the amiloride-sensitive Na⁺ current (Fig. 2). The fusion protein containing the C terminus of mouse α-ENaC, however, did not have this effect (Fig. 2).

Identification of the Motif Responsible for the Feedback-independent Inhibitory Action of the C Terminus of β-ENaC—We first investigated the basis of the inhibitory action of the C terminus of β-ENaC. We found that a fusion protein containing the C terminus of human β-ENaC, in which the PY motif (PPNY) had been mutated (PPNA) so that it no longer interacted with Nedd4 (29), was still inhibitory (Fig. 3). Furthermore, inclusion of the C terminus of mouse β-ENaC in the pipette solution together with a dominant negative mutant of ubiquitin (K48R), which we have previously shown to block inhibition of Na⁺ channels by increased intracellular Na⁺ in these cells (14), failed to prevent the inhibition produced by the C terminus of mouse β-ENaC. Because inclusion of the K48R mutant of ubiquitin in zero Na⁺ pipette solution is not itself inhibitory (14), we concluded that the inhibition produced by the C terminus β-ENaC does not involve ubiquitination of the Na⁺ channel. Deletion of 10 amino acids from the C terminus of the mouse β-ENaC fusion protein, however, prevented the inhibitory effect of the fusion protein (Fig. 3), suggesting that the inhibitory motif is present in the last 10 amino acids of the β-subunit.

We confirmed these findings by using synthetic peptides corresponding to the final 10 amino acids of mouse β-ENaC (Fig. 4A). We found that the inclusion of 20 μM βC10 peptide in the zero Na⁺ pipette solution inhibited the amiloride-sensitive Na⁺ current almost completely (Fig. 4B). We then investigated whether the action of the peptide was mediated by a pertussis toxin-sensitive G protein, because these are known to inhibit amiloride-sensitive Na⁺ channels (37, 39). We found, however, that the inclusion of activated pertussis toxin in the peptide solution did not prevent its inhibitory action on the Na⁺ channel (Fig. 4B).

RESULTS

The C Termini of β-ENaC and γ-ENaC, but Not α-ENaC, Reduce Na⁺ Channel Activity Independently of Feedback Inhibition—Prior to examining the effect of the C-terminal fusion proteins (Fig. 1) on Na⁺ feedback inhibition of the Na⁺ channel, we checked whether they affected Na⁺ channel activity when the Na⁺ feedback system is inactive. We did so by including 300 μg of each GST fusion protein ml in the zero Na⁺ pipette solution and then observing the effect of the fusion protein on the amiloride-sensitive Na⁺ current. To our surprise, we found that both the fusion protein containing the C terminus of human β-ENaC and that containing the C terminus of mouse γ-ENaC inhibited the amiloride-sensitive Na⁺ current (Fig. 2). The fusion protein containing the C terminus of mouse α-ENaC, however, did not have this effect (Fig. 2).

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found that a peptide in which Ser640 was substituted by glycine inhibited the action of the peptide (Fig. 4). We then investigated whether a chemically synthesized peptide corresponding to this region (Fig. 4) was without inhibitory activity (Fig. 4C). We further found that the inhibitory effect of the βC10 peptide was completely abolished by substituting glycine for the serine residues at positions 3 (peptide βS631G in Fig. 4A) or 5 (peptide βS633G in Fig. 4A). Substituting glycine for the aspartate residue at position 4 (βD632G in Fig. 4A), which lies between the two serine residues, reduced (p < 0.05), but did not abolish (p < 0.05) the inhibitory action of the peptide (Fig. 4C).

Identification of the Motif Responsible for the Feedback-independent Inhibitory Action of the C terminus of γ-ENaC—We then investigated the basis of the inhibitory action of the C terminus of mouse γ-ENaC. We found that mutation of the PY motif of mouse γ-ENaC (PPRA) did not alter the inhibitory activity of the fusion protein (Fig. 5), indicating that the inhibition was not caused by activation of the feedback inhibitory system mediated by Nedd4. In contrast to our findings with the C terminus of mouse β-ENaC, deletion of the terminal 10 amino acids of mouse γ-ENaC did not remove the inhibitory effect of this fusion protein on the Na⁺ channel (Fig. 5). Only when we removed 19 amino acids from the C terminus of mouse γ-ENaC did the fusion protein cease to inhibit the amiloride-sensitive Na⁺ current (Fig. 5).

The experiments with GST fusion proteins suggested that the region between 10 and 19 residues from the C terminus of mouse γ-ENaC contained the inhibitory motif. We therefore investigated whether a chemically synthesized peptide corresponding to this region (γmC9 in Fig. 6A) was inhibitory when included in the zero Na⁺ pipette solution, and we found that it was (Fig. 6B). Substituting the three serine residues (γSer640, γSer642, and γSer644) in this peptide by glycines (γSer → Gly) removed this inhibitory activity (γS-G in Fig. 6B). We then found that a peptide in which Ser640 was substituted by glycine (γS640G in Fig. 6A) was without inhibitory activity (Fig. 6B), whereas a peptide in which serines 643 and 644 are replaced by glycines (γS643G/γS644G in Fig. 6A) was still inhibitory (Fig. 6B). Thus, serine 640 is required for the inhibitory activity of the C terminus of mouse γ-ENaC.

The Basis for the Feedback-independent Inhibitory Activity of Human γ-ENaC—Serine 640 in mouse γ-ENaC is not present in human γ-ENaC where it is replaced by an arginine residue (Fig. 7A). We thus expected that a GST fusion protein corresponding to the C terminus of human γ-ENaC would not be inhibitory when included in the zero Na⁺ pipette solution (Fig. 7A). We found, however, that it did inhibit the amiloride-sensitive Na⁺ current (Fig. 7B). We thus investigated the effect of mutating the sole serine in the C terminus of human γ-ENaC to glycine (γhS637G). We found that this mutant no longer inhibited the amiloride-sensitive Na⁺ current when included in the zero Na⁺ pipette solution (Fig. 7B). Mutation of this serine in the C terminus of mouse γ-ENaC, however, did not remove the inhibitory activity of the C terminus of mouse γ-ENaC (Fig. 7B). This was consistent with our earlier findings using chemically synthesized peptides (see above and Fig. 6B).

A Peptide Including the β-ENaC PY Motif Blocks Na⁺ Feedback Inhibition—As outlined in the Introduction, we wished to determine whether mouse Nedd4 interacts with all three subunits of the epithelial Na⁺ channel or just two of them. We decided to do this by investigating whether inclusion in the
patch pipette of GST fusion proteins of the C termini of α-ENaC, β-ENaC, or γ-ENaC (Fig. 1) could block the inactivation of Na+ channels induced by raised intracellular Na+. This experimental strategy was based on the idea that Nedd4 in salivary duct cells would bind exogenous PY motifs, which would then prevent its normal interaction with, and inhibition of, Na+ channels. We tested this idea by examining whether a synthetic peptide that included the PY motif to an alanine residue of γ-ENaC had been deleted, or 300 μg/ml GST-γENaC fusion protein in which the final 19 amino acids of γ-ENaC had been deleted. The number of experiments is given in parentheses, and the chord conductance observed with the control zero Na+ pipette solution is shown as a broken line.

The C Termini of β- and γ but not α-ENaC Inhibit Regulation of Na+ Channels by Increased Intracellular Na+.—We then proceeded to investigate whether the ENaC C termini interrupted control of the Na+ channels by intracellular Na+. We found that we could prevent the inhibitory action of the 70 mM Na+ pipette solution by including in it the C terminus of mouse β-ENaC with the last 10 amino acids deleted (Fig. 9). To confirm that this effect was due to the PY motif in this construct, we also investigated the effect of this construct following mutation of the tyrosine in the PY motif to an alanine residue (βmENaC-10 PY-mut). We have previously shown that this mutation eliminates the binding of the C terminus of mouse β-ENaC to Nedd4 (29). We found that the mutant construct no longer prevented feedback inhibition of the amiloride-sensitive Na+ current (Fig. 9). We also found that the C terminus of mouse γ-ENaC with the last 19 amino acids deleted prevented the inhibition of the amiloride-sensitive Na+ current by raised intracellular Na+ (Fig. 9). Once again, mutation of the con-
that we have previously shown that the sites recognized by WW2 and WW3 in ENaCs from mouse salivary cells are distinct and are able to distinguish between WW2 and WW3 (29), it would thus appear likely that the WW2 of Nedd4 binds \( \beta \)-ENaC and the WW3 of Nedd4 binds \( \gamma \)-ENaC or vice versa. From the present data we cannot distinguish these possibilities.

The other major finding of our study is that the exogenous C terminus of both the \( \beta \)- and \( \gamma \)-subunits of the \( Na^+ \) channel inhibit \( Na^+ \) channel activity. As argued below, this probably occurs because the exogenous C termini compete with the \( Na^+ \) channels to interact with a protein, perhaps a kinase, that is required to maintain channel activity. In the case of the C terminus of mouse \( \beta \)-ENaC, this motif is located in the final 10 amino acids and requires the presence of both of the serine residues present in this peptide, \( \beta \text{Ser}^{631} \) and \( \beta \text{Ser}^{633} \), as well as the aspartate residue between them, \( \beta \text{Asp}^{632} \). In the case of the C terminus of mouse \( \gamma \)-ENaC, the motif is in the region between 10 and 19 residues from the C terminus, and requires the presence of only one of the three serine residues present in this region, \( \gamma \text{Ser}^{640} \). We further found that the C terminus of human \( \gamma \)-ENaC inhibits the amiloride-sensitive \( Na^+ \) current. This inhibition requires the presence of the serine \( \gamma \text{Ser}^{637} \), the only serine present in the C terminus of human \( \gamma \)-ENaC. Interestingly, the corresponding serine in mouse \( \gamma \)-ENaC is not involved in inhibiting the amiloride-sensitive \( Na^+ \) current (Fig. 7).

Consistent with our present findings, it has been reported previously that deletion of the C-terminal 8 amino acids of \( \beta \)-ENaC increases \( Na^+ \) channel activity in Xenopus oocytes (21), although a subsequent study in which the final 10 amino acids were deleted from \( \beta \)-ENaC failed to observe any stimulation (33). It has also been reported that the constitutively activated \( Na^+ \) channels obtained from lymphocytes of subjects with Liddle’s syndrome can be blocked by the application of peptides that include the final 10 amino acids of the C terminus of \( \beta \)-ENaC (42). This inhibition was attributed to the marked negative charge of the terminal decapeptide of \( \beta \)-ENaC, because a peptide in which the four negatively charged residues found in it had been substituted by uncharged residues was inactive (42). The four negative charges mutated in the lymphocyte studies included, however, \( \beta \text{Asp}^{632} \), which we have shown in the present study to be required for the inhibitory activity of the C terminus of \( \beta \)-ENaC. Furthermore, we have found that mutation of \( \beta \text{Glu}^{636} \) to an uncharged residue does not impair the inhibitory activity of the C-terminal peptide of \( \beta \)-ENaC (42).

The mechanism by which the C termini of the \( \beta \)- and \( \gamma \)-C subunits inhibit the \( Na^+ \) channels is unclear. For the C termini of both \( \beta \)-ENaC and \( \gamma \)-ENaC, we can exclude any role for binding of Nedd4. In the case of \( \beta \)-ENaC, we can also exclude any role for ubiquitination or pertussis toxin-sensitive G proteins, which are known to inhibit \( Na^+ \) channels in these and other cell types (14, 37, 39). Finally, the small size of the inhibitory regions of the C termini make it unlikely that they are forming a \( \beta \)-pleated sheet and hence blocking the channel pore (43). Given the requirement for the presence of serine residues in the C termini of both \( \beta \)-ENaC and \( \gamma \)-ENaC and the report that these regions are constitutively phosphorylated in Madin-Darby canine kidney cells (44), it is tempting to postulate that the addition of exogenous \( \beta \)-ENaC or \( \gamma \)-ENaC C termini to the cytosol may be interfering in the phosphorylation processes required to maintain the channels in an active state. Consistent with this possibility is the observation that the C terminus of \( \alpha \)-ENaC, which in the present studies is without inhibitory activity, is not phosphorylated in vivo (44).
