Proteolytic Processing of the Epithelial Sodium Channel γ Subunit Has a Dominant Role in Channel Activation*

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Maturation of the epithelial sodium channel (ENaC) involves furin-dependent cleavage at two extracellular sites within the α subunit and at a single extracellular site within the γ subunit. Channels lacking furin processing of the α subunit have very low activity. We recently identified a prostasin-dependent cleavage site (RKRR186) in the γ subunit. We also demonstrated that the tract αD206-R231, between the two furin cleavage sites in the α subunit, as well as the tract γE144-K186, between the furin and prostasin cleavage sites in the γ subunit, are inhibitory domains. ENaC cleavage by furin, and subsequently by prostasin, leads to a stepwise increase in the open probability of the channel as a result of release of the α and γ subunit inhibitory tracts, respectively. We examined whether release of either the α or γ inhibitory tract has a dominant role in activating the channel. Co-expression of prostasin and either wild-type channels or mutant channels lacking furin cleavage of the α subunit (αR205A,R208A,R231Aγ186) in Xenopus laevis oocytes led to increases in whole cell currents to values significantly higher than those from oocytes expressing wild type ENaC. When channels lacked the α and γ subunit inhibitory tracts, α subunit cleavage was required for channels to be fully active. Channels lacking both furin cleavage and the inhibitory tract in the γ subunit (αβγR143A,δE144-K186) showed a significant reduction in the efficacy of block by the synthetic α-26 inhibitory peptide representing the tract αD206-R231. Our data indicate that removal of the inhibitory tract from the γ subunit, in the absence of α subunit cleavage, results in nearly full activation of the channel.

Epithelial sodium channels (ENaCs)2 mediate Na+ transport across apical membranes of cells lining the distal nephron, airway, and alveoli, and distal colon. These channels are required for the maintenance of extracellular fluid volume, blood pressure, and airway surface liquid volume. ENaCs are composed of three structurally similar subunits termed α, β, and γ (1). Each subunit has two transmembrane domains connected by a large ectodomain with short intracellular N and C termini. ENaC is assembled in the endoplasmic reticulum where the three subunits undergo Asn-linked glycosylation (2–4). The recently resolved structure of chicken acid-sensing ion channel 1 (cASIC1), an ENaC-related family member, revealed that members of this family of ion channels are likely homo- or hetero-trimmers (5).

We previously reported that α and γ ENaC subunits are processed within their extracellular domains by furin, a serine protease that resides primarily in the trans-Golgi network (6). The α subunit is cleaved twice by furin immediately following Arg-205 and Arg-231, and the γ subunit at a single site after Arg-143 (6). Two pools of ENaC subunits are expressed at the plasma membrane, an immature pool with endoglycosidase H-sensitive non-processed N-glycans that lacks proteolytic processing, and a mature pool with endoglycosidase H-resistant terminally processed N-glycans and proteolytically processed α and γ subunits (7). Channels with non-cleaved α subunits or with α subunits that are cleaved at only one furin cleavage site display low open probability when expressed in Xenopus laevis oocytes (6, 8–10). However, channels carrying mutations in the first α subunit furin cleavage site as well as a deletion of the connecting tract (αR205A,ΔD206-R231γ186) lack cleavage in the α subunit, but are active when expressed in oocytes. These observations suggest that the release or removal of an inhibitory tract (D206-R231) from the extracellular domain of the α subunit, rather than cleavage per se is required for normal channel activity (9).

ENaC activity has been shown to be modulated by several serine proteases, including a group of “channel-activating proteases” (CAPS) such as prostasin (CAP-1), TMPRSS4 (CAP-2), and matriptase (CAP-3 or MT-SP1). Other proteases that active ENaC are chymotrypsin, neutrophil elastase, pancreatic elastase, kallikrein, and trypsin (11–20). We recently identified a prostasin-dependent cleavage site in the γ subunit of ENaC (RKRR186) that is distal to the furin cleavage site (RKRR143) (14). The tract E144-K186, between the furin and prostasin cleavage sites in the γ subunit, encompasses an inhibitory domain (14). Channels proteolytically processed by both furin and prostasin or channels lacking the γ inhibitory tract exhibit a very high open probability (14).

We have proposed that sequential release of the α and γ subunit inhibitory tracts lead to a stepwise increase in the open

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2 The abbreviations used are: ENaC, epithelial Na+ channel; cASIC1, chicken acid-sensing ion channel 1; TEV, two-electrode voltage clamp; HA, hemagglutinin; ANOVA, analysis of variance; CAP, channel-activating protease.

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We dedicate this report to the memory of James B. Bruns.
probability of the channel (9, 14). In this report, we investigated whether loss of either the α subunit or γ subunit inhibitory tract has a dominant role in activating ENaC. In the absence of α subunit cleavage, we found that removal of the γ subunit inhibitory domain leads to near full activation of the channel, supporting the concept that proteolysis of the γ subunit with release of its inhibitory domain has a major role in the modulation of channel gating.

**EXPERIMENTAL PROCEDURES**

**Oocyte Expression**—cRNAs for α, β, and γ mouse ENaC (wild-type and mutant) subunits and mouse prostasin were synthesized with T3 or T7 mMessage mMachine™ (Ambion, Austin, TX). α and γ subunits had N-terminal HA and C-terminal V5 epitope tags (6). Mutants of mouse ENaC subunits used in this work were previously described (6, 9, 14). Stage V-VI *X. laevis* oocytes were injected with 1–2 ng of cRNA for each subunit and 3 ng of prostasin cRNA.

**Peptides**—The peptide DLRGALPHPLQRLRTPPPNPAR-SAR (α-26) was synthesized and HPLC-purified by GenScript Corporation (Piscataway, NJ).

**Two-electrode Voltage Clamp (TEV)**—Electrophysiological measurements were performed 20–28 h after injection. The extracellular solution was (in mM): 110 NaCl, 2 KCl, 1.5 CaCl₂, 10 HEPES, pH 7.4. The amiloride-insensitive component of the whole cell current was determined by perfusion with an extracellular solution supplemented with 10 μM amiloride. The amiloride-sensitive component of the whole cell Na⁺ current, at −60 mV, was defined as the ENaC-mediated current. The α-26 mer inhibitory peptide was dissolved in the extracellular solution, where indicated.

**Data and Statistical Analyses**—Each set of experiments were performed with at least two batches of oocytes obtained from different frogs. Data were not normalized and were expressed as the mean ± S.E. (n), where n equals the number of independent experiments analyzed. Statistical comparisons were performed with GraphPad 3.0 (GraphPad Software, San Diego, CA). One-way ANOVA followed by Student-Newman-Keuls test were used for multiple comparisons. Repeated measures ANOVA were used to compare multiple treatments in the same cell. p < 0.05 was considered statistically significant.

**RESULTS**

We previously demonstrated that sequential removal of the α and γ subunit inhibitory domains leads to a stepwise increase in ENaC open probability (9, 14). Functional changes observed at the macroscopic level by removal of subunit inhibitory domains were directly associated with changes in the open probability of the channel (8, 9, 14). To gain insight into the regulation of channel gating by proteolytic processing, we examined whether sequential removal of the α and γ inhibitory domains is required for activation of the channel by prostasin. Wild-type ENaC (αβγ) or channels carrying mutations in the α subunit furin consensus cleavage sites (αR205A,R208A,R231Aβy (αRtripleAβy)) were expressed in *X. laevis* oocytes with or without prostasin. As we previously observed, oocytes expressing channels that lacked α subunit cleavage had currents that were reduced by 92.1±1.0%, compared with oocytes expressing wild type channels. However, co-expression of prostasin with channels containing either wild type or mutant α subunits led to an increase in whole cell currents to a similar level (Fig. 1). We previously demonstrated that channels carrying mutations in the γ subunit within the tract RKR186 are not activated by prostasin (14). In agreement with our previous observations, mutation of both the furin and prostasin cleavage sites in the γ subunit (γR143A,RKRK186QQQQ) prevented prostasin-dependent activation of channels that also contained either a wild type or mutant α subunit (αRtripleA) (Fig. 2). As channels with the αRtripleA mutant α subunits retain their α inhibitory tract (6), our data suggest that removal of the γ inhibitory tract is sufficient to activate the channel in the presence or absence of the α inhibitory tract.

To further validate our findings, we expressed four different channels that had α and γ subunits with inhibitory domains that were either retained or removed (see Fig. 3). Wild type channels that are processed by furin lack the α subunit inhibitory domain, but retain the γ subunit inhibitory domain as the
E\text{NaC} \text{ Inhibitory Tracts}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{enac-inhibitory-tracts.png}
\caption{Deletion of the \(\gamma\) subunit inhibitory tract E144-K186 increases the activity of channels lacking \(\alpha\) subunit furin cleavage. A, models for wild type and furin-insensitive (\(\alpha R205A, R208A, R231A\) (\(\alpha\text{tripleA}\)) \(\alpha\) subunits, and wild type, furin-, and furin-prostasin-insensitive (\(\gamma R143A, RKR R186\)QQQQ (\(\gamma R/A, RKR R/Q4\)), and prostasin-processed \(\gamma\) subunits. The sites of furin- and prostasin-dependent cleavage are denoted in gray and mutations in black. B, TEV was performed with oocytes expressing either wild-type (\(\alpha R\)), \(\alpha R205A, R208A, R231A\) (\(\alpha\text{tripleA}\)), \(\alpha R143A, RKR R186\)QQQQ (\(\alpha R R/A, RKR R/Q4\)) and \(\alpha R205A, R208A, R231A\) (\(\alpha R\)). The dotted line denotes a deletion.}
\end{figure}

\(\gamma\) subunit is only cleaved once. Channels with \(\alpha\) subunits lacking furin cleavage sites (\(\alpha\text{tripleA}\)) retain both the \(\alpha\) and \(\gamma\) subunits. The \(\gamma R143A, R114-E186\) mutant (\(\gamma R/\Delta\)) lacks both the \(\gamma\) subunit furin cleavage site and the inhibitory tract. \(\alpha R/\Delta\) channels that are processed by furin will lack both the \(\alpha\) and \(\gamma\) subunits. \(\alpha\text{tripleA}\) channels will retain the \(\alpha\) subunit inhibitory domain, but lack the \(\gamma\) subunit inhibitory domain. As we previously reported, oocytes expressing channels that lacked both inhibitory tracks (\(\alpha R/\Delta\)) exhibited whole cell currents that were \(2.55 \pm 0.29 \text{ fold} \) significantly greater than oocytes expressing wild type \(\alpha R\) (Fig. 3). Whole cell currents measured in oocytes expressing channels that lacked only the \(\gamma\) subunit inhibitory track (\(\alpha\text{tripleA}\)\(\gamma R/\Delta\)) were similar in magnitude to that measured in oocytes lacking both inhibitory tracks (\(\alpha R/\Delta\)). These results provide additional support for the concept that release of the \(\gamma\) subunit inhibitory domain has a dominant role in activating the channel.

We previously demonstrated that \(\alpha\) subunit proteolysis was not necessary to activate channels lacking the \(\alpha\) subunit inhibitory tract (9). In agreement with our previous findings, we observed that oocytes expressing wild type channels exhibited currents that were similar in magnitude to channels that lacked both furin cleavage sites and the inhibitory tract in the \(\alpha\) subunit (\(\alpha R205A, R208A, R231A\) (\(\alpha\text{tripleA}\)) (Fig. 4). The difference between the wild type \(\alpha\) subunit and \(\alpha R/\Delta\) is that one is cleaved (wild type), while the other is not (\(\alpha R/\Delta\)). As both constructs lack the \(\alpha\) subunit inhibitory tract, we postulated that expression of channels with either \(\alpha\) subunit (wild type or \(\alpha R/\Delta\)) with a \(\gamma\) subunit that lacked its inhibitory domain and furin cleavage site (\(\gamma R/\Delta\)) would result in channels with significantly enhanced activity. Surprisingly, oocytes expressing \(\alpha R/\Delta\)\(\gamma R/\Delta\) had whole cell currents that were significantly lower than oocytes expressing \(\alpha R/\Delta\) channels (Fig. 4). Fur-
FIGURE 4. Channels lacking both furin cleavage and the inhibitory tracts in the α and γ subunits display reduced activity. A, models for wild type α, furin-insensitive α (αR205A, R208A, R231A (αRtripleA)) and γ subunits lacking both furin cleavage sites and the inhibitory tract D206-R231 (γR/AΔ), wild type γ, furin-insensitive γ (γR143A (γR/A)), and mutant γ subunits lacking both the furin cleavage site and the inhibitory tract E144-K186 (γR143A, E144-K186 (γR/AΔ)). Furin-dependent cleavage sites are indicated in gray. The dotted line denotes a deletion. B, TEV was performed with oocytes expressing either wild-type (αβγ), αR205A, R208A, R231A, (αRtripleAγR/AΔ), and γR143A, E144-K186 (γR/AΔ). Aβγ current amplitudes were also statistically different between oocytes expressing αβγR/R/AΔ and αβγR/AΔ channels (p < 0.001), and αβγR/R/AΔ (p < 0.01). Amiloride-sensitive currents were also statistically different between oocytes expressing αβγR/R/AΔ and αβγR/AΔ channels (p < 0.001, one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test). Experiments were performed with 20 oocytes for each group. The presence or absence of an inhibitory domain is indicated by (+) or (−), respectively.

thermore, when the α subunit lacked its inhibitory tract while retaining a furin cleavage site (αD206-R231 (αΔ)), deletion of the γ subunit inhibitory tract led to a large 3.14 ± 0.31 fold increase in ENaC activity when compared with αβγ (Fig. 5). Oocytes expressing αβγR/R/AΔ exhibited whole cell currents that were significantly greater than currents measured in oocytes expressing αβγR/R/AΔ. These results suggest that when channels lack the α and γ subunit inhibitory tracts, α subunit cleavage is required for channels to be fully active.

We previously reported whole cell amiloride-sensitive Na⁺ currents of 9.68 ± 2.06 μA in oocytes expressing channels lacking both furin cleavage and the inhibitory tract in the γ subunit (αβγR143A, E144-K186) (n = 16), whereas currents in oocytes expressing wild type ENaC were 2.63 ± 0.48 μA (n = 16) (14). Within the same experimental group, oocytes expressing channels solely lacking the γ inhibitory tract (αβγE144-K186) exhibited currents of 9.72 ± 2.24 μA (n = 16). Our results indicate that furin cleavage of the γ subunit does not influence the activity of channels lacking the γ inhibitory tract.
Our results suggest that channels are activated by excising an inhibitory domain from the γ subunit. In this setting, the presence of an α subunit inhibitory domain did not appear to reduce channel activity (Fig. 3). The mutant α subunit in this experiment (Fig. 3) lacked both furin sites, raising the possibility that the α subunit inhibitory domain might be in an unfavorable conformation to interact with the channel. To address this possibility, we examined the activity of channels lacking the γ subunit inhibitory tract (γR/Δ) while retaining the α subunit inhibitory tract in the setting where the α subunit was either not cleaved (αRtripleA) or was cleaved only once (αR205A). Surprisingly, oocytes expressing αR205AβγR/Δ had whole cell currents that were significantly lower than oocytes expressing αRtripleAβγR/Δ (Fig. 6).

We previously demonstrated that wild type ENaCs expressed in oocytes are inhibited by a synthetic peptide (α-26) with the sequence of the α subunit inhibitory tract (D206-R231) in the low micromolar range (9). However, the peptide was a relatively poor blocker of transepithelial Na⁺ transport in cortical collecting duct cells (mKCCD₁₆) and human airway cells (IC₅₀ ~ 50–100 μM), and of whole cell Na⁺ currents in oocytes co-expressing ENaC and prostasin (IC₅₀ > 50 μM) (9). These data suggest that release of the γ subunit inhibitory tract may affect the interaction of the α inhibitory peptide with the channel. We therefore examined whether the α-26 peptide was an effective inhibitor of channels lacking the γ subunit inhibitory tract. While whole cell currents in oocytes expressing wild type αβγ were inhibited by 1 and 10 μM α-26 in a dose-dependent manner, only a small inhibition of current was observed in oocytes expressing αβγR/Δ, which lacked the furin cleavage site and the inhibitory tract in the γ subunit (Fig. 7).

**DISCUSSION**

We previously reported that both the α and γ subunits of ENaC contain inhibitory tracts that can be liberated by proteolysis. The release of the α subunit inhibitory tract led to partial channel activation, whereas the release of both inhibitory tracts resulted in channels with a very high open probability (9, 14). We now report that channels lacking furin cleavage of the α subunit are still near fully activated by co-expression with prostasin. Furthermore, we observed a significantly increased activity of channels with a retained α subunit inhibitory tract and deleted γ subunit inhibitory tract E144-K186. These observations suggest that the release or removal of the inhibitory tract in the γ subunit, even in presence of the α inhibitory tract, results in nearly full activation of the channel.
Our results suggest that pools of channels that have not been proteolytically processed, or channels that have been cleaved by furin and have lost their α subunit inhibitory tract will be fully activated by release of the γ subunit inhibitory tract. For channels with γ subunits that have been processed by furin, cleavage of the channel within the vicinity of the prostatasin cleavage site should be sufficient to fully activate the channel. Several proteases have been reported to cleave the γ subunit in the region near the prostatasin cleavage site. ENaC activation by the proteases neutrophil elastase, prostatasin and kallikrein is associated with proteolytic processing of the γ subunit. This suggests that the α inhibitory tract (D206-R231), when present within the α subunit, is unable to efficiently block channels that lack the γ subunit inhibitory tract. In agreement with this finding, we also observed a reduced efficacy of the α-26 mer peptide to inhibit channels that lacked the γ subunit inhibitory tract (Fig. 7).

In conclusion, our work indicates that the contributions of inhibitory domains within the α and γ subunits in the modulation of channel gating are not equal. Removal of the γ subunit inhibitory tract results in nearly full activation of the channel in the absence of α subunit furin processing.

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