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TMPRSS4-dependent activation of the epithelial sodium channel requires cleavage of the \(\gamma\)-subunit distal to the furin cleavage site

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Passero CJ, Mueller GM, Myerburg MM, Carattino MD, Hughey RP, Kleyman TR. TMPRSS4-dependent activation of the epithelial sodium channel requires cleavage of the \(\gamma\)-subunit distal to the furin cleavage site. Am J Physiol Renal Physiol 302: F1–F8, 2012. First published October 12, 2011; doi:10.1152/ajprenal.00330.2011.—The epithelial sodium channel (ENaC) is activated by a unique mechanism, whereby inhibitory tracts are released by proteolytic cleavage within the extracellular loops of two of its three homologous subunits. While cleavage by furin within the biosynthetic pathway releases one inhibitory tract from the α-subunit and moderately activates the channel, full activation through release of a second inhibitory tract from the \(\gamma\)-subunit requires cleavage once by furin and then at a distal site by a second protease, such as prostan, plasmin, or elastase. We now report that coexpression of mouse transmembrane protease serine 4 (TMPRSS4) with mouse ENaC in Xenopus oocytes was associated with a two- to threefold increase in channel activity and production of a unique \(~70\)-kDa carboxyl-terminal fragment of the \(\gamma\)-subunit, similar to the \(~70\)-kDa \(\gamma\)-subunit fragment that we previously observed with prostan-dependent channel activation. TMPRSS4-dependent channel activation and production of the \(~70\)-kDa fragment were partially blocked by mutation of the prostan-dependent cleavage site (γK173Q, γK175Q, and γR177Q), in addition to γK173R175Q. Mutation of the four basic residues associated with the furin cleavage site (γK173R175Q) also prevented TMPRSS4-dependent channel activation. We conclude that TMPRSS4 primarily activates ENaC by cleaving basic residues within the tract \(\gamma\)K173-K186 distal to the furin cleavage site, thereby releasing a previously defined key inhibitory tract encompassing γR158-F168 from the \(\gamma\)-subunit.

The extent of ENaC proteolysis has an important role in determining channel activity. Channels that bypass Golgi complex processing events are not cleaved and are found on the cell surface as near-silent channels that exhibit a very low open probability \((P_o=0.1)\), but can be fully activated by exogenously added proteases, such as trypsin or elastase (5, 6, 18, 23). Furin-cleaved channels exhibit a moderate \(P_o=0.3–0.4\). Furin-cleaved channels can also be cleaved by a second protease that produces channels with a very high \(P_o\) approaching 1. This correlation of proteolysis with \(P_o\) is consistent with the observation that coexpression of ENaC with prostan in Xenopus oocytes, or treatment of oocytes expressing ENaC with exogenous plasmin, activates amiloride-sensitive currents by two- to threefold (3, 23, 29, 30). Sites of activating cleavage by these nonfurin proteases have been consistently found in the \(\gamma\)-subunit distal to the furin cleavage site, thereby releasing a peptide of \(~43\) residues. For example, prostan induces cleavage of the \(\gamma\)-subunit at a basic tract, γRKRKR186, plasmin...
ceases after the tract γIHK194, pancreatic elastase cleaves after γA195, and neutrophil elastase cleaves after γV198 (1, 3, 29, 30).

Altogether, these data support the hypothesis that ENaC activation by proteases is due to the release of inhibitory tracts from the α- and γ-subunits. In support of this theory, we found that deletion of either the α-26-mer or γ-43-mer tract (with or without adjacent proteolytic cleavage sites) activates the channel, while exogenous addition of synthetic peptides based on either one of these two excised tracts inhibits the channel (3, 10, 28). Serial deletions within either the α- or γ-inhibitory tracts and short synthetic peptides have further defined the key inhibitory sites in the α- (8-mer tract) and γ-subunits (11-mer tract) (9, 28). Modeling of the α-subunit based on the crystal structure of the related acid-sensing ion channel 1 (19) allowed us to characterize the binding site for the α-subunit-derived 8-mer inhibitory peptide between the “finger” and “thumb” subdomains of the extracellular loop. We proposed that the α-subunit inhibitory peptide affects channel gating by constraining motions within these two major subdomains (20, 21).

García-Caballerò et al. (15) recently suggested that human TMPRSS4 (CAP2) activated rat ENaC in Xenopus oocytes primarily via TMPRSS4-dependent cleavage at the γ-subunit furin cleavage site (rat γRKRR138). The author’s conclusion that TMPRSS4 activated ENaC primarily by cleaving the γ-subunit furin cleavage site is contrary to our current hypothesis of ENaC activation by a second cleavage event distal to the γ-subunit furin cleavage site and thereby releasing an inhibitory tract from the channel. The authors also noted TMPRSS4-dependent cleavage of the γ-subunit distal to the furin cleavage site. However, mutation of both the prostasin-dependent cleavage site (rat γRKRR181QQQQ) and four distal basic residues (rat K185Q, K189Q, K200Q, and K201Q) did not block channel activation by TMPRSS4 coexpression (15). These results are consistent with TMPRSS4-dependent cleavage at a site distal to the furin cleavage site, but distinct from the sites examined by García-Caballerò and coworkers. We have tested our “second cleavage” hypothesis by coexpression of mouse TMPRSS4 and mouse αβ-ENaC. Our results indicate that TMPRSS4-dependent channel activation is dependent on γ-subunit cleavage at a site (or sites) distal to the furin cleavage site. TMPRSS4 induces cleavage of the γ-subunit at sites distal to the furin cleavage site within a tract, including seven basic residues that are proximal to or within the prostasin-dependent cleavage site, thereby releasing the γ-subunit inhibitory tract. In agreement with García-Caballerò and coworkers, our results also suggest that TMPRSS4 induces cleavage of the γ-subunit at sites in the vicinity of the furin cleavage site.

MATERIALS AND METHODS

DNA constructs, site directed mutagenesis, and cRNAs. The generation of cDNAs and cRNAs for mouse α-, β-, and γ-ENaC was previously described (3, 30). Using a PCR-based approach, we generated mutant γ-subunits (γΔ172–182, γRKRR186QQQQ and γK173Q, K175Q, R177Q, RRKR186QQQQ) resistant to prostasin-dependent cleavage at RKRR186, and lacking either the tract between G172 and G182 (Δ172–182) or the three basic residues (K173, K175, R175) proximal to the prostasin-dependent cleavage site (K173Q, K175Q, R177Q) (3, 36). Wild-type γ, the furin consensus site mutants γR143A and γRKRR143QQQQ, the prostasin-dependent cleavage mutant γRKRR186QQQQ, and the mutant γK173Q,K175Q,R177Q,RRKR186QQQQ were double-epitope tagged [aminog terminal hemagglutinin (HA) and carboxyl terminal V5]. The cDNA for the mouse ortholog of human TMPRSS4 was obtained from Open Biosystems (Huntsville, AL).

EXPERIMENTAL DESIGN. Stage V-VI oocytes from Xenopus laevis were harvested according to a protocol approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee. Oocytes were injected with mouse TMPRSS4 cRNA (3 ng) and ENaC α-, β-, and γ-subunit cRNAs (1 ng each) (3, 8). Two-electrode voltage clamping was performed using a recording solution containing the following (in mM): 110 NaCl, 2 KCl, 1.54 CaCl2, 10 HEPES, pH 7.4, as previously described (8). At 29–41 h after injection, currents were measured at −60 mV before and after the addition of amiloride (10 μM). ENaC-mediated Na+ currents were determined as the amiloride-sensitive component of the current.

Detection of the surface pool of ENaC in oocytes. Biochemical characterization of the surface pool of ENaCs was performed as previously described (3, 17, 30). Oocytes were injected with cRNAs for wild-type α- and β-subunits, and an epitope-tagged mutant or wild-type γ-subunit (HA-γ-V5) with or without TMPRSS4, as described above. At 31–43 h postinjection, oocytes (15–30 per group) were placed in ice-cold modified Barth’s saline (in mM): 88 NaCl, 1 KC1, 2.4 NaHCO3, 15 HEPES, 0.3 Ca(NO3)2, 0.41 CaCl2, 0.82 MgSO4, pH 7.4. Surface proteins were biotinylated with the membrane-impermeant sulfo-NHS-SS-biotin, and surface biotinylated proteins were recovered with streptavidin-conjugated beads (Thermo Scientific Pierce, Rockford, IL). Surface γ-subunits were detected by immunoblotting with anti-V5 antibodies, as described previously (3, 17, 30).

Data and statistical analyses. Functional data are expressed as means ± SE (n), where n is the number of independent experiments analyzed. Statistical comparisons between groups were performed using Student’s t-test. A P < 0.05 was considered statistically significant.

RESULTS

TMPRSS4-dependent activation of ENaC and cleavage of the γ-subunit are reduced by mutation of the prostasin cleavage site at RKRR186. To determine whether mouse TMPRSS4 coexpression activates mouse ENaC, Xenopus laevis oocytes were injected with cRNAs for mouse ENaC (non-tagged α and β, HA-γ-V5), with or without mouse TMPRSS4. Measurement of amiloride-sensitive currents the following day revealed a 2.0 ± 0.1-fold (P < 0.0001 Student’s t-test) increase in ENaC activity when coexpressed with TMPRSS4 compared with ENaC expressed alone, consistent with previous reports of TMPRSS4-dependent activation of ENaC (Fig. 1) (15, 42).

To determine whether TMPRSS4 induces cleavage of the γ-subunit at a site distal to the furin cleavage site at RKRR143, oocytes expressing ENaC (non-tagged α and β, HA-γ-V5), with or without TMPRSS4, were treated with a membrane-impermeant biotinylation reagent, and biotinylated proteins were recovered with streptavidin-conjugated beads for analysis of the epitope-tagged γ-subunit. Immunoblot analyses of biotinylated proteins from oocytes expressing ENaC alone revealed the expected full-length γ-subunit (93 kDa) and the furin-cleaved carboxyl terminal fragment (75 kDa) when probed with anti-V5 antibodies, as well as a broad heterogeneous band just below the 75-kDa fragment (Fig. 2). Analysis of biotinylated ENaCs from oocytes coexpressing TMPRSS4 revealed the 93- and 75-kDa forms of the γ-subunit, as well as an additional, clearly demarcated band migrating at ∼70 kDa. No bands were detected from noninjected oocytes. Interestingly, the ∼70-kDa form of the γ-subunit produced by coexpression with TMPRSS4 was similar in size to the unique carboxyl
pressing TMPRSS4 and αβγRKRK186/QQQQ, we found that the well-defined ~70-kDa fragment of γ was notably reduced compared with levels in oocytes coexpressing wild-type ENaC and TMPRSS4 (Fig. 2, A and B, compare lanes 2 and 4). However, the broad heterogeneous band just below the 75-kDa fragment was still present. As the activation of αβγRKRK186/QQQQ by TMPRSS4 coexpression was significantly less than that of wild-type ENaC, we considered the possibility that TMPRSS4-dependent cleavage of the γ-subunit also occurred at adjacent basic residues proximal to the prostasin-dependent cleavage site (γRKRK186).

**F3**

**TMPRSS4-dependent activation and cleavage of ENaC is fully blocked by the mutation of basic residues in the tract K173-K186 in the γ-subunit.** Our laboratory has previously shown that combined furin and prostasin-dependent cleavage of the γ-subunit activates the channel by releasing an inhibitory tract of 43 residues (3): E144AGSMRSTWEGTPPR158FLNLIP LLVF168NE173GK175AR177DFFTGRKRK186.

**Fig. 1.** Transmembrane protease serine 4 (TMPRSS4)-dependent activation of the epithelial sodium channel (ENaC) is reduced by the mutation γRKRK186/QQQQ. Xenopus oocytes were injected with cRNAs for nontagged mouse α- and β-subunits and a double-epitope-tagged wild-type (WT) or mutant γ-subunit, with or without TMPRSS4. Amiloride-sensitive currents were measured the following day by two-electrode voltage clamp at −60 mV. The average current for WT ENaC was −2.2 ± 0.1 μA (n = 59) and for αβγRKRK186/QQQQ was −2.9 ± 0.2 μA (n = 71). Coexpression of TMPRSS4 and αβγ-ENaC resulted in an average 2.0 ± 0.1-fold increase in normalized current compared with WT ENaC alone (***P < 0.0001, Student’s t-test, ENaC + TMPRSS4 vs. ENaC alone). Currents were normalized to the average amiloride-sensitive current for WT or mutant channels in the absence of TMPRSS4 each experimental day. Coexpression of TMPRSS4 and αβγRKRK186/QQQQ resulted in an average 1.2 ± 0.7-fold increase in normalized current compared with αβγRKRK186/QQQQ alone (***P < 0.05 Student’s t-test). The TMPRSS4-dependent fold activation of WT αβγ-ENaC was significantly greater than TMPRSS4-dependent fold activation of αβγRKRK186/QQQQ (P = 2.5 × 10−6, Student’s t-test; n = 67–69).

To determine whether mouse TMPRSS4 coexpression activates the channel by inducing cleavage of mouse ENaC at the prostasin-dependent cleavage site, we coexpressed TMPRSS4 with ENaC containing the mutant epitope-tagged γ-subunit that blocks prostasin-dependent cleavage (γRKRK181/186/QQQQ). Measurement of amiloride-sensitive currents the following day revealed a modest, but significant 1.2 ± 0.7-fold (P < 0.05, Student’s t-test) increase in ENaC activity when αβγγRKRK186/QQQQ was coexpressed with TMPRSS4, compared with mutant channel expressed alone (Fig. 1). When we analyzed the surface biotinylated γ-subunits from the same batches of oocytes coexpressing TMPRSS4 and αβγγRKRK186/QQQQ, we found that the well-defined ~70-kDa fragment of γ was notably reduced compared with levels in oocytes coexpressing wild-type ENaC and TMPRSS4 (Fig. 2, A and B, compare lanes 2 and 4). However, the broad heterogeneous band just below the 75-kDa fragment was still present. As the activation of αβγγRKRK186/QQQQ by TMPRSS4 coexpression was significantly less than that of wild-type ENaC, we considered the possibility that TMPRSS4-dependent cleavage of the γ-subunit also occurred at adjacent basic residues proximal to the prostasin-dependent cleavage site (γRKRK186).

**TMPRSS4-dependent cleavage of the γ-subunit is reduced by the mutation γRKRK186/QQQQ.** Mouse αβγ or αβγγRKRK186/QQQQ was expressed with or without TMPRSS4 in *Xenopus* oocytes, as described in the legend to Fig. 1. Both the WT and mutant (Mut) γ-subunits had amino-terminal hemagglutinin (HA) and carboxy-terminal V5 epitope tags. Oocytes were treated with the membrane-impermeant sulfo-NHS-SS-biotin before solubilization. Surface proteins were precipitated with streptavidin-conjugated beads and analyzed by immunoblotting with anti-V5 antibodies. A: an immunoblot representative of three experiments is shown. Bands representing full-length (93 kDa) and furin-cleaved (75 kDa) γ-subunits were present in all lanes. An additional band at 70 kDa was present when TMPRSS4 was coexpressed with WT channels. In contrast, a broad heterogeneous band just below the 75 kDa fragment was present when TMPRSS4 was coexpressed with αβγγRKRK186/QQQQ. Apparent molecular mass (in kDa) on the left indicates carboxy-terminal V5-tagged cleavage fragments. UI refers to un.injected oocyte controls (lane 5). Numbers on the right refer to the mobility of the Bio-Rad mass markers in kDa. B: a side-by-side scan of lanes 1–4 from the same immunoblot using Bio-Rad Quantity One software is provided with alignment of the major bands representing the three forms of γ at 93 kDa (γ93), 75 kDa (γ75), and 70 kDa (γ70). The x-axis is the relative mobility of the bands within the scanned portion of the blot. Rf, retention factor.
Our laboratory previously reported that the key residues for channel inhibition resided within an 11-mer tract, \( \gamma_{172-182} \), \( \gamma_{172} \), \( \gamma_{173-176} \), and distal to the key 11-residue inhibitory tract, we generated a mutant where \( \gamma_{172-182} \) was K173Q,K175Q,R177Q,RKRK186QQQQ. \(
\begin{align*}
\text{Normalized current} \\
\text{TMPPRSS4} & \quad \text{a\(\beta\)\(\gamma\) (WT)} \\
\text{a\(\beta\)\(\gamma\)172-182, RKRK186QQQQ} & \quad \text{a\(\beta\)\(\gamma\)172-182, RKRK186QQQQ}
\end{align*}
\)

Fig. 3. TMPRSS4-dependent activation of ENaC is abolished by the \( \gamma_{172-182}/\text{RKRK186QQQQ} \) mutation. Xenopus oocytes were injected with cRNAs for nontagged \( \alpha \) and \( \beta \)-subunits and nontagged WT or mutant \( \gamma \)-subunit, with or without TMPRSS4. Amiloride-sensitive currents were measured the following day by two-electrode voltage clamp at \(-60\) mV. Currents were normalized to the average amiloride-sensitive current for WT or mutant channels in the absence of TMPRSS4 each experimental day. The average current for WT \( \alpha\beta\gamma \) was \(-2.1 \pm 0.2\) \( \mu \)A (\( n = 10 \)) and for \( \alpha\beta\gamma_{172-182} \) \( \text{RKRK186QQQQ} \) was \(-3.1 \pm 0.3\) \( \mu \)A (\( n = 20 \)). Coexpression of TMPRSS4 and \( \alpha\beta\gamma \) resulted in an average 1.9 \pm 0.2-fold increase in normalized current compared with WT ENaC alone (\( **P < 0.001 \), Student’s \( t \)-test, ENaC + TMPRSS4 vs. ENaC alone). Coexpression of TMPRSS4 did not significantly alter whole cell currents in oocytes expressing \( \alpha\beta\gamma_{172-182} \) \( \text{RKRK186QQQQ} \) (\( n = 16 \)). The fold-change in current observed with coexpression of TMPRSS4 was 0.72 \pm 0.07 (\( *P < 0.01 \), Student’s \( t \)-test). The TMPRSS4-dependent fold activation of WT \( \alpha\beta\gamma \)-ENaC was significantly greater than TMPRSS4-dependent fold change in current observed with \( \alpha\beta\gamma_{172-182} \) \( \text{RKRK186QQQQ} \) (\( P = 6.2 \times 10^{-5} \), Student’s \( t \)-test; \( n = 16 \)).

We found that the channel was modestly reduced 0.7 \pm 0.1-fold (\( P = 0.01 \), Student’s \( t \)-test) compared with expression of \( \alpha\beta\gamma_{172-182} \) \( \text{RKRK186QQQQ} \) ENaC alone (Fig. 3). In parallel control experiments, coexpression of TMPRSS4 with wild-type ENaC significantly increased channel activity 1.9 \pm 0.2-fold (\( P < 0.001 \), Student’s \( t \)-test) compared with expression of wild-type ENaC alone (Fig. 4). In contrast, coexpression of TMPRSS4 with wild-type ENaC significantly increased channel activity 3.2 \pm 0.7-fold (\( P = 0.01 \), Student’s \( t \)-test) compared with expression of ENaC alone.

Given the lack of activation of the channel mutated at seven basic residues (\( \gamma_{173Q,K175Q,R177Q,RKRK186QQQQ} \)) by TMPRSS4 coexpression, we examined whether TMPRSS4 induced cleavage of this mutant \( \gamma \)-subunit at sites distal to the furin cleavage site (Fig. 5). When surface biotinylated proteins were analyzed by immunoblotting with anti-V5 antibodies, we found the 93-kDa full-length and 75-kDa furin-cleaved carboxyl terminal fragment in all extracts. The TMPRSS4-dependent \(~70\) kDa cleavage fragment was present only when TMPRSS4 was coexpressed with wild-type ENaC, but not when it was coexpressed with \( \alpha\beta\gamma_{K173Q,K175Q,R177Q,RKRK186QQQQ} \).

We conclude from these data that TMPRSS4 induces cleavage of the \( \gamma \)-subunit at multiple sites distal to both the furin cleavage site and the 11-mer inhibitory tract \( (\gamma_{R158-F168}) \), thereby releasing the inhibitory \( \gamma \)-subunit and activating the channel.

**TMPRSS4-dependent activation of ENaC is blocked by mutation of four basic residues associated with the furin consensus site.** García-Caballero et al. (15) previously reported that human TMPRSS4-dependent activation of rat ENaC was blocked by the mutations \( \gamma_{R138A} \) or \( \gamma_{R138Q} \), but not \( \gamma_{R138K} \), at the P1 position of the furin-consensus site \( (\gamma_{RKRR138}) \) in rat ENaC. They also observed that the mutation \( \gamma_{R138A} \) blocked a TMPRSS4-dependent increase in levels of the 75-kDa COOH-terminal fragment of \( \gamma \) normally associated with cleavage by furin, as
activity when either wild-type ENaC (2.0 ± 0.6-fold) or αβγR143A (3.0 ± 1.3-fold) was coexpressed with TMPRSS4, compared with channel expressed alone (Fig. 6, A and B, P < 0.01 for both, Student’s t-test). However, mutant ENaC containing γRKRR143QQQQ was not significantly activated by coexpression of TMPRSS4 compared with channel expressed alone (0.84 ± 0.2-fold, P = 0.1, Student’s t-test), indicating that additional basic residues in the furin consensus site could represent sites for TMPRSS4-dependent cleavage.

**DISCUSSION**

We observed a two- to threefold activation of mouse ENaC by coexpression of mouse TMPRSS4 in *Xenopus* oocytes, which correlated with the production of a unique ~70-kDa carboxyl-terminal fragment from the γ-subunit. Similar analyses of channels with mutant γ-subunits led us to conclude that TMPRSS4 activates ENaC by inducing cleavage of γ distal to the furin cleavage site within a polylbasic tract, γK173-K186, thereby releasing peptides containing the key 11-mer inhibitory tract γR158-F168 from the γ-subunit (see Fig. 7). These channels presumably have γ-subunits that have also been cleaved within the vicinity of the furin cleavage site.

Our laboratory previously observed that mutation of a polylastic tract (mouse γKRR186) blocked prostasin-dependent activation of mouse ENaC and γ-subunit cleavage when mouse prostasin was coexpressed with mouse αβγRKRR186QQQQ (3). Our data suggest that TMPRSS4 preferentially cleaves at γRKRR186, but also cleaves the γ-subunit at adjacent basic

![Fig. 5. TMPRSS4-dependent cleavage of γ-ENaC is reduced by the mutation γK173Q,K175Q,R177Q,RRKR186QQQQ. WT αβγ or mutant (αβγK173Q,K175Q,R177Q,RRKR186QQQQ) channels were expressed with or without TMPRSS4 in *Xenopus* oocytes, as described in the legend to Fig. 4. Both WT and mutant γ-subunits had amino-terminal HA and carboxyl-terminal V5 epitope tags. Oocytes were treated with the membrane-impermeant sulfo-NHS-biotin before solubilization. Surface proteins were precipitated with streptavidin-conjugated beads and analyzed by immunoblotting with anti-V5 antibodies. A: an immunoblot representative of three experiments is shown. Bands representing full-length (93 kDa) and furin-cleaved (75 kDa) γ were present in all lanes. An additional band at 70 kDa was present when TMPRSS4 was coexpressed with WT channels, but was absent when TMPRSS4 was coexpressed with αβγK173Q,K175Q,R177Q,RRKR186QQQQ. Apparent mass (in kDa) on the left indicates carboxyl-terminal V5-tagged cleavage fragments of γ. Numbers on the right refer to the mobility of the Bio-Rad mass markers in kDa. UI refers to un.injected control oocytes. B: a side-by-side scan of lanes 1–4 from the same immunoblot using Bio-Rad Quantity One software is provided with alignment of the major bands representing the three forms of γ at 93 kDa (γ93), 75 kDa (γ75), and 70 kDa (γ70). The x-axis is the relative mobility of the bands within the scanned portion of the blot. C: an immunoblot of whole cell lysates from A were probed with an anti-β-actin antibody as a loading control.

![Fig. 6. TMPRSS4-dependent cleavage of γ-ENaC is reduced by the mutation γRKRR143QQQQ, but not by γR143A. *Xenopus* oocytes were injected with cRNAs for nontagged α- and β-subunits and a double-epitope-tagged WT or mutant γ-subunit, with or without TMPRSS4. Amiloride-sensitive currents were measured the following day by two-electrode voltage clamp at −60 mV. Currents were normalized to the average amiloride-sensitive current for WT or mutant channels in the absence of TMPRSS4 each experimental day. The average current for WT αβγ was −2.8 ± 0.4 μA (n = 36), −1.7 ± 0.4 μA (n = 20) for αβγR143A, and −1.9 ± 0.4 μA (n = 30) for αβγRKRR143QQQQ. Coexpression of either WT channels (n = 35) or αβγR143A (n = 24) with TMPRSS4 resulted in an average 2.0 ± 0.6-fold or 3.0 ± 1.3-fold increase in normalized current compared with WT or mutant channel alone, respectively (*P < 0.01, Student’s t-test, ENaC + TMPRSS4 vs. ENaC alone). Coexpression of TMPRSS4 did not significantly alter whole cell currents in oocytes expressing αβγRKRR143QQQQ channels (n = 22). The fold change in current observed with coexpression of TMPRSS4 was 0.84 ± 0.2 (P = 0.1, Student’s t-test).
residues, including γK173, γK175, or γR177. The well-defined ~70-kDa γ fragment associated with TMPRSS4-dependent activation of ENaC is consistent with TMPRSS4-dependent cleavage of wild-type channels at γRKRK186. In contrast, the broad heterogeneous band below the 75-kDa band that we observed with coexpression of the αβγRKRK186QQQQ mutant and TMPRSS4 (Fig. 2) is consistent with TMPRSS4-dependent cleavage at one or more basic residues preceding the γRKRK186 tract, including γK173, γK175, or γR177.

García-Caballero et al. (15) previously reported that human TMPRSS4-dependent activation of rat αβγ-ENaC correlated with production of a unique ~73-kDa carboxy-terminal fragment of the γ-subunit, but the site of cleavage distal to the furin cleavage site (γRKRK138) was not defined. In fact, the authors suggested that mutation of the furin site (γRKRK181QQQQ) and basic residues distal to this site (γK185Q, γK189Q, γK200Q, and γK201Q) did not block channel activation by TMPRSS4 activation. They observed that channels with a mutation of the γRKRK181 tract in the rat γ-subunit were robustly activated by coexpression with human TMPRSS4 (15). This observation contrasts with the very modest activation of mouse αβγRKRK186QQQQ channels that we noted with mouse TMPRSS4 and suggests that there may be some degree of species specificity regarding the efficacy of TMPRSS4-dependent cleavage of the basic residues immediately preceding the γRKRK186 tract.

Our laboratory previously reported that the γ-subunit is cleaved by the TGN-resident protease furin and suggested that this likely occurs as ENaCs transit through the TGN (18). We found that channels with a mutation of the furin consensus site P1 position (γR143A) were activated in oocytes coexpressing TMPRSS4 (Fig. 6), despite that fact that our laboratory has previously shown that this mutation prevents furin-dependent processing of the γ-subunit (18). As γR143 is within a polybasic tract, we postulated that TMPRSS4 is inducing cleavage within this polybasic tract, even in the presence of the γR143A mutant. Our observation that a mutation of all four basic residues in the mouse γ-subunit (γRKRK143QQQQ) blocked mouse TMPRSS4-dependent activation of ENaC suggests that TMPRSS4 induces cleavage within the polybasic tract (γRKRK143) encompassing the furin cleavage site. These results suggest that the key 11-mer inhibitory tract could be released by a TMPRSS4-dependent cleavage of γ at two polybasic sites in the absence of furin cleavage. In this regard, immature channels that lack cleavage by furin have been previously identified at the surface of intact rat kidney tubules (14).

García-Caballero et al. (15) also suggested that TMPRSS4 induces processing of the γ-subunit at the furin cleavage site. They observed that a mutation of the furin cleavage site in the rat γ-subunit (γR138Q or γR138A) blocked channel activation by TMPRSS4, although channels with a γR138K mutation were activated by the protease. The γR138K mutant was not processed by furin, but was processed by TMPRSS4 when coexpressed with ENaC. The difference between the observations of García-Caballero et al. (15) and our data regarding TMPRSS4-dependent activation of channels with the γR138A mutant may simply reflect minor differences due to species variation in channel structure in the vicinity of the furin consensus site. Nevertheless, the fact that a furin site mutation prevented channel activation is not surprising and supports our hypothesis that cleavage at two distinct sites in the γ-subunit is required for channel activation.

TMPRSS4 is one of a growing number of proteases with the ability to activate ENaC by direct or indirect cleavage of the γ-subunit at sites distal to the key inhibitory tract (mouse γR158-F168). This group of proteases includes plasmin, prostatin, pancreatic elastase, and neutrophil elastase (1, 3, 17, 30). The region distal to the key inhibitory tract is a protease-sensitive region that is highly conserved across mammalian species. This region is nearly identical in rats, mice, and humans (Fig. 7) and may be a target for aberrant proteolytic activation of ENaC in human disease. For instance, the activation of ENaC by the presence of plasmin in the urinary space has been implicated in the pathogenesis of volume overload seen in proteinuric states (22, 28–30, 40). In cystic fibrosis, mucociliary dysfunction is thought to result from enhanced ENaC activity, reflecting the presence of elastase and possibly other proteases within the airway (6, 26, 40). Furthermore, an alteration of the balance of proteases and protease inhibitors may contribute to channel activation in cystic fibrosis (26, 41). Proteases that have a role in the “normal” physiological regulation of ENaC activity by cleaving the γ-subunit and releasing the γ inhibitory peptide remain to be established.

TMPRSS4 has been implicated in a wide range of biological processes. Morpholino knockdown of the homolog of TMPRSS4 in zebrafish embryos results in severe defects in organogenesis and cell adhesion (27), while knockdown of the protease inhibitor HAI-1 in a lung carcinoma cell line produced an epithelial-to-mesenchymal transition that was mediated by both TMPRSS4 (CAP2) and matr_transfected (CAP3) (11). Vuagniaux et al. (42) previously reported that CAP1...
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(prostasin), CAP2 (TMPRSS4), CAP3 (matriptase), and the α-subunit of ENaC are all expressed in epithelia known to exhibit amiloride-sensitive Na⁺ transport. This group speculated that these three proteases constitute a catalytic cascade (2). Future studies should reveal if cleavage of ENaC by a TMPRSS4-dependent pathway has a role in the proteolytic processing of channels under normal physiological conditions or in specific pathological states.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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