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INVITED REVIEW

Regulation of the epithelial Na^+ channel and airway surface liquid volume by serine proteases

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Abstract Mammalian airways are protected from infection by a thin film of airway surface liquid (ASL) which covers airway epithelial surfaces and acts as a lubricant to keep mucus from adhering to the epithelial surface. Precise regulation of ASL volume is essential for efficient mucus clearance and too great a reduction in ASL volume causes mucus dehydration and mucus stasis which contributes to chronic airway infection. The epithelial Na^+ channel (ENaC) is the rate-limiting step that governs Na^+ absorption in the airways. Recent in vitro and in vivo data have demonstrated that ENaC is a critical determinant of ASL volume and hence mucus clearance. ENaC must be cleaved by either intracellular furin-type proteases or extracellular serine proteases to be active and conduct Na^+, and this process can be inhibited by protease inhibitors. ENaC can be regulated by multiple pathways, and once proteolytically cleaved ENaC may then be inhibited by intracellular second messengers such as cAMP and PIP_2. In the airways, however, regulation of ENaC by proteases seems to be the predominant mode of regulation since knockdown of either endogenous serine proteases such as prostasin, or inhibitors of ENaC proteolysis such as SPLUNC1, has large effects on ENaC activity in airway epithelia. In this review, we shall discuss how ENaC is proteolytically cleaved, how this process can regulate ASL volume, and how its failure to operate correctly may contribute to chronic airway disease.

Keywords Na^+ channel · Cystic fibrosis · Epithelial transport · Fluid absorption · Lung liquid

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Introduction

The epithelial Na^+ channel (ENaC) is expressed in the apical plasma membrane of several tissues including the colon, kidney, and lung and constitutes the rate-limiting step for Na^+ absorption [84, 122]. ENaC in the airways is distinguished from ENaC expressed in other tissues by insensitivity to aldosterone [136]. Instead, ENaC in airway epithelia is regulated by glucocorticoids and so its expression is linked to changes in inflammation rather than changes in blood pressure/volume [136, 151]. ENaC can also be controlled by intracellular factors including pH, lipids (PIP_2 and PIP_3), and protein kinase A [91, 112, 166] which may affect the number of ENaCs inserted into the plasma membrane as well as affect gating [91, 156, 166]. The conducting airways are the primary interface between the extracellular environment and the lung and play a major role in host defense [9, 34, 48]. Mucus clearance is a central component of the innate defense system of the lung [35, 76]. During normal mucus clearance, inhaled

pathogens and particles become trapped in the mucus layer and are then expelled before they can colonize the airways [30]. The mucus layer is of variable height, and mucus is kept away from the predominantly ciliated airway epithelia by the presence of a ~7 μm periciliary liquid layer which, as the name suggests, surrounds the beating cilia and acts as a lubricant to keep mucus away from the epithelial cell surface. Together, these layers make up the airway surface liquid (ASL) [14]. The rate of mucociliary clearance is strongly influenced by the hydration state of the ASL and mucus layers [142]. Mucus hydration is set by the volume of liquid present on airway surfaces, which in turn may be modified by active ion transport processes in both the superficial epithelium and glands [7, 14, 157, 159]. Secretion is mediated by the apical membrane Cl^- channels TMEM16A and CFTR [3, 47] whilst absorption requires ENaC [123]. Intracellular second messengers play a large role in regulating these channels [3, 47, 63, 85, 90, 112]. However, in the last decade, soluble and epithelial membrane bound proteases have been found to regulate ENaC, which has opened up a whole new avenue of research [122, 123]. Channel-activating protease 1 (CAP1; prostasin) was first identified in *Xenopus laevis* oocytes [150] and, subsequently, CAP2 (transmembrane protease serine 4 [TMPRSS4]), CAP3 (matriptase or epithin), and human neutrophil elastase [22] as well as trypsin [150] were all found to activate ENaC. This review will focus on recent discoveries regarding the ability of these proteases and their inhibitors to affect ASL volume by varying the ENaC activity and on their potential clinical significance.

Structure of ENaC and non-proteolytic regulation

ENaC consists of three separate subunits termed α, β, and γ that are ~30% homologous to each other. These subunits form a channel that is highly selective for Na^+ over K^+ and is characterized by sensitivity to amiloride in the sub-micromolar range [122, 123]. Each subunit is composed of a large extracellular loop, which has been likened to a hand (Fig. 1). In addition, ENaC also has two transmembrane domains termed M1 and M2, respectively, and two short intracellular tails (Fig. 1). Site-directed mutagenesis has revealed that residues in the pre-M2 region of α, β, and γENaC subunits affect the channel conductance and amiloride binding [126]. Based on the recent crystal structure of the related acid-sensing channel (ASIC), the issue of ENaC subunit stoichiometry appears to be settled with ENaC presumably consisting of a 1α:1β:1γ complex [69]. However, ASIC is a homotrimer, and previous functional studies with ENaC subunits have suggested a heterotetrameric or even variable stoichiometry [2, 41, 80, 133]. When the αENaC subunit is injected into *Xenopus* oocytes alone, ~2% of current is seen relative to α, β, γENaC co-expression. In contrast, when the α, β or α, γ subunit combinations are co-injected, up to 15% of full activity is seen. The β or γ subunits do not produce currents when expressed alone and β, γ together induce ~2% of full activity, but only at several days post-injection [12]. Mueller et al. have reported that the C-terminus of

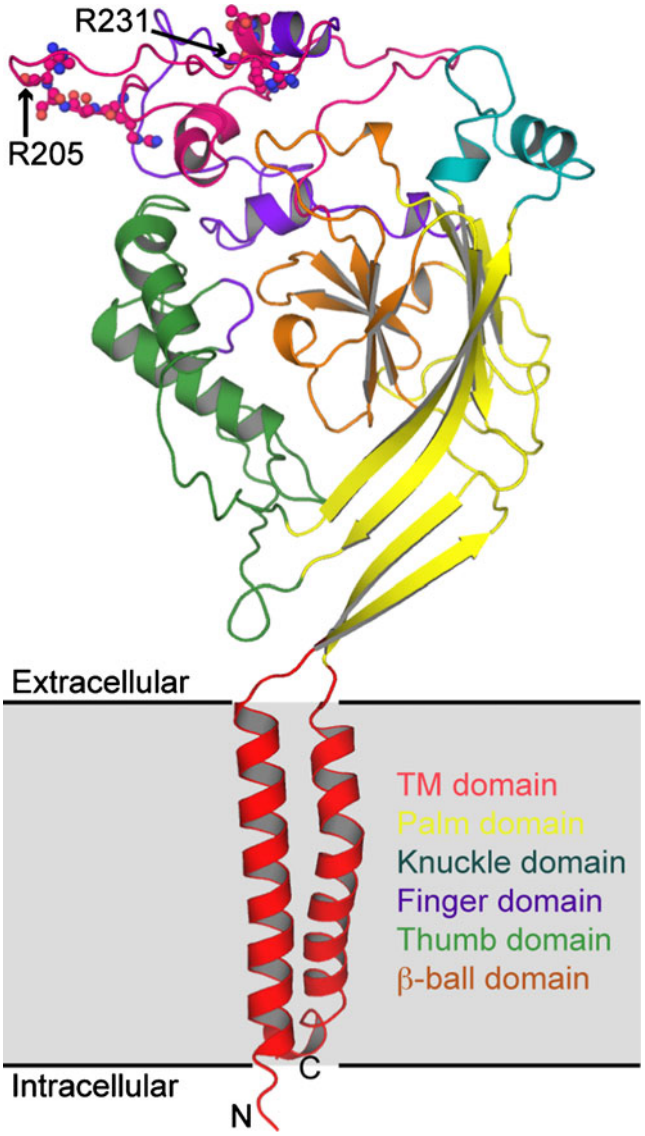


Fig. 1 Cartoon representation of the structural model of the rat αENaC subunit. Each ENaC subunit has two transmembrane regions, a large extracellular loop and a small "Pre-TM2" region (not represented in the model) that may form part of the selectivity filter [126]. For all the subunits, the C- and N-termini are intracellular. The sequence alignment for modeling the structure of rat αENaC was generated by extending the one provided by Stockand et al. [135]. A portion of the 'hypervariable region' was included in our alignment to account for the furin sites in this model [135]. The structural model was generated using Medusa [42, 163] with the crystal structure of cASIC as the template [69]. Regions that do not have a template were modeled as loops using Modeller [46]

αENaC plays a critical role in permitting exit of this channel from the endoplasmic reticulum [97]. Thus, αENaC may have an essential role in bringing the β and γ subunits to the plasma membrane, while the β and γ subunits are essential for stabilization of the multimer in the plasma membrane.

In addition to the well-characterized α, β, and γ subunits, a fourth ENaC subunit, termed δ, has been described [18, 155]. δENaC is highly expressed in the testis, ovary, pancreas, and brain and is also expressed in the nasal epithelium. The δENaC subunit shares considerable sequence similarity with the α subunit and forms functional ion channels together with the β and γ subunits in pancreas, testes, ovaries, and brain and in smaller amounts in heart, placenta, lung, liver, kidney, thymus, prostate, colon, and lymphocytes [8, 18, 71, 155, 161]. In *Xenopus* oocytes, expression of δ, β, γENaC results in an approximately tenfold increase in amiloride-sensitive current compared to α, β, γENaC. Consequently, differential expression of the α and δ subunits may provide an additional way of regulating ENaC activity and the δ subunit seems to promote constitutive cleavage of the γ subunit, which may favor the presence of active channels at the cell surface [56]. Thus, replacement of the α subunit with the δ subunit in the heterotrimeric channel may favor the constitutive activation of ENaC and may reduce the pool of near-silent plasma membrane channels that can be activated by extracellular proteases [56].

ENaC gating kinetics are characterized by long opening and closing times, [113] and structural studies have revealed two regions that are important for gating. The HG motif, so named because it possesses a glycine and a histidine in the amino terminus, is present in every ENaC/degenerin family member known to conduct ions and is involved in channel opening. Mutation of this residue in the βENaC subunit causes pseudohypoaldosteronism, a disease characterized by inactivity of ENaC [73, 134]. The DEG gating domain in the extracellular loop, close to the M2 region, was first described in degenerins channels of *Caenorhabditis elegans*, which are related to ENaC, and involves a critical serine residue [126]. Mutations in this region cause an increase in the open probability [72, 129].

Regulation of ENaC by intracellular 2nd messengers

Although it is perhaps surprising that ENaC is the sole ion channel responsible for apical Na^+ entry into airway epithelial cells, it is clear that this channel can undergo multiple modes of regulation. For example, in addition to being cleaved and activated by several different types of proteases, ENaC can also be regulated by intracellular second messengers. In non-cystic fibrosis (CF) human airways, ENaC is inactivated by agonists that raise cAMP, such as adenosine, forskolin, or isoproterenol [16, 75, 78]. In CF airways, which lack functional CFTR, increases in cAMP stimulate ENaC [16, 75, 78], suggesting that CFTR acts to limit ENaC activity in the airways. Using the patch clamp technique, Stutts et al. demonstrated that CFTR expression directly altered ENaC sensitivity to forskolin-induced changes in intracellular cAMP/PKA [137, 138]. Using the *Xenopus* oocyte expression system, it was reported that the intracellular domains of CFTR directly interacted with ENaC sufficiently to alter ENaC's sensitivity to cAMP [70, 86, 127]. However, since CFTR and ENaC are linked to the cytoskeleton, there may also be indirect interactions between these two ion channels [10, 68, 102].

A reduction in the intracellular Cl^- concentration following stimulation of either CFTR or ClC Cl^- channels has also been reported to lower ENaC activity, suggesting that ENaC is regulated by the intracellular Cl^- concentration [6, 70, 96]. Despite a precedence for this type of regulation of ENaC in mouse salivary duct epithelia [32], this hypothesis remains to be tested in the airways, and other investigators using *Xenopus* oocytes have reported that ENaC is still regulated appropriately following co-injection of αβγENaC and a non-Cl^--conducting CFTR mutant [139]. The interaction between these two ion channels may be variable, and in the sweat glands, where Cl^- absorption rather than Cl^- secretion occurs under basal conditions, ENaC is activated in parallel with CFTR rather than being inactivated [115]. Surprisingly, more than 20 years after the initial discovery that ENaC's sensitivity to cAMP is CFTR dependent, the molecular basis underlying this phenomenon has not been fully understood.

Activation of $P2Y_2$ receptors on the apical membrane of airway epithelial cells with ATP or UTP raises intracellular IP_3 and Ca^{2+}, in parallel with a fall in intracellular PIP_2 [36]. These changes then stimulate Ca^{2+}-mediated Cl^- secretion and inhibit ENaC-mediated Na^+ absorption [39, 67, 95]. The underlying Cl^- channel has only recently been cloned and is likely to be TMEM16A/Ano1 [25, 60, 128, 162]. TMEM16A is sensitive to changes in intracellular Ca^{2+} levels [25, 128, 162]. However, ENaC is not directly Ca^{2+} sensitive and instead is activated by anionic lipids [91, 165]. Phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 3,4,5-trisphosphate (PIP_3), and phosphatidylserine were shown to prevent ENaC run-down in excised patches of plasma membrane [91] and PIP_2 was shown to bind to all the three ENaC subunits [165], resulting in ENaC activation. Tong and Stockland demonstrated that the epidermal growth factor receptor decreases plasma membrane PIP_2 levels to inhibit ENaC [148], whilst Kunzelmann et al. demonstrated that the stimulation of purinergic receptors decreases PIP_2 levels to inhibit ENaC [82]. PIP_2 is the substrate that is used by phospholipase C

to form IP_3 following stimulation of $P2Y_2$-R [152]. In addition, numerous other regulators of ENaC exist, including, but not limited to, the epidermal growth factor receptor [148], endothelin receptors [52], epinephrine [110], as well as bacterial proteins and respiratory viruses [83, 87, 132].

Types of proteases that can cleave ENaC

In contrast to intracellular 2nd messengers, which can affect either the number of ENaC channels in the plasma membrane (n) or their open probability (P_o), proteolytic cleavage of ENaC is thought to have a greater effect on P_o. Three different types of proteases have been shown to cleave ENaC: (1) intracellular convertase-type proteases such as furin [64], (2) extracellular, cell-attached proteases, which can either be membrane spanning (with either the C or N terminus located intracellularly) [110] or glycosyl-phosphatidylinositol (GPI)-anchored [150], and (3) soluble proteases including trypsin and neutrophil elastase [22, 150]. A schema of how proteases may cleave ENaC to increase P_o is shown in Fig. 2, whilst an overview of how ENaC may be cleaved by different proteases is shown in Fig. 3. Extracellular proteases may be more influenced by changes in the ASL microenvironment, especially during chronic airway disease, since the activity of these proteases can be affected by changes in ASL pH and/or the presence of extracellular protease inhibitors. However, intracellular proteases may also be affected by such changes as the lack of CFTR [103].

Furin-type proteases Convertases are so named since they process precursor proteins into biologically active forms. Furin is a Ca^{2+}-dependent serine endoprotease that cleaves precursor proteins at their paired basic amino acid processing sites [104]. The minimal consensus sequence for furin cleavage is R/K-X-X-R where X is any amino acid [65]. Endogenous, furin-dependent intracellular cleavage of ENaC was first suggested by Hughey et al. [64]. These investigators demonstrated that expression of α, β, or γENaC alone in MDCK cells showed full-length subunits by Western blot (~93–96 kDa). In contrast, co-expression of these three subunits resulted in the appearance of smaller ~65-kDa bands of α and γENaC subunits, but not the βENaC subunit, as well as the full-length bands [64]. The mass of the α and γ fragments matched the predictions for cleavage at two recognized furin sites in the α subunit and a single site in the γ subunit (see Fig. 1 for the location of the furin cleavage sites). These investigators then demonstrated by mutagenesis that cleavage of the α subunit had the greatest effects on macroscopic ENaC currents [65]. Paradoxically, cleavage of primarily the γ subunit by extracellular serine proteases also exerts large effects on ENaC function, and the

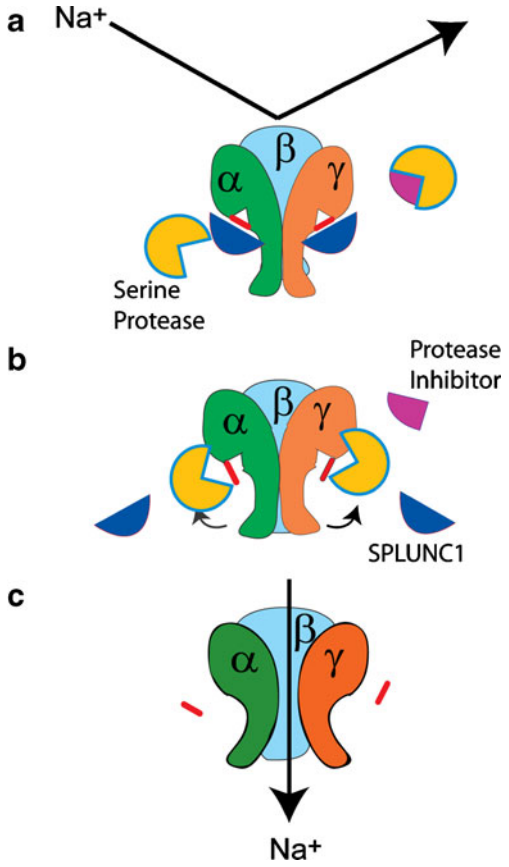


Fig. 2 Proteolytic cleavage allows Na^+ conduction through ENaC. ENaC is a heterotrimer consisting of α, β, and γ subunits that together form the functional channel. **a** In the absence of proteolysis, ENaC is uncleaved and cannot conduct Na^+. **b** After proteolytic cleavage, the "inhibitory" tracts from the extracellular loops of the α and γ subunits are removed, possibly leading to a conformational change in the channel, which can now conduct Na^+ (**c**)

difference between these two findings has yet to be explained [59]. Furin is predominantly located in the Golgi apparatus (Fig. 2). However, furin family convertases may also be active at the cell surface and could theoretically cleave ENaC whilst it is in or near the plasma membrane [147]. Thus, furin-dependent activation of ENaC has been suggested to provide a pool of ENaCs, whose activity can be further modified by membrane-associated CAPs and/or by intracellular second messengers such as PIP_2 and PKA [109].

GPI-anchored proteases Serine proteases are named because at least one of the amino acids in their active site is a serine and this does not refer to their target amino acid sequence, which can vary considerably depending on the serine protease in question. The first channel-activating protease (CAP1) discovered to activate ENaC was ultimately identified as murine prostasin (TMPRSS8) [154], and human prostasin was found to similarly activate ENaC [44]. Co-expression of the serine protease CAP1/prostasin,

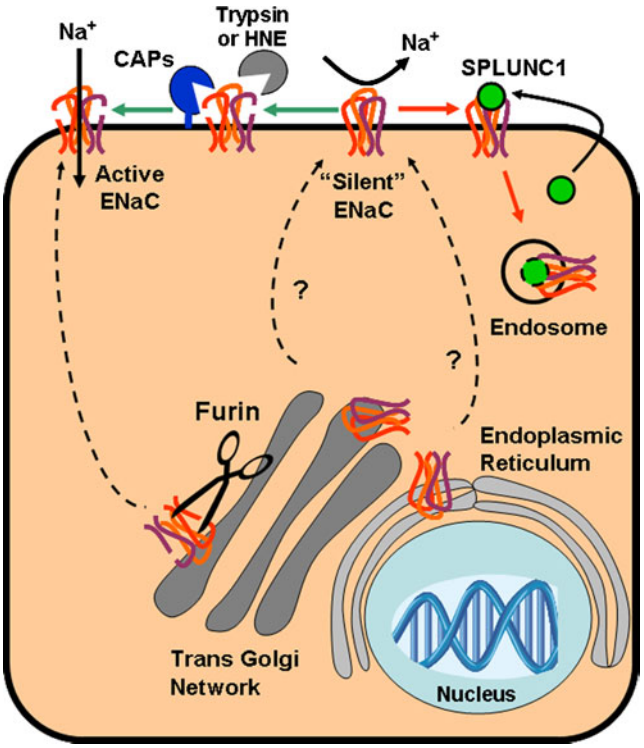


Fig. 3 Speculation on ENaC proteolysis and how SPLUNC1 may inhibit this process. Uncleaved ENaCs can be cleaved in the trans-Golgi network by furin. These cleaved ENaCs are then transported to the plasma membrane in a highly regulated fashion (not shown, see [20, 131]) where they are ready to conduct Na^+. An additional pool of newly formed ENaCs are able to bypass the furin cleavage step through an as-yet undetermined mechanism and directly inserted into the plasma membrane as uncleaved, inactive "silent" channels. These silent ENaCs can then be protetolytically cleaved by cell-attached, extracellular, channel-activating proteases (CAPS) or by soluble proteases such as trypsin or human neutrophil elastase and converted into active ENaC channels. Alternatively, silent ENaCs may bind to SPLUNC1, which is secreted from some epithelial cells in an autocrine/paracrine fashion. SPLUNC1 then triggers the internalization of ENaC through an unknown process, making ENaC unavailable for cleavage by CAPs or soluble proteases. Whether these internalized ENaCs continue to bind to SPLUNC1 when internalized and whether they can be recycled to the plasma membrane or are targeted for lysosomal degradation is not known

cloned from the X*enopus* kidney epithelial cell line (A6 cells), with ENaC in *Xenopus* oocytes, resulted in several fold increases in ENaC-mediated whole cell currents [150]. Subsequently, prostasin has been soundly implicated by siRNA-mediated knockdown [149] or by gene disruption in mice [111] in the regulation of ENaC in the lung. Interestingly, this effect may not rely on extracellular CAP1 activity, because in other studies catalytically inactive prostasin mutants fully stimulated ENaC [153]. However, incubation in media containing the Kunitz-type serine protease inhibitor aprotinin prevented ENaC activation by CAP1, perhaps indicating that CAP1 plays a non-catalytic role in a proteolytic cascade that targets ENaC (see below) [150]. This study found no effect of CAP1 on the number of channels at the plasma membrane, suggesting that CAPs increase ENaC activity by changing the open probability (P_O). Exogenous application of purified trypsin can mimic the actions of CAPs by activating ENaC in the oocyte expression model [150].

The human ortholog of CAP1 was found to be prostasin [44], which was originally cloned from bicarbonate-rich seminal fluid [164]. Prostasin is present in normal and CF airway epithelia [44], and Tong et al. demonstrated that knockdown of prostasin in a CF airway epithelial cell line lowered basal amiloride-sensitive current [149]. Since prostasin is GPI-anchored, it is cleaved by GPI-specific phospholipase C and can be secreted in addition to being membrane bound [29]. Thus, the (possibly indirect) activation of ENaC by prostasin may be relieved if prostasin is itself cleaved by phospholipase C and removed from the airway surface. While such a mode of regulation has been demonstrated in renal epithelia [140], it remains to be tested in the airways. If this happens in the airways, PLC-dependent regulation of ENaC may provide for an additional mode of regulation.

A polybasic stretch of residues in γ-ENaC was recently identified by mutagenesis as being required for CAP1/prostasin stimulation of ENaC [19]. Interestingly, Bruns et al. [19] reported that γ-ENaC processing occurs in oocytes expressing catalytically "dead" prostasin that could not directly cleave proteins following site-directed mutagenesis. Furthermore, this protein is maximally active at pH 9 and essentially inactive at pH 7 [164]. However, ASL pH is generally thought to be ≤pH 7, suggesting that prostasin may not be proteolytically active in the airway lumen [31]. Taken together, these data suggest that either (1) the catalytic mutant prostasin retains a portion of its protease activity or (2) prostasin may facilitate the activation of other channel-activating protease(s) by means other than its proteolytic capability. What is left unresolved from these studies, however, is the mechanism by which CAP1/prostasin stimulates ENaC. Bruns et al. proposed that prostasin cleaves γ-ENaC at a polybasic tract (RKRK) downstream from the γ-furin site and that prostasin worked in conjunction with furin to remove an inhibitory peptide from the γ-ENaC extracellular domain [19]. Interestingly, it has now been recognized that CAP1/prostasin stimulates ENaC independently of its own catalytic activity [150, 153]. Questions have been raised, but not settled, about the effectiveness of mutagenesis of the prostasin catalytic triad as a maneuver to completely eliminate prostasin catalytic activity [19], which would influence the interpretation of the earlier experiments. Most recently, Svenningsen and colleagues found that plasmin binds to prostasin and that this interaction is important for ENaC activation [140]. Although not tested or discussed by these authors, perhaps this role of prostasin does not require its catalytic activity

and could reveal that prostasin plays multiple roles in proteolytic regulation of ENaC, either as a protease targeting ENaC or as an anchor in a complex of ENaC and other proteases.

Trans-membrane serine proteases ENaC is also regulated by a variety of type II membrane-bound serine proteases whose N-termini are intracellularly located. CAP2 (TMPRSS4), CAP3 (matriptase), and human airway trypsin-like protease are all expressed in the airways [146]. CAPs 2 and 3 are known to regulate ENaC [153]. In contrast to CAP1, both CAP2 and CAP3 must possess catalytic activity to stimulate ENaC [4]. CAP2 cleaves ENaC at multiple sites in all three subunits, including cleavage at a conserved basic residue located in the vicinity of the degenerin site (α-K561, β-R503, and γ-R515) [49]. Multiple sites including the furin consensus sites in αENaC (R205/R231) and γENaC (R138) are responsible for the ENaC fragments observed in *Xenopus* oocytes co-expressing CAP2 [49]. However, the only cleavage site absolutely required for channel activation by CAP2 is the γENaC site R138 [49].

Soluble proteases The first soluble serine protease shown to affect ENaC was trypsin [150]. Pretreatment with this enzyme diminished the size of γ-ENaC from 87 kDa (full length) or 76 kDa (furin-cleaved) to 67 kDa in *Xenopus* oocytes. Diakov et al. [40] found that cleavage of the γENaC subunit was important for changes in overall ENaC conductance and that a single-point mutation at position 181 (γK181A) removed the stimulatory effect of serine proteases [40]. Interestingly, in oocytes expressing α, β, and γK181A ENaC, the 67-kDa band was present even in the absence of extracellular trypsin, suggesting that this mutant was spontaneously cleaved and it is likely that this mutation increases the specificity of γENaC for endogenous proteases. However, when the furin-insensitive mutation was introduced into γENaC along with the γK181A mutation (α, β, γP138A, K181AENaC), the gain of function associated with the γK181A mutation alone was absent, indicating that cleavage at the furin site must occur before cleavage at position 181 by serine proteases [40].

In *Xenopus* oocytes expressing α, β, γENaC or in airway epithelia natively expressing ENaC, a brief exposure to the soluble serine protease neutrophil elastase increased amiloride-sensitive currents approximately fivefold [22, 58]. The effects of neutrophil elastase were not potentiated by trypsin, suggesting that ENaC is maximally activated by either serine protease. Harris et al. found that brief exposure of ENaC expressing *Xenopus* oocytes to neutrophil elastase generated a new fragment of γ-ENaC in the surface pool, consistent with cleavage downstream of the γ-ENaC furin site [58]. Elastase inhibitors prevented this effect and the elastase-induced fragment appeared only in the pool of proteins biotinylated at the cell surface [58]. However, a contemporaneous study identified the specific residues V182 and V193 in human γENaC as being essential for ENaC stimulation by human neutrophil elastase, a location consistent with the γENaC fragment generated by elastase in ENaC-expressing *Xenopus* oocytes [49].

Mast cells can release soluble, chymotrypsin-like serine proteases [141], which may also be able to activate ENaC [40]. However, little is known about the effect of this protease on ENaC activity in the airways. In *Xenopus* oocytes expressing δ, β, γENaC, exposure to chymotrypsin resulted in a twofold increase in whole cell current, which was less than the effect on α, β, γENaC (fivefold), consistent with the hypothesis that δENaC is more likely to be constitutively cleaved than αENaC [56]. The increase in whole-cell current following chymotrypsin application coincided with the appearance of an approximately 20-kDa δENaC cleavage fragment. Similar to αENaC, the δ subunit requires co-expression with the γENaC subunit for activation by external proteases.

Proteolytic activation of near silent ENaC channels

It has been suggested that a subpopulation of ENaCs somehow bypass furin cleavage in the Golgi apparatus, and following their insertion into the plasma membrane these channels display very little or no activity. Accordingly, these channels have been termed ‘near silent’ ENaCs (Fig. 3) [21]. Distinct, basally active, silent and/or near-silent ENaC populations were distinguished by patch clamp [22]. The application of exogenous trypsin increased the open probability of near-silent ENaC more than 50-fold in outside-out patch recordings, increasing their activity towards that of basally active ENaCs [21]. Extensive washing did not reverse this process, suggesting that the activation was irreversible, as one would expect after proteolytic cleavage [21]. The increase in open probability occurred within seconds to several minutes after application of trypsin and could be inhibited by soybean trypsin inhibitor [21]. Blockade of trypsin-sensitive G protein-coupled proteinase-activated receptors did not affect the increase in open probability of ENaC. Taken together, these results suggested a direct effect of trypsin on P_o [21, 40].

In similar experiments, concentrations of neutrophil elastase aimed to reprise those seen in diseased lungs increased the activity of near-silent ENaCs approximately 100-fold in patches from mammalian NIH-3T3 fibroblast cells [22]. No significant effects of neutrophil elastase were observed on basally active ENaCs, again suggesting that neutrophil elastase only activates ENaCs which have not

been cleaved. Trypsin exposure following neutrophil elastase did not increase the amiloride-sensitive short-circuit current further, suggesting that these proteases share a common mode of action for increasing Na^+ transport, likely through proteolytic activation of ENaC. Great variability in the extent of ENaC stimulation by application of exogenous proteases has been reported and partly reflects different extents of proteolytic stimulation of ENaC existing in different experimental systems. In addition, varying extents of proteolytic stimulation of ENaC or of Na^+ absorption are consistent with the hypothesis that both intracellular, aprotinin-insensitive activation of ENaC prior to membrane insertion and extracellular activation of ENaC by serine proteases can occur. Since acute neutrophil elastase exposure rapidly diminishes mucus clearance rates in sheep [124], it is possible that a reserve of silent ENaCs exist in the plasma membrane of superficial airway epithelia under normal physiological conditions.

The relative contributions of α vs. γ subunit cleavage to macroscopic currents

The initial work of Hughey and Kleyman detected fragments of ENaC that matched the size of fragments predicted to arise from cleavage at furin consensus sequences in α and γENaC [64, 66]. Moreover, mutagenesis of these sites to prevent cleavage by furin reduced basal ENaC-mediated current which was then recovered by exogenous trypsin. The contributions of α and γ sites to this effect were disproportionate, with mutation of the cleavage sites in αENaC having the greatest effect on macroscopic currents. Although the validity of these observations has not been challenged (our unpublished data is the same), they are nonetheless difficult to reconcile with the overall body of subsequent work on non-furin type serine proteases that activate ENaC. For example, neutrophil elastase fully stimulates quiescent ENaC [22], and in two independent studies cleavage of γENaC at specific residues was found to be the requisite cleavage event for this activation [1, 58]. Prostasin-mediated stimulation of ENaC was also traced to cleavage of the γENaC subunit [19]. In addition, we reported that although CAP2 co-expression with ENaC generated fragments of α-ENaC predicted by cleavage at its two furin consensus sequences, mutation of those sites and prevention of cleavage was without effect on CAP2 stimulation of ENaC. In contrast, preventing cleavage of γENaC at its consensus furin sequence eliminated CAP2 activation of ENaC. Subsequently, much, if not all of the ability of trypsin [40] and plasmin [105] to stimulate ENaC have been traced to cleavage of γENaC. These findings with multiple non-furin serine proteases, each of which likely cleave ENaC only at the cell surface, are difficult to reconcile with the demonstrated importance of the α-subunit furin sites in earlier studies [26]. These findings clearly indicate that broad gaps in our knowledge exist, and the data may indicate as-yet, poorly understood contributions of αENaC to overall ENaC activity, perhaps relating to channel assembly, trafficking, or surface dwell time.

Protease inhibitors

Kunitz-type inhibitors In their seminal 1997 *Nature* paper, Vallet et al. [150] demonstrated that CAP1-dependent activation of ENaC could be prevented by pretreatment with the 6.5-kDa protease inhibitor aprotinin [150]. Bridges et al. found that, out of several serine protease inhibitors tested in human airway epithelial cultures, only inhibitors containing naturally occurring Kunitz domains inhibited ENaC currents [17]. Aprotinin, also known as bovine pancreatic trypsin inhibitor, is a reversible Kunitz-type inhibitor of several serine proteases including trypsin and CAPs 1–3 [123]. Using single-channel noise analysis, Adebamiro et al. found that aprotinin caused an apparent decrease in the number of ENaC channels in the plasma membrane, which accounted for the decrease in macroscopic ENaC currents [1]. In fact, the noise technique correctly detected a decrease in the number of open (active) channels but could not recognize that they were replaced by near-silent channels. The rates of onset and offset for inhibition of ENaC by aprotinin and other protease inhibitors is much slower than for pore-blocking small molecules such as amiloride. The reason for this difference is that protease inhibitors act to prevent the conversion by proteolysis of uncleaved, near-silent ENaC to actively gating channels. In this schema, the slow onset of aprotinin inhibition of ENaC-mediated current reflects active channels being replaced by near-silent channels. The rate of onset of ENaC current inhibition by aprotinin or other broad-specificity inhibitors is expected to approximate the rate of retrieval of ENaC from the surface. Similarly, the gradual recovery from inhibition by aprotinin likely reflects the rate of insertion of new ENaC channels into the plasma membrane [123].

The Bayer pharmaceutical compound BAY 39-9437 is also a Kunitz-containing serine protease inhibitor that is similar to placental bikunin. BAY 39-9437 is considered a potential therapeutic agent for CF lung disease that would act by ameliorating the Na^+ hyperabsorption in CF airways. EPI-hNE4 (DX-890) is a low molecular mass (6.2 kDa) inhibitor of neutrophil elastase that was modified from the second Künitz domain of inter-α-trypsin inhibitor [55, 118]. Replacement of the critical arginine residue at position 297 with an isoleucine makes this domain a potent

neutrophil elastase inhibitor that has been demonstrated to protect the lungs of rats against neutrophil elastase and from CF sputum [38]. This compound also prevents ENaC from being activated by neutrophil elastase, but not by trypsin or CAP1 [58].

Serpins Serpins are a group of proteins first identified as protease inhibitors. The acronym serpin was originally coined because many family members inhibited chymotrypsin-like serine proteases [27]. The first members of the serpin superfamily to be extensively studied were the human plasma proteins anti-thrombin and anti-trypsin, which play key roles in controlling blood coagulation and inflammation, respectively [53]. SerpinE2, also known as protease nexin 1 (PN-1), has been shown to regulate Na^+ absorption in airway epithelia [99]. Myerberg et al. reported that PN-1 complexed with and inhibited prostasin on the apical surface of cultured airway epithelia, leading to inhibition of Na^+ absorption. Although PN-1 has been referred to as the "cognate prostasin inhibitor", Myerberg et al. discovered that PN-1 also inhibited matriptase on the surface of airway epithelia [46]. Since matriptase can activate both prostasin and ENaC, PN-1, and very likely other soluble, serpins are predicted to influence the proteolytic regulation of ENaC.

Small molecule inhibitors In addition to protein serine protease inhibitors, low molecular weight chemically synthesized serine protease inhibitors have also been developed (125). Some of these inhibitors, including camostat, have been shown to inhibit proteolytic activation of ENaC in vitro and in vivo [33]. Many covalent and non-covalent protease inhibitors have been developed as anti-coagulators [125]. While the effect of these compounds on ENaC activity is not currently known, they may represent a library of potentially useful compounds that could inhibit ENaC in vivo.

Non-classical inhibitors of ENaC proteolysis Short palate lung and nasal epithelial clone 1 (SPLUNC1) was recently identified as a regulator of ENaC using a non-biased, proteomic screen designed to find ASL proteins capable of interacting with serine proteases [50]. SPLUNC1 is a secreted protein of ~28 kDa which shares homology with anti-bacterial proteins but has no obvious homology with Kunitz-type inhibitors or serpins [11]. SPLUNC1 has previously been identified in human airway epithelial cultures and this protein comprises up to 10% of the total protein content in their ASL [23]. SPLUNC1 was found to prevent ENaC proteolysis and inhibited ENaC-mediated currents in both *Xenopus* oocytes and in human bronchial epithelial cultures [50]. In addition to being structurally different from traditional protease inhibitors, which typically prevent cleavage of ENaC, its mode of action also appears to differ. For example, despite preventing ENaC proteolysis and activation by serine proteases such as trypsin, CAP1, and CAP2, SPLUNC1 does not inhibit protease activity directly and rather binds to ENaC, preventing it from being cleaved [50]. SPLUNC1 also reduces basal ENaC currents in *Xenopus* oocytes [50], and SPLUNC1 may actually reduce the number of ENaCs that are inserted into the plasma membrane, thus reducing both basal ENaC activity and the number of ENaCs that are available for proteolytic cleavage [121]. Whilst the nature of this internalization remains to be elucidated, SPLUNC1 has several predicted adaptor protein binding-domains. Such adaptor proteins have previously been shown to play a role in the internalization of ENaC [130, 158] and may be for a macromolecular complex with SPLUNC1 and α,β,γENaC to facilitate ENaC's internalization.

Regulation of ASL volume by ENaC, extracellular proteases, and anti-proteases

Airway epithelial cells constitutively release extracellular purine nucleotides including ATP and UTP at levels sufficient to activate $P2Y_2$ receptors on the apical membrane, which stimulate rises in intracellular IP_3 and Ca^{2+}, in parallel with a fall in intracellular PIP_2 [36]. ATP can also be broken down to adenosine (ADO) by a series of extracellular enzymes including the 5′ ectonucleotidase CD73, and ADO can then stimulate A2B-R to raise cAMP which can activate CFTR and also regulate ENaC [88, 89, 106–108]. However, based on the observation that ENaC is basally active, airway epithelia were thought to be primarily absorptive, with little or no gradient for Cl^- secretion, even when CFTR was activated [13]. Furthermore, Cl^- secretion was only predicted to occur when ENaC was inhibited by molecules such as amiloride, which would hyperpolarize the apical membrane and provide an electrical gradient for Cl^- secretion against the unfavorable chemical gradient [Cl_o^- ~130 mM; Cl_i^- ~40 mM] [13]. These initial studies were performed under "thick film conditions" where ASL was washed away and replaced with unphysiologically large volumes of Ringer solution. Thus, whilst the native ASL volume on a 1 cm^2 airway epithelial culture is measured in microliters, such a culture may be bathed in up to 10 ml of Ringer solution during the course of an Ussing chamber-type experiment. However, studies of ASL volume regulation under thin film conditions, where native ASL is preserved, have demonstrated that ENaC can be spontaneously inactivated, permitting Cl^- (and liquid) secretion to occur. For example, Widdicombe et al. demonstrated that ASL secretion could be induced in freshly excised bovine trachea [160]. Similarly, we have

shown that purine nucleotides and nucleosides induced ASL secretion in the absence of pre-inhibition of ENaC with amiloride [142, 145]. These nucleotides and nucleosides may act as soluble volume sensors whose concentration or dilution can directly regulate ASL volume by changing the rates of Cl^- secretion [120, 143, 145]. Whilst changes in nucleotides/nucleosides have been shown to directly affect ENaC under closed-circuit conditions, when these compounds are directly applied by the investigator at relatively high micromolar concentrations, it seems under open circuit/thin film conditions when endogenous ATP and ADO are much lower, and closer to concentrations seen in vivo (i.e., nanomolar levels) that ENaC is primarily under the control of the protease/protease inhibitor system despite the presence of endogenous ATP and ADO [50, 144, 146].

Human airway epithelial cultures were found to shift their phenotype from predominantly absorptive when excess solution was added to the culture surface to predominantly secretory when ASL height was similar to ciliary height (i.e., ~7 μm) [144]. Building upon this observation, further research indicated that ENaC was acutely sensitive to the overlying ASL volume and was activated when ASL volume was raised [98, 146], a termed coined "ASL volume expansion" [98]. Following ASL volume expansion, it is likely that silent ENaCs become rapidly activated, allowing for a rapid return to a 7 μm ASL height. Whilst ENaC is not itself likely to be volume sensitive, washing cultures with Ringer solution resulted in a brisk activation of ENaC, whilst allowing ASL to accumulate resulted in ENaC inhibition [98, 146]. Airway cultures express (1) membrane-attached serine proteases that are not affected by washing and/or dilution and (2) soluble protease inhibitors that could be removed/diluted [98, 146]. Thus, based on these observations, it was proposed that when ASL volume is high, soluble protease inhibitors are removed/diluted and ENaC is activated by cell-attached proteases (Fig. 4a) [28]. This is analogous to the situation seen in Ussing chambers when ASL is washed away or diluted by the Ringer solution which bathes the mucosal membrane. A physiological parallel may occur following glandular secretions onto airway surfaces. In contrast, when ASL is undisturbed, protease inhibitors are present in sufficient concentrations to inhibit ENaC (Fig. 4b).

The importance of soluble protease inhibitors as volume-sensing regulators of ENaC has recently been confirmed and knockdown of the soluble ENaC inhibitor SPLUNC1 prevented spontaneous, ASL volume-dependent inactivation of ENaC in human airway cultures [50]. In the absence of SPLUNC1, ASL volume continued to be absorbed following the addition of a test solution and was not slowed as ASL height approached 7 μm, resulting in ASL volume collapse to CF-like levels [50]. These data indicate that SPLUNC1 plays a crucial role in ASL volume regulation [50]. SPLUNC1 is most highly expressed in glands of the proximal airways in vivo [11] which may reflect a more secretory phenotype of these regions. SPLUNC1 is less well expressed in the distal airways, consistent with Kilburn's hypothesis that converging airways need to absorb excess solution to maintain constant ASL height [74]. Importantly, when soluble inhibitors or proteins such as SPLUNC1 increase their concentration in the ASL sufficiently to inhibit ENaC, this will facilitate Cl^- secretion by hyperpolarizing the apical membrane, providing an electrical gradient for Cl^- exit into the lumen [13]. Whether or not other highly expressed protease inhibitors such as the secretory leukocyte peptidase inhibitor can affect ENaC activity remains to be tested. However, other PLUNC family members that are expressed in the airways, including SPLUNC2 and LPLUNC1, do not appear to inhibit ENaC to the same degree as SPLUNC1 [121].

The majority of protease/protease inhibitor regulation dependent of ENaC occurs, by its nature, at the non-genomic level. However, normal airway epithelia exposed to prolonged 24 h ASL volume expansion showed increased prostasin expression [99]. This increase in prostasin expression was more pronounced after 12 h of ASL volume expansion whereas the effect of excess ASL on ENaC activation is immediate [98, 146]. It is likely therefore that, whilst the bulk of volume-induced ENaC regulation happens non-genomically, the epithelia also has an extensive reserve capacity and can increase expression of proteins that activate ENaC if required to do so.

ENaC and mucus clearance in vivo

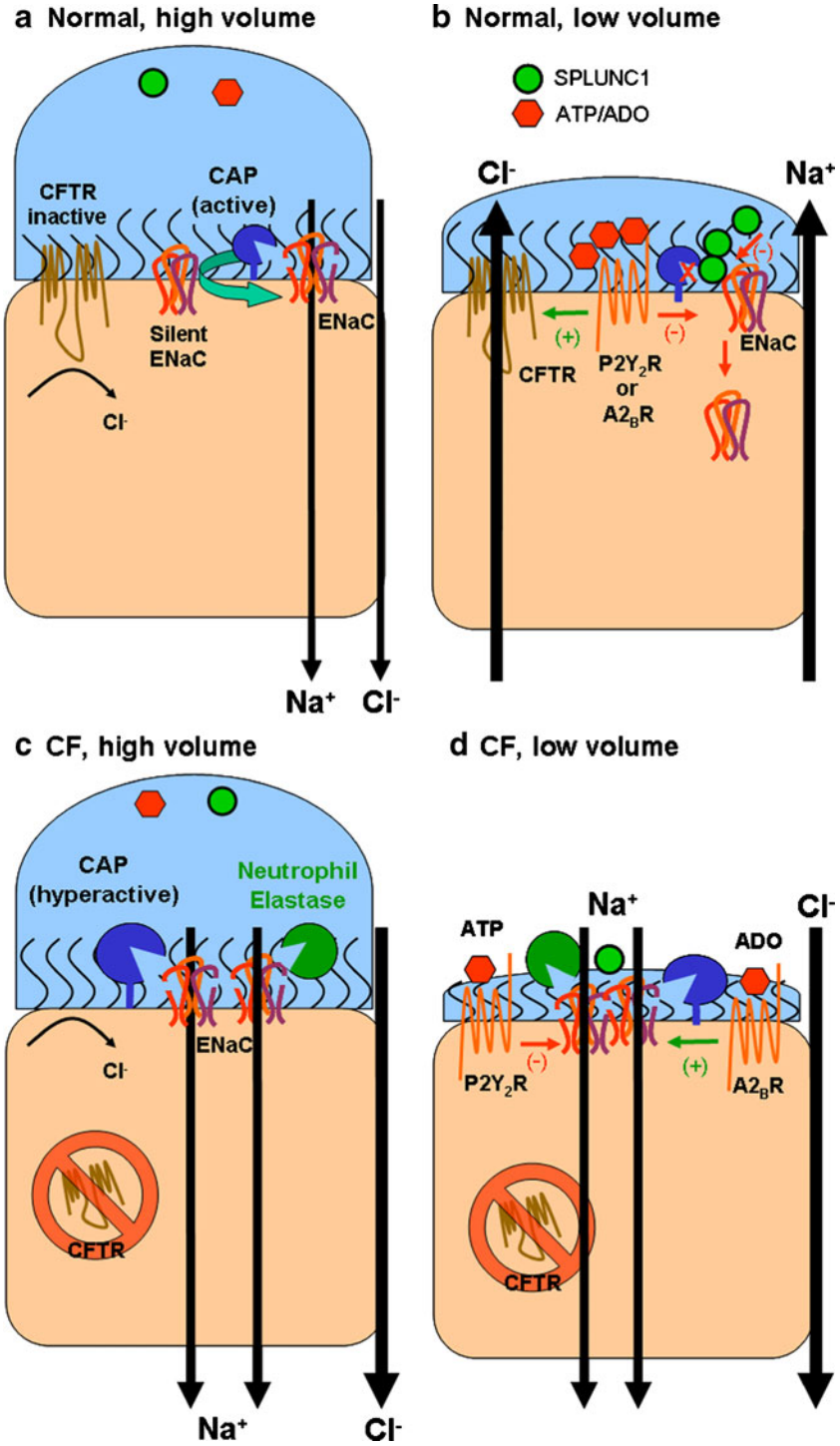
The alveolar region of the lung has a surface area that is approximately the size of one side of a tennis court (i.e., ~75 m^2) vs. the proximal airways, which have a surface area comparable to a tea towel (i.e., ~30 cm^2). During this transition, there are 23 generations of airways, from the respiratory bronchioles to the trachea. As ASL and mucus move up the respiratory tract, excess liquid must be absorbed in a regulated fashion as two airways converge to keep ASL height/volume relatively constant. Failure to absorb excess ASL would result in an increase in ASL height with every generation of airways that would become greater than the diameter of the airways within a few generations, something that is clearly not seen in vivo. This model of regulated absorption was first proposed over 40 years ago by Kay Kilburn [74]. Kilburn's hypothesis predicts that regulated absorption is active throughout the airways to help remove the excess liquid which occurs as two airways converge into one, and thus far is the only

proposed model of mucus clearance that accounts for the movement of liquid up the airways vs. the huge reduction in surface area presented to this anterograde-moving liquid. Recently, in vivo experiments have been performed that support this hypothesis. For example, acute inhalation of the ENaC antagonists amiloride or Parion552, a highly specific ENaC antagonist with a Kd 100 times greater than amiloride (~7 nM), acutely increased mucus clearance rates in unanesthetized sheep [61, 62]. The inhibition of ENaC with either of these compounds in vivo likely results in an increase in mucus hydration followed by a speeding up of mucus clearance rates. A similar correlation between mucus hydration and mucus transport rates has previously been reported in vitro [144]. Protease inhibitors do not affect ENaC directly but block activation of near-silent channels arriving at the cell surface leading to a decrease in Na^+ absorption. Inhalation of the serine protease inhibitor camostat decreases Na^+ absorption and increases mucus clearance rates in vivo, suggesting that ENaC is likely to be active and proteolytically cleaved under normal physiological conditions in vivo [33]. As will be discussed later, neutrophil elastase, which also cleaves and activates ENaC, acutely decreases mucus clearance rates when inhaled, suggesting that ENaC can also be further activated beyond normal levels to increase fluid absorption and decrease mucus clearance rates [124]. This data also indicates that there is a pool of silent ENaCs that are present in the airways that are ready to be activated by this enzyme. Since neutrophil elastase levels are elevated in both CF and chronic obstructive pulmonary disease (COPD), an understanding of how this protease can affect ENaC levels and mucus clearance rates is of particular interest in the treatment of these diseases.

The contribution of ENaC to airway disease

CF reflects a spectrum of more than 1,600 mutations in the cystic fibrosis conductance regulator (CFTR) gene [37]. The CF gene product, CFTR, is an ATP-binding cassette family member [117]. The most common mutation, which occurs on 70% of all CF chromosomes, is the deletion of phenylalanine at position 508 of the CFTR protein, which is designated as ΔF508. The ΔF508 CFTR protein has a folding defect and is retained by the quality control system in the endoplasmic reticulum, falls short of the processing pathways, and is degraded by endoplasmic reticulum-associated degradation. CFTR functions as an apical, cAMP-activated chloride channel and thus the molecular pathogenesis of ΔF508 CFTR is reflected by the absence of functioning CFTR proteins in the apical plasma membrane of airway epithelia. Other mutations can produce a non-functioning CFTR Cl^- channel at the apical membrane and/or a mutant CFTR with abnormal ion permeation characteristics. Despite relatively simple genetics, multiple mechanisms have been evoked to describe CF pathogenesis, beyond the lack of the apical cAMP-regulated Cl^- conductance. For example, defective HCO_3^- secretion through CFTR has been suggested to cause abnormal mucin release, which may lead to mucus obstruction in CF airways [51, 114]. Furthermore, persistent airway inflammation and infection may be caused by a lack of CFTR [57, 92, 100]. However, although not universally accepted, an increasing body of evidence suggests that ASL volume regulation and mucociliary clearance are abnormal in CF due to defective ion transport processes [15] (Fig. 4c, d). Thus, CF patients, who exhibit Cl^- hyposecretion, Na^+ hyperabsorption, and reduced glandular secretions, have reduced mucus clearance [119], which can be partially restored by inhalation of nebulized hypertonic saline [43, 45] or by inhalation of the ENaC antagonist amiloride [5, 77]. In contrast, adult patients with pseudohypoaldosteronism have downregulated rates of Na^+ transport and, consequently, an abundance of ASL which results in clearance rates greater than those seen in either normal controls or CF patients [73]. Unlike CF patients, who lack ASL volume, it seems that too much ASL does not compromise lung defense and pseudohypoaldosteronism patients are infection-free in adulthood [73]. The importance of ENaC in maintaining mucus clearance has recently been demonstrated (1) in a murine model overexpressing βENaC, which results in a decrease in ASL volume and mucus clearance and the emergence of chronic inflammation and lung disease

Fig. 4 ENaC and ASL volume regulation. **a** In normal airways under high volume conditions, which may occur following glandular secretions, soluble inhibitors of ENaC proteolysis such as SPLUNC1 or purinergic inhibitors such as adenosine or ATP may be diluted to such an extent that ENaC is fully active, leading to Na^+-led isotonic ASL absorption with Cl^- following through the paracellular pathway. Most non-cleaved, silent ENaCs are likely to be converted to cleaved active ENaCs by endogenous cell surface CAPs. Due to low levels of ATP and adenosine, CFTR and TMEM16A (not shown) are inactive and no Cl^- secretion occurs. **b** As ASL volume returns to steady-state levels, SPLUNC1 accumulates in the ASL in sufficient quantities to prevent the conversion of silent ENaCs to active ENaCs and SPLUNC1 may also reduce surface ENaC levels. Adenosine also accumulates to sufficient levels to activate CFTR (likely by stimulation of A2B adenosine receptors). This leads to a steady-state ASL height which approximates the height of outstretched cilia (7 μm). **c** High ASL volume conditions may occur in CF airways after inhalation of hypertonic saline. Under these conditions, Na^+ absorption dominates, as in normal airways. **d** However, as ASL volume is reduced in CF airways, regulatory molecules such as SPLUNC1 are predicted to be ineffective at inhibiting ENaC, leading to an inappropriate reduction in ASL volume. In the absence of CFTR, active $A2_BR$ increases cAMP levels to stimulate rather than inactivate ENaC, further driving the reduction in ASL volume. Thus, unlike NL airways, CF airways are unable to make the switch from an absorbing to a secreting epithelia and ASL volume depletion results



[94], and (2) in sheep acutely exposed to neutrophil elastase, which results in a rapid decrease in mucus clearance rates [124]. Perhaps surprisingly, patients with Kartagener's syndrome, who have immotile cila, exhibit near-normal rates of mucus clearance [101, 116]. This likely occurs in the face of an absence of ciliary beating due to increased rates of cough clearance in these patients as compared to normal subjects. Airway epithelial cultures derived from these patients undergo normal ASL volume homeostasis, suggesting that their mucus is normally hydrated [146]. Taken together, these data suggest that, even in the absence of beating cilia, mucus clearance can be preserved if the mucus layer is appropriately hydrated.

Boucher et al. first demonstrated that ENaC hyperactivity was at least in part due to abnormal stimulation of Na^+ absorption by cAMP/PKA in CF airway epithelia [16], something that was subsequently confirmed by Mall et al. [93]. However, it is also possible that abnormal proteolytic cleavage of ENaC contributes to Na^+ hyperabsorption. Due to secondary defects, which may include abnormal local pH environments and/or chronic inflammation, furin, prostasin, and CAP2 have been shown to be upregulated in the absence of CFTR [99, 103, 146]. This upregulation may contribute to a failure of CF airways to affect suitable ASL volume regulation, leading to ASL volume collapse which is indicative of a failure of mucus clearance in vivo. Evidence for this failure comes from studies performed under thin film conditions. For example, the ENaC-mediated transepithelial potential difference in airway epithelial cultures derived from non-CF donors was trypsin insensitive when ASL volume was high and ENaC-led absorption was underway, indicating that ENaC was fully active. The potential difference then spontaneously underwent a shift and became trypsin sensitive when ASL volume was low, suggesting that ENaC had been inactivated by the cultures [146]. In contrast, ENaC in CF-derived airway cultures remained trypsin insensitive, irrespective of ASL volume status, indicating a failure to regulate ENaC proteolysis. Interestingly, ENaCs in CF airway cultures remained aprotinin sensitive, suggesting that the defect lies at the level of the protease/anti-protease balance in the ASL and that CF ENaCs are inherently normal in their ability to be proteolytically cleaved [146].

As mentioned above, ENaC retains the ability to be cleaved in CF airway epithelia in an identical fashion to non-CF ENaCs. However, protease levels are elevated in diseased lungs, which may lead to abnormal ENaC activity. For example, whilst healthy individuals do not have detectable levels of neutrophil elastase in the airways [79], concentrations of active neutrophil elastase in excess of 1 μM or 200 μg/ml have been reported in CF lungs [38, 79]. Such an increase may occur not only in CF airway disease but also in COPD. Cigarette smoke exposure can induce downregulation of CFTR [24, 81], but as yet there is no evidence that ENaC is hyperactive in COPD airways. However, the high concentrations of neutrophil elastase measured in COPD airways make this a likely symptom of the disease. In addition, neutrophil elastase may overwhelm the ability of endogenous Kunitz-type anti-proteases or SPLUNC1 to inhibit ENaC. It should also be noted that since Kunitz-type proteases inhibitors do not inhibit neutrophil elastase and vice versa, therapies based around Kunitz-type anti-proteases may not be fully effective at inhibiting ENaC in inflamed/diseased airways which have elevated levels of neutrophil elastase.

ENaC-related therapies and clinical trials

In keeping with the hypothesis that abnormal CF airway ion transport results in mucus dehydration, improving the hydration of CF airway surfaces with inhaled hypertonic saline has been shown to improve lung function and decreases the number of acute exacerbations [43, 45]. However, since Na^+ is the natural substrate for ENaC, these therapies have a relatively short duration of action [145]. To improve hydration, either alone or in combination with hypertonic saline, drugs directly blocking ENaC-mediated Na^+ absorption are undergoing clinical evaluation. Early trials using nebulized amiloride revealed mixed benefits for CF patients [54, 77], and it was suggested that a weak affinity of amiloride for its receptor and the short half-life of amiloride (~10 min) [145] contributed to amiloride's failure to consistently improve lung function in the clinic. It is also possible that the therapeutic benefit of amiloride was impeded by factors related to the chronic disease process such as the systematic destruction of CF small airways and that preventive amiloride therapy initiated shortly after birth, when CF lungs are structurally normal, may be the most effective treatment of CF lung disease [54, 77]. Support for this hypothesis was recently obtained in preclinical studies in βENaC-overexpressing mice by comparing therapeutic effects of preventive versus late amiloride treatment in this model of CF lung disease [167]. More recently, however, long-acting and highly potent ENaC blockers have been developed which may circumvent the pharmacokinetic limitations of amiloride [62]. As such, the compound Gilead GS9411, which is 100 times more potent than amiloride, has completed a phase 1 CF clinical trial, although the results have yet to be published (http://www.cff.org/treatments/Pipeline/).

In addition to ENaC antagonists, protease inhibitors may also be clinically effective. In a proof of concept study, Coote et al. reported that inhibition of ENaC function by the Kunitz-type serine protease inhibitor camostat increased mucus clearance [33]. This has led to a search for low molecular weight protease inhibitors as potential pharmacological agents to modulate ENaC function and improve mucociliary clearance and it may not be long before low molecular weight Kunitz-type serine protease inhibitors enter phase 1 clinical trials. Neutrophil elastase inhibitors have also been developed with a view towards inhibiting neutrophil elastase-induced ENaC hyperactivity in the lung [58]. Inhibition of neutrophil elastase by specific inhibitors may have additional beneficial effects on CF airways as this treatment will not only block ENaC activity but also prevent the detrimental effects of neutrophil elastase as mediator of tissue inflammation and destruction. Future clinical trials may soon reveal the benefits of elastase inhibitors in the treatment of CF patients.

Conclusions

Proteolytic cleavage is required for ENaC activation and controlled inhibition of this process is vital for limiting Na^+ absorption rates and for efficient ASL volume homeostasis (Fig. 4). However, little is known about the physiological regulation of proteases and their inhibitors in the airways, and their contribution to ENaC disregulation in chronic lung disease is only now being fully appreciated. For example, unchecked activation of ENaC in CF airway epithelia likely contributes to the depletion of ASL volume and impaired mucociliary clearance in CF airways, yet the mechanism responsible for the protease–protease inhibitor imbalance in CF airways remains uncertain (Fig. 4). Thus, a better understanding of this complex regulatory process is warranted before we can develop effective treatments against ENaC hyperabsorption in chronic lung disease.

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