AIRWAY SURFACE LIQUID VOLUME REGULATES ENaC BY ALTERING THE SERINE PROTEASE-PROTEASE INHIBITOR BALANCE: A MECHANISM FOR SODIUM HYPERABSORPTION IN CYSTIC FIBROSIS

Mike M. Myerburg1, Michael B. Butterworth2, Erin E. McKenna1, Kathryn W. Peters2, Raymond A. Frizzell2, Thomas R. Kleyman3,2, and Joseph M. Pilewski1,2

From the 1Division of Pulmonary, Allergy, and Critical Care Medicine, 2Department of Cell Biology and Physiology, and 3Renal-Electrolyte Division, University of Pittsburgh, Pittsburgh, PA 15213

Running Title: Regulation of ENaC by Protease Inhibitors in the ASL

Address correspondence to: Joseph Pilewski, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh Medical Center, 628 NW Montefiore University Hospital, 3459 Fifth Avenue, Pittsburgh, PA 15213, Tel (412) 692-2210, Fax (412) 692-2260, E-Mail: pilewskijm@upmc.edu

Efficient clearance of mucus and inhaled pathogens from the lung is dependent on an optimal airway surface liquid (ASL)1 volume, which is maintained by the regulated transport of sodium and chloride across the airway epithelium. Accumulating evidence suggests that impaired mucus clearance in cystic fibrosis (CF) airways is a result of ASL depletion caused by excessive Na+ absorption through the epithelial sodium channel (ENaC). However, the cellular mechanisms that result in increased ENaC activity in CF airways are not completely understood. Recently, proteases were shown to modulate the activity of ENaC, but the relevance of this mechanism to the physiologic regulation of ASL volume is unknown. Using primary human airway epithelial cells, we demonstrate that: (i) protease inhibitors are present in the ASL and prevent the activation of near-silent ENaC, (ii) when the ASL volume is increased, endogenous protease inhibitors become diluted, allowing for proteolytic activation of near-silent channels, and (iii) in CF, the normally present near-silent pool of ENaC is constitutively active and the α subunit undergoes increased proteolytic processing. These findings indicate that the ASL volume modulates the activity of ENaC by modification of the serine protease - protease inhibitor balance and that alterations in this balance contribute to excessive Na+ absorption in cystic fibrosis.

Mucociliary clearance is the primary innate defense mechanism of the conducting airways, enabling inhaled particulate matter and pathogens to be expelled (1). Accumulating evidence indicates that mucus clearance is dependent on the presence of a thin layer of fluid, known as the airway surface liquid (ASL), which acts as a low viscosity medium that allows the cilia to beat effectively. Because the airway epithelium is relatively permeable to water, the ASL is roughly isotonic to plasma. Thus ASL volume is dictated by the osmotic driving force established by the oppositely directed transport of Na+ and Cl− across the airway epithelium (2-5). When the balance between Na+ absorption, through the epithelial sodium channel (ENaC), and Cl− secretion, through the cystic fibrosis transmembrane conductance regulator (CFTR), is disrupted, as in cystic fibrosis (CF), ASL volume is reduced and mucus clearance is impaired, resulting in mucus obstruction, chronic airway infection, and inflammation (2-7). Recent evidence suggests that unregulated ENaC activity is central to the development of ASL dehydration in CF (6-13). Despite the immense importance of properly regulated ion transport, the cellular mechanisms that result in increased ENaC activity in CF airways are not known.

In order for ENaC to be maximally activated, the α and γ subunits require proteolytic processing (14). Thus, two distinct pools of channels are present at the apical membrane: (i)
mature channels with complex N-glycans and cleaved \( \alpha \) and \( \gamma \) subunits; and (ii) immature channels with high mannose type N-glycans and uncleaved subunits. Proteolytic processing in the biosynthetic pathway increases the channel’s activity and is mediated by furin. Channels that bypass proteolytic processing are believed to provide a reserve pool for activation in post-Golgi compartments (15-17). In agreement with this hypothesis, an inactive or “near-silent” ENaC population can be activated at the cell surface by proteases, such as channel activating proteases (CAPs) or trypsin (14,18-20). Furthermore, treatment with aprotinin, a Kunitz-type serine protease inhibitor, decreases the amiloride-sensitive short circuit current \( (I_{SC}) \) (21,22). A number of serine protease inhibitors containing a Kunitz domain are expressed in the lung and are present in glandular secretions (23-28). Under physiological conditions, the importance of the near-silent ENaC population and the role of endogenous protease inhibitors in the proteolytic regulation of ENaC are unknown.

We reasoned that endogenous protease inhibitors in the ASL might prevent proteolytic activation of near-silent ENaC by CAPs. Accordingly, during periods when the ASL is diminished, the high concentration of protease inhibitors would prevent activation of uncleaved ENaC and further depletion of the ASL. Conversely, when the ASL volume is high, protease inhibitors would be diluted, allowing for CAP mediated activation of a pool of inactive ENaC, and a resultant increase in \( Na^+ \) and water absorption. Here, we demonstrate that these mechanisms contribute to the regulation of ENaC activity in primary human airway epithelial cells (HAEC) and that an altered protease-protease inhibitor balance contributes to \( Na^+ \) hyperabsorption in CF epithelium.

**EXPERIMENTAL PROCEDURES**

**Primary human airway epithelial cell culture.** HAEC were cultured from excess pathological tissue following lung transplantation and organ donation under a protocol approved by the University of Pittsburgh Investigational Review Board. HAEC were cultured on human placental collagen-coated Costar Transwell filters (0.33 cm\(^2\)) as previously described (29), and used for experimentation following 4-6 weeks of culture at an air-liquid interface. Non-CF HAECs were obtained from 2 normal donors and 12 donors with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, obliterative bronchiolitis, or primary pulmonary hypertension. Qualitative differences due to disease state were not observed. CF HAECs were obtained from 5 donors with the following CF genotypes; ΔF508, G551D, and 2789+5G→A. Qualitative differences due to genotype were not observed.

**Airway surface liquid (ASL) volume expansion.** In order to expand the ASL volume, 5 to 100 µl of Ringers or PBS was gently pipetted onto the apical surface of differentiated HAEC. Where indicated, aprotinin (Sigma) was added to the apical fluid. Cells were then returned to a humidified incubator for the indicated time prior to \( I_{SC} \) measurement. HAEC apical secretions were collected by incubating 100 µl of PBS on the apical surface of HAEC for 72 hours. 300 µl of pooled conditioned apical fluid was concentrated by precipitation in 1 ml acetone overnight at -20ºC. The apical secretions were then pelleted by centrifugation and resuspended in 100 µl PBS for subsequent ASL volume expansion experiments.

**\( I_{SC} \) Recordings.** Short circuit currents were measured as previously described (29,30). In brief, cells cultured on filter supports were mounted in modified Ussing chambers, and the cultures were continuously short circuited with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance was measured by periodically applying a 2.5-mV bipolar voltage pulse and was calculated using Ohm’s law. The bathing Ringers solution was composed of 120 mM NaCl, 25 mM NaHCO\(_3\), 3.3 mM KH\(_2\)PO\(_4\), 0.8 mM K\(_2\)HPO\(_4\), 1.2 mM MgCl\(_2\), 1.2 mM CaCl\(_2\), and 10 mM glucose. Chambers were constantly gassed with a mixture of 95% O\(_2\) / 5% CO\(_2\) at 37º C, which maintained the pH at 7.4. Following a 5 minute equilibration period, the baseline \( I_{SC} \) was recorded. To determine the amiloride-sensitive \( I_{SC} \) \( (I_{ENaC}) \), amiloride (Sigma) was added to the apical cell chamber to a concentration of 10 µM. Alternatively, 1 µM trypsin (Sigma) was added to the apical surface for 5 minutes prior to amiloride addition, providing a measure of protease
activatable channels (ΔTrypsin) (20,28,31-33). To
determine stimulated CFTR currents ($I_{CFTR}$), after
addition of amiloride, 10 µM forskolin (Sigma)
and then 10 µM CFTRinh172 (Calbiochem) were
added.

**Surface Biotinylation and Western Blotting.** Surface biotinylation and western blotting was performed as previously described (30). Briefly, differentiated HAEC cultures grown on filter supports were placed on ice and the apical surface was washed with ice cold PBS plus 1 mM CaCl₂, to remove cellular debris. Subsequently, the apical surface of the HAEC filters was incubated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce) in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na₂B₄O₇, pH 9). After 20 minutes, the biotinylation reaction was quenched with PBS plus 10% fetal bovine serum and the cells were rinsed with ice cold PBS + Ca²⁺. The cells were then lysed in cell lysis buffer (10mM Tris-Cl, 50mM EGTA, 0.4% Sodium Deoxycholate, 1% NP-40, pH 7.4). Cellular debris was removed by centrifugation and protein concentration was determined using the Bradford method (Bio-Rad). The biotinylated proteins in 100 µg of cellular lysate were recovered by incubation with streptavidin beads (Pierce) overnight at 4ºC. The proteins were resolved using standard SDS-PAGE and transferred to nitrocellulose. The membrane was then immunoblotted using antisera from rabbits immunized with an N-terminal αENaC peptide, as previously described (30,34-36). Subsequently the blots were stripped and re-probed with monoclonal β actin antibodies (Sigma). Band intensity was quantified by densitometry (Quantiscan). To demonstrate antigen specificity, peptide competition was performed by incubating 10 µg/ml of the immunizing peptide with the antisera for 2 hours at 37ºC prior to immunoblotting.

**Statistics.** Results are expressed as mean ± S.E. The percent of baseline $I_{ENaC}$ was determined by normalizing amiloride-sensitive current to values obtained from matched HAEC from the same donor on the same day of experimentation. Significance was determined by ANOVA with Bonferroni post-hoc analysis, or by students t-test where appropriate. Results were confirmed in at least 3 independent experiments using HAEC cultured from different lung tissues.

### RESULTS

**ASL volume expansion increases the amiloride sensitive $I_{SC}$.** The baseline $I_{SC}$ for HAEC at air-liquid interface averaged 34.3 ± 1.9 µA/cm² (n=81). Following 24 hours of ASL volume expansion with 100 µl Ringers, the mean $I_{SC}$ increased to 77.1 ± 4.1 µA/cm² (n=60, p<0.001 versus control). Following the addition of 10 µM amiloride, the residual $I_{SC}$ was the same in control and ASL expansion conditions (12.2 ± 0.5 µA/cm² vs 13.4 ± 0.7 µA/cm², NS). This indicates that the increased $I_{SC}$ observed with ASL volume expansion reflects conductive ENaC mediated sodium absorption. $I_{CFTR}$, defined as the CFTRinh172 sensitive current following forskolin stimulation, was not significantly altered by ASL volume expansion (9.8 ± 0.7 vs 9.9 ± 0.6 µA/cm², n=15 and 11). Transepithelial potential difference increased from 12.1 ± 0.6 mV to 26.2 ± 1.3 mV following ASL volume expansion (p<0.001). Transepithelial resistance decreased from 492 ± 18 Ω·cm² to 407 ± 20 Ω·cm² (p=0.002). Thus, ASL volume expansion increased the amiloride-sensitive $I_{SC}$ ($I_{ENaC}$) 2-3 fold, consistent with increased ENaC activity.

We next determined the influence of time and dose on the changes in ENaC activity following ASL volume expansion. To assess the kinetics, the ASL volume of matched HAEC was expanded with 100 µl of Ringers for 0, 24, 48, and 72 hours prior to measurement of the amiloride-sensitive $I_{SC}$. Representative $I_{SC}$ tracings and mean $I_{ENaC}$ are shown in figure 1 A+B. In this series of experiments, the air-liquid baseline $I_{ENaC}$ was 23.7 ± 5 µA/cm², and increased to 87.7 ± 3.8 µA/cm² at 24 hours (p=0.0005). Following the initial rise at 24 hours, $I_{ENaC}$ declined to 41.4 ± 5 µA/cm² at 72 hours (p=0.26 compared to baseline). The $I_{SC}$ of matched control filters at air-liquid interface increased by 2.6 ± 3.3 µA/cm² over the 72-hour interval (p> 0.05, n=3). Thus, the increase in $I_{ENaC}$ due to ASL volume expansion is maximal at 24 hours, after which time $I_{ENaC}$ declines to near baseline.

In order to further define the kinetics of the $I_{ENaC}$ response to ASL expansion, the ASL volume was increased with 100 µl of Ringers solution ½, 1, 4, and 24 hours prior to measurement of amiloride sensitive $I_{SC}$. The $I_{ENaC}$ was normalized to that of matched air-liquid
HAEC and fitted to a 1st order exponential equation (% baseline = y0 + ae-kt). As shown in Figure 1C, there is a rapid increase in I_{ENaC} following ASL volume expansion (t1/2 = 0.5 ± 0.09 hours). Additionally, the rate of I_{ENaC} decline following the removal of excess apical fluid was assessed. For this series of experiments, the ASL volume was expanded for 24 hours, and the excess apical fluid was subsequently aspirated at 0, 4, 12, and 24 hours prior to measurement of I_{ENaC}. Following the removal of excess ASL, I_{ENaC} returned to baseline over ~12 hours (t1/2 = 2.8 ± 0.8 hours, see Figure 1C). Because of the rapidity of these changes, all ISc measurements were obtained following a brief 5 minute equilibration period in the Ussing chamber.

To assess the effect of increasing ASL volume, 0, 5, 50, or 100 µl of Ringers solution was added to the apical surface of HAEC for 24 hours. As shown in Figure 1D, I_{ENaC} increased proportionately with increasing ASL volume. Volume increases above 100 µl had no further effect (not shown). Thus, the increase in I_{ENaC} following ASL volume expansion was both time-dependent and dose responsive.

**ASL volume expansion alters the serine protease balance with endogenous protease inhibitors, allowing for activation of near-silent ENaC.** The increase in amiloride-sensitive current following treatment with trypsin observed in airway epithelium is thought to reflect the presence of a near-silent pool of ENaC (20,28,31-33). Because endogenous serine proteases, such as CAPs, are proposed to activate silent channels present on the apical membrane of the epithelium (14,18), we reasoned that endogenous protease inhibitors must be present in the ASL. To assess this, the effects of aprotinin and trypsin on HAEC with and without expanded ASL volumes were compared (see Figure 2A). 30 µM aprotinin was administered in either 5 or 100 µL of PBS to the apical surface of HAEC for 24 hours prior to ISc measurement. These apical volumes were selected because 5 µl did not alter I_{ENaC} at 24 hours (see Figure 1D) and 100 µl caused the maximal ASL volume expansion response (data not shown). In air-liquid conditions (5 µL added), exposure to aprotinin for 24 hours did not significantly alter baseline ISc, suggesting that an aprotinin-like protease inhibitor is endogenously present in the ASL. However, under ASL volume expansion, aprotinin had a profound effect, decreasing baseline ISc from 84.8 ± 8.9 to 41.4 ± 8.6 µA/cm² (p=0.006). This suggests that the increase in I_{ENaC} following ASL volume expansion is due to dilution of a protease inhibitor. We then reasoned that if ASL volume expansion is diluting a protease inhibitor, thereby allowing for protease mediated activation of near-silent ENaC, the activating effect of trypsin would be lost following 24 hours of ASL volume expansion. To evaluate this, the ΔISC induced by 1 µM trypsin (ΔTRYPsin) was compared in HAEC maintained at air-liquid and after ASL volume expansion (see Figure 2A). The addition of trypsin increased the ISC in air-liquid conditions from 34.9 ± 7.5 to 56.9 ± 8.4 µA/cm² reflecting the presence of protease susceptible channels (p<0.001). Conversely, the mucosal addition of trypsin did not affect the ISC of cultures under ASL volume expansion (95.9 ± 4.5 vs. 101.7 ± 5.2 µA/cm², p=0.4), suggesting that the pool of silent channels had previously been activated by endogenous proteases.

To further characterize the relationship between ASL volume expansion and aprotinin sensitivity, aprotinin dose titrations were performed in HAEC under basal and expanded ASL volumes. As shown in figure 2B, aprotinin had virtually no effect on I_{ENaC} in HAEC under air-liquid conditions at all concentrations examined (p=0.57, n=3). Conversely, HAEC with ASL volume expansion exhibited a classic dose response to increasing aprotinin concentration. When fit to the Hill equation, the IC50 was 13.5 ± 2.1 µM (R²=0.85, p<0.0001) and I_{ENaC} decreased to the level of air-liquid cultures with 75 µM aprotinin (17.9 ± 4.1 vs 17.1 ± 0.79 µA/cm², p=0.857, n=3). While the data in figure 2B are representative of the relationship between ASL volume expansion and aprotinin sensitivity, the aprotinin IC50 varied among the different cell lines that we tested (mean IC50 9.1 ± 6.8 µM aprotinin, n=54, from 9 tissue donors).

To determine whether protease susceptible ENaC activity exhibits a dose response relationship to increasing ASL volume, the ΔISC following mucosal trypsin exposure was measured in HAEC across a range of ASL volumes. As shown in figure 2C+D, increasing apical volume induced a stepwise increase in ISC. In parallel, the ΔISC induced by trypsin decreased stepwise from
26.8 ± 5.4 µA/cm² at basal ASL depth to -0.4 ± 1.31 µA/cm² with 100 µl of excess apical volume (p<0.001, n=3). The findings that Na⁺ conductance increases and that trypsin susceptible $I_{sc}$ decreases with escalating ASL volume suggest that a pool of near-silent ENaC is activated by endogenous CAPs when the ASL volume increases.

Protease inhibitors are present in the ASL that inhibit Na⁺ conductance by preventing proteolytic activation of ENaC. To directly determine whether the ASL contains protease inhibitors which inhibit ENaC, we examined whether the apical secretions from HAEC could prevent the proteolytic activation of ENaC following ASL expansion. The ASL was expanded for 24 hours with 100 µl PBS ± the apical secretions collected from parallel filters prior to measurement of $I_{ENaC}$ and trypsin stimulated $I_{sc}$ (Figure 3). The presence of an inhibitory factor in the ASL is supported by the finding that apical secretions markedly attenuated the increase in $I_{ENaC}$ induced by ASL volume expansion toward the current level observed for air-liquid interface cultures (p<0.001, n=14). Additionally, when the ASL was expanded with reconstituted HAEC apical secretions, the pool of trypsin susceptible channels was restored (p<0.001, n=6-14). The findings that the apical secretions of HAEC inhibit ENaC and maintain a pool of protease activated channels strongly suggest that endogenous protease inhibitors in the ASL regulate channel activating proteases.

Excessive proteolysis of ENaC in cystic fibrosis epithelium. Next, we evaluated whether altered proteolytic regulation of ENaC contributes to excessive Na⁺ absorption in CF. To assess this, the effects of aprotinin and trypsin were compared between normal and CF HAEC (Figure 4). Overnight exposure to 10 µM aprotinin in 5 µl PBS caused a 39.4 ± 6.3% decrease in $I_{ENaC}$ in CF HAEC, whereas Na⁺ absorption was only inhibited by 18.2 ± 7.7% in normal epithelium (p=0.043, n=15). This suggests that there is a protease inhibitor deficiency in the ASL of CF epithelium that permits constitutive activation of ENaC by CAPs. Furthermore, the activating effect of 1 µM trypsin on $I_{ENaC}$ in CF HAEC was half of that seen in normal HAEC (62.6 ± 8.3% vs 115.5 ± 9.2% increase, p<0.001, n>27), suggesting that fewer near-silent channels are present on the apical surface of CF epithelium. Likewise, CF HAEC had a diminished ability to increase $I_{ENaC}$ following ASL volume expansion for 24 hours (238.2 ± 16.1 % increase in normal vs 77.9 ± 25.7 % increase in CF, p=0.002, n=6). Therefore, the finding that the inducible pool of near-silent ENaC, present in normal HAEC, is constitutively activated in CF cells suggests that unregulated proteolytic activation of ENaC leads to Na⁺ hyperabsorption and promotes ASL depletion in CF.

To confirm that the differences in protease and protease inhibitor susceptibility in CF HAEC are due to proteolysis of ENaC, we performed western blotting on normal and CF HAEC lysate using a rabbit polyclonal antibody against αENaC (30,34-36). This antibody recognizes specific bands corresponding to the ~97 kDa full length channel and a ~75 kDa N-terminal cleavage fragment in HAEC (Figure 5A). To date the following αENaC fragments have been reported: full length (~97 kDa) (15-17,37), furin cleaved fragments (~30 kDa N-terminal and ~65 kDa C-terminal) (15-17,37), and an uncharacterized long N-terminal fragment (~65 kDa) (37-40). As demonstrated in figure 5B-D, there is a substantial increase in the amount of cleaved ENaC in CF HAEC compared to normal. Furthermore, surface biotinylation demonstrates that both the full length and the 75 kDa fragment exist on the cell surface (Figure 5C). The blots were then stripped and re-probed for β actin to assure equal protein loading and assure that intracellular biotinylation had not occurred. There was no consistent appreciable increase in surface expression of full length αENaC in CF cells. However, as shown in Figure 5D, densitometric quantification of the normalized band intensity from 3 independent experiments using HAECE derived from different tissue donors demonstrated a >3 fold increase in the percentage of cleaved channels present in CF HAEC (p=0.038, n=8). These data provide direct evidence for excessive proteolysis of the αENaC subunit in CF human airway epithelium.

**DISCUSSION**

Significant progress has been made towards defining the regulation of airway surface
liquid volume; however, the cellular mechanisms that result in Na⁺ hyperabsorption in CF airways have not been fully defined. Using primary cultures of human airway, we provide evidence that (i) protease inhibitors are present in the ASL and prevent the activation of near-silent ENaC in normal HAEC, (ii) when the ASL volume is increased, endogenous protease inhibitors become diluted, allowing for proteolytic activation of near-silent channels, and (iii) in CF, the normally present near-silent pool of ENaC is constitutively active and the α subunit undergoes increased proteolytic processing. These finding indicate that, in normal airways, a balance between CAPs and endogenous protease inhibitors regulates ENaC activity and provides a plausible mechanistic explanation for HAEC’s ability to auto-regulate ASL volume. Therefore, the concentration of protease inhibitor present in the ASL appears to be a signal that conveys information on the ASL depth to the epithelium to alter its Na⁺ transport properties, in agreement with a recent report from Tarran et al. (28). In addition to elucidating a novel mechanism of ASL auto-regulation, our findings suggest that the activity of CAPs is unregulated in CF, and promotes excessive Na⁺ absorption.

These results differ from that of previous reports of the effect of aprotinin on bronchial epithelial cells (21,22). In our studies (i) trypsin increased I_SC without prior treatment with aprotinin, and (ii) aprotinin had a negligible effect on I_SC when the epithelium was maintained at physiological air-liquid conditions. We speculate that these discrepancies are caused by the common practice of delaying I_SC measurement 20-30 minutes as an equilibration period and the prolonged voltage clamping used in previous studies. In these experiments, the effect of aprotinin was measured following 90 minutes in an Ussing chamber, during which time the cells are submerged. Additionally, the effects of trypsin were assessed following 20-90 minutes of voltage clamping. Based on our kinetic data (Figure 1C), this time interval significantly alters the properties of ENaC on the cell surface. Therefore, our approach was to begin our experiments after a 5 minute equilibration period, in an effort to minimize these potential obscuring effects. Furthermore, Tarran et al. recently reported (28) similar aprotinin and trypsin susceptibility in HAEC when studied under “thin-film” conditions, supporting our results that were obtained in rapid Ussing chamber experiments.

In addition to the dilution of protease inhibitors, it is possible that additional mechanisms serve to increase I_ENaC following ASL volume expansion. When air-liquid HAEC were exposed to trypsin, the I_SC did not increase entirely to the level seen following volume expansion (Figure 2C); this suggests that ASL expansion may also increase channel density at the cell surface, irrespective of serine protease activity. However, cell surface biotinylation of ENaC has not demonstrated a change in channel number following acute trypsin or aprotinin exposure (41-43). Alternatively, endogenous proteases may be more effective than trypsin in activating the channel’s activity. Recently, perturbations to the apical surface of epithelium, such as pressure and shear stress, have been demonstrated to regulate I_ENaC (32,44,45). The mechanical forces induced by ASL volume expansion may be an additional stimulus for I_ENaC. Alternatively, other factors that inhibit ENaC, such as ATP or adenosine (28,45,46), may be similarly diluted following ASL volume expansion.

The serine protease inhibitors HAI-1 and HAI-2 (placental bikunin) are expressed in lung tissue (26-28,47), and have been demonstrated to inhibit prostasin and ENaC activity (21,23). Because primary airway cultures are a mixed population of cell type, including columnar ciliated cells, serous cells, and basal cells, it is unclear whether the critical serine protease inhibitors originate from glandular secretions or from the surface epithelium. Glandular secretions contain these and other serine protease inhibitors and have been suggested to regulate the activity of ENaC in surface epithelium and in submucosal glands (24,25). Our results are consistent with this hypothesis, and provide direct evidence that one or more soluble proteins secreted by human airway epithelial cells regulates ENaC activity (Figure 3).

The significance of the 75 kDa N-terminal αENaC cleavage product found in abundance in CF HAEC remains to be determined. Cleavage of αENaC by furin near the 1st membrane spanning domain increases the channel’s activity (15,33,48). Others have also observed long N-terminal fragments indicating that the channel is cleaved in a region closer to the 2nd membrane spanning
region (37-40). However, at the current time it is not known whether proteolysis in this region of the subunit also results in an increase in channel activity. Interestingly, we did not find evidence of increased αENaC proteolysis following ASL volume expansion (data not shown). This may be the result of our antibody’s inability to detect the known 30 kDa N-terminal product of proteolytic processing or because the proteolytic regulation of ENaC’s activity during ASL volume homeostasis occurs via another subunit. Additional studies are needed to determine whether the 75 kDa N-terminal fragment abundant in CF HAEC constitutes an activated channel and to determine which protease cleaves αENaC near the 2nd membrane spanning region.

The extent to which altered regulation of CAP activity by protease inhibitors in cystic fibrosis contributes to the Na⁺ hyper-absorption in vivo remains to be determined. While our data demonstrates a deficiency of near-silent ENaC in CF HAEC in vitro, the protease-protease inhibitor balance may be different in native CF airways as (i) protease inhibitor containing glandular secretions are aberrant in cystic fibrosis (25,49,50), and (ii) neutrophil elastase, which is abundant in CF airways and absent in cultured HAEC, activates near-silent ENaC channels (20). Therefore, our results may underestimate the contribution of abnormal proteolytic regulation of ENaC on excessive Na⁺ absorption in the airways of CF patients. As aprotinin significantly decreases Na⁺ conductance in CF epithelium, our studies indicate that Kunitz-type serine protease inhibitors are likely to augment airway surface liquid and mucociliary clearance, and may prove therapeutically useful.

REFERENCES


**FOOTNOTES**

*We would like to we thank Dr. Kenneth McCurry and the Lung Transplant Program at the University of Pittsburgh Medical Center for facilitating tissue acquisition and Joseph Latoche for technical assistance. This work was supported by an Institutional National Research Services Award (T32 HL007653), NIH DK56490, DK54814 and DK065161, DK72506 and Cystic Fibrosis Foundation Research Development Program Grant to the University of Pittsburgh, R883CR02.

The abbreviation used are: ENaC, epithelial Na⁺ channel; ASL, airway surface liquid; HAEC, human airway epithelial cells; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CAP, channel-activating protease; $I_{SC}$, short-circuit current; $I_{ENaC}$, amiloride sensitive $I_{SC}$; HAI, hepatocyte growth factor activator inhibitor; NS, not significant*
FIGURE LEGENDS

FIGURE 1. ASL volume expansion increases $I_{ENaC}$. (A,B) Matched HAEC were placed in an Ussing chamber at 0, 24, 48, and 72 hours following ASL volume expansion with Ringers solution. A. Representative $I_{SC}$ tracings demonstrate the increase in $I_{ENaC}$ following ASL expansion. Following a 2.5-mV bipolar pulse, 10 µM amiloride was added to the apical bath. B. Mean $I_{ENaC}$ following ASL volume expansion for 0, 24, 48, and 72 hours. *Significantly different from air-liquid control. C. Kinetics of $I_{ENaC}$ following the addition and removal of excess ASL volume. There is an exponential rise in $I_{ENaC}$ at 0, 0.5, 1, 4, and 24 hours following ASL expansion with Ringers solution (solid line), and an exponential decay in $I_{ENaC}$ following aspiration of expanded ASL volume (dashed line). Matched HAEC were submerged for 24 hours and the excess apical fluid was aspirated at 4, 12, and 24 hours prior to measurement of amiloride sensitive $I_{SC}$. D. Mean percent of air-liquid baseline $I_{ENaC}$ following ASL volume expansion with 0, 5, 50, or 100 µl Ringers solution for 24 hours. *Significantly different from all other conditions. Data shown are mean ± S.E., n=3.

FIGURE 2. ASL volume expansion activates ENaC by altering the protease – protease inhibitor balance. A. Comparison of the $\Delta I_{SC}$ induced by aprotinin and trypsin in HAEC in air-liquid vs ASL volume expansion conditions. Matched HAEC were exposed to either 5 µl (air-liquid) or 100 µl (ASL volume expansion) of Ringers solution ± 30 µM aprotinin for 24 hours. Subsequently, basal $I_{SC}$ and change in $I_{SC}$ following exposure to 1 µM trypsin were measured. Data shown are the mean ± S.E. change in $I_{SC}$ attributable to aprotinin ($\Delta$Aprotinin) or trypsin ($\Delta$Trypsin) for air-liquid and ASL volume expansion conditions; n=6-15 from ≥2 tissue donors. *Significant difference from air-liquid condition. B. Aprotinin dose-response in air-liquid vs ASL volume expansion conditions. Increasing concentrations of aprotinin in either 5 µl or 100 µl was apically applied to matched HAEC for 24 hours. Data shown are mean $I_{ENaC}$ ± S.E, n=3. C. Representative $I_{SC}$ tracings demonstrate the loss of trypsin-stimulated current with increasing ASL volume. ASL volume was expanded with 0, 5, 50, or 100 µl Ringers solution for 24 hours. Following stabilization of the $I_{SC}$, 1 µM trypsin was added to the apical chamber. D. Mean $I_{SC}$ and $\Delta$Trypsin following increasing ASL volume expansion. Data shown are mean $I_{SC}$ ± S.E, n=3. *Significantly different from air-liquid condition.

FIGURE 3. Endogenous protease inhibitors are present in the ASL that inhibit ENaC. Mean amiloride sensitive $I_{SC}$ ($I_{ENaC}$) and $\Delta I_{SC}$ induced by 1 µM trypsin ($\Delta$Trypsin) following ASL volume expansion with 100 µl PBS ± reconstituted apical secretions. Data shown are the mean ± S.E., n=6-14. *Significantly different from air-liquid condition. τSignificantly different from PBS.

FIGURE 4. The protease-protease inhibitor balance is altered in cystic fibrosis epithelium. Matched normal and CF HAEC cultures were exposed 5 µl of PBS ± 10 µM aprotinin for 24 hours. Subsequently, basal $I_{SC}$ and change in $I_{SC}$ following exposure to 1 µM trypsin were measured. Data shown are the mean ± S.E. change in $I_{SC}$ attributable to aprotinin ($\Delta$Aprotinin) or trypsin ($\Delta$Trypsin), normalized to baseline $I_{ENaC}$, from normal and CF HAEC; n>15 from 5 tissue donors. *Significant difference between CF and normal.

FIGURE 5. Excessive proteolysis of $\alpha$ENaC in cystic fibrosis HAEC. A. Immunoblot of CF HAEC lysate demonstrates peptide specificity of the $\alpha$ENaC antisera. The accompanying schematic demonstrates the furin cleavage site and the region of the immunizing peptide. When the antisera was pre-incubated with 10 µg/ml of the immunizing peptide, the intensity of the 97 kDa and 75 kDa bands decreases demonstrating antigen competition. B. Western blot demonstrates an increase in a 75 kDa band of $\alpha$ENaC in CF HAEC lysate, compared to normal, consistent with increased proteolysis in CF. Accompanying $\beta$ actin blot reveals equal protein loading. C. Western blot of the biotinylated apical proteins, from CF and normal HAEC, confirming that the full length and cleaved $\alpha$ENaC molecular species are present on the cell surface. Accompanying $\beta$ actin blot demonstrates that the cellular proteins
were not biotinylated. D. Mean normalized densitometry of the 97 kDa and 75 kDa αENaC bands in normal and CF HAEC. Data shown are mean ± S.E., n=8. *Significantly different from normal HAEC.
FIGURE 1
FIGURE 2

A

\[ \frac{\Delta J_{SC}}{} \text{ (mA/cm}^2\text{)} \]

- Aprotinin
- Trypsin

B

\[ \text{I}_{\text{EKC}} \text{ (mA/cm}^2\text{)} \]

Aprotinin Concentration (µM)

C

\[ \text{I}_{\text{SC}} \text{ (mA/cm}^2\text{)} \]

Time (minutes)

- 100 µL
- 50 µL
- 5 µL
- Air-Liquid

D

\[ \text{I}_{\text{SC}} \text{ (mA/cm}^2\text{)} \]

Volume ASL Expansion (µl)

- Baseline
- \[\Delta\text{Trypsin}\]
FIGURE 3

\[
\begin{array}{c}
\text{Air-Liquid} \\
\text{PBS} \\
\text{PBS + Apical Secretions}
\end{array}
\]

\[
\begin{array}{c}
I_{ENaC} \\
\Delta \text{Trypsin}
\end{array}
\]

\[
\text{ISC (\mu A/cm^2)}
\]

* \text{Significant difference}

\text{\(\tau\)} \text{Not significant difference}
FIGURE 4

% I\(\text{ENaC}\) Change

- Normal
- Cystic Fibrosis

\(\Delta\) Aprotinin
\(\Delta\) Trypsin

*
Airway surface liquid volume regulates ENaC by altering the serine protease-protease inhibitor balance: A mechanism for sodium hypersabsorption in cystic fibrosis
Mike M. Myerburg, Michael B. Butterworth, Erin E. McKenna, Kathryn W. Peters, Raymond A. Frizzell, Thomas R. Kleyman and Joseph M. Pilewski

J. Biol. Chem. published online July 26, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M606449200

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