

Airway Surface Liquid Volume Regulates ENaC by Altering the Serine Protease-Protease Inhibitor Balance

A MECHANISM FOR SODIUM HYPERABSORPTION IN CYSTIC FIBROSIS*

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Efficient clearance of mucus and inhaled pathogens from the lung is dependent on an optimal airway surface liquid (ASL) 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),² 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.

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 α and γ subunits; and (ii) immature channels with high mannose type N-glycans and uncleaved subunits. Proteolytic processing in the biosynthetic pathway increases the activity of the channel 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 in-active 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 pre-

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² The abbreviations used are: ASL, airway surface liquid; ENaC, epithelial Na⁺ channel; HAEC, human airway epithelial cells; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CAP, channel-acti-

vating protease; I_{SC} , short circuit current; I_{ENaC} , amiloride-sensitive I_{SC} ; HAI, hepatocyte growth factor activator inhibitor; PBS, phosphate-buffered saline.

vent 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 described previously (29) and used for experimentation following 4–6 weeks of culture at an air-liquid interface. Non-CF HAECs were obtained from two 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 five donors with the following CF genotypes: ΔF508 , G551D , and $2789 + 5\text{G} \rightarrow \text{A}$. Qualitative differences due to genotype were not observed.

ASL Volume Expansion—To expand the ASL volume, 5–100 μl of Ringer's 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 h. 300 μl of pooled conditioned apical fluid was concentrated by precipitation in 1 ml acetone overnight at -20°C . The apical secretions were then pelleted by centrifugation and resuspended in 100 μl of 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 Ringer's solution was composed of 120 mM NaCl, 25 mM NaHCO_3 , 3.3 mM KH_2PO_4 , 0.8 mM K_2HPO_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 37°C , which maintained the pH at 7.4. Following a 5 min equilibration period, the base-line 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 min prior to amiloride addition, providing a measure of protease activable channels ($\Delta\text{trypsin}$) (20, 28, 31–33). To determine stimulated CFTR currents (I_{CFTR}), after addition of amiloride, 10 μM forskolin (Sigma) and then 10 μM CFTR_{inh}172 (Calbiochem) were added.

Surface Biotinylation and Western Blotting—Surface biotinylation and Western blotting was performed as described previously (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 CaCl_2 , 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 $\text{Na}_2\text{B}_4\text{O}_7$, pH 9). After 20 min, the biotinylation reaction was quenched with PBS plus 10% fetal bovine serum and the cells were rinsed with ice-cold PBS + Ca^+ . The cells were then lysed in cell lysis buffer (10 mM Tris-Cl, 50 mM EGTA, 0.4% sodium deoxycholate, 1% Nonidet P-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 4°C . 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 described previously (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 $\mu\text{g}/\text{ml}$ of the immunizing peptide with the antisera for 2 h at 37°C prior to immunoblotting.

Statistics—Results are expressed as mean \pm S.E. The percent of base-line 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 analysis of variance with Bonferroni post hoc analysis or by Student's t test where appropriate. Results were confirmed in at least three independent experiments using HAEC cultured from different lung tissues.

RESULTS

ASL Volume Expansion Increases the Amiloride-sensitive I_{SC} —The base-line I_{SC} for HAEC at air-liquid interface averaged $34.3 \pm 1.9 \mu\text{A}/\text{cm}^2$ ($n = 81$). Following 24 h of ASL volume expansion with 100 μl of Ringer's, the mean I_{SC} increased to $77.1 \pm 4.1 \mu\text{A}/\text{cm}^2$ ($n = 60$, $p < 0.001$ versus control). Following the addition of 10 μM amiloride, the residual I_{SC} was the same in control and ASL expansion conditions ($12.2 \pm 0.5 \mu\text{A}/\text{cm}^2$ versus $13.4 \pm 0.7 \mu\text{A}/\text{cm}^2$, not significant). This indicates that the increased I_{SC} observed with ASL volume expansion reflects conductive ENaC-mediated sodium absorption. I_{CFTR} , defined as the CFTR_{inh}172-sensitive current following forskolin stimulation, was not significantly altered by ASL volume expansion (9.8 ± 0.7 versus $9.9 \pm 0.6 \mu\text{A}/\text{cm}^2$, $n = 15$ and 11). Transepithelial potential difference increased from $12.1 \pm 0.6 \text{ mV}$ to $26.2 \pm 1.3 \text{ mV}$ following ASL volume expansion ($p < 0.001$). Transepithelial resistance decreased from $492 \pm 18 \Omega \cdot \text{cm}^2$ to $407 \pm 20 \Omega \cdot \text{cm}^2$ ($p = 0.002$). 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

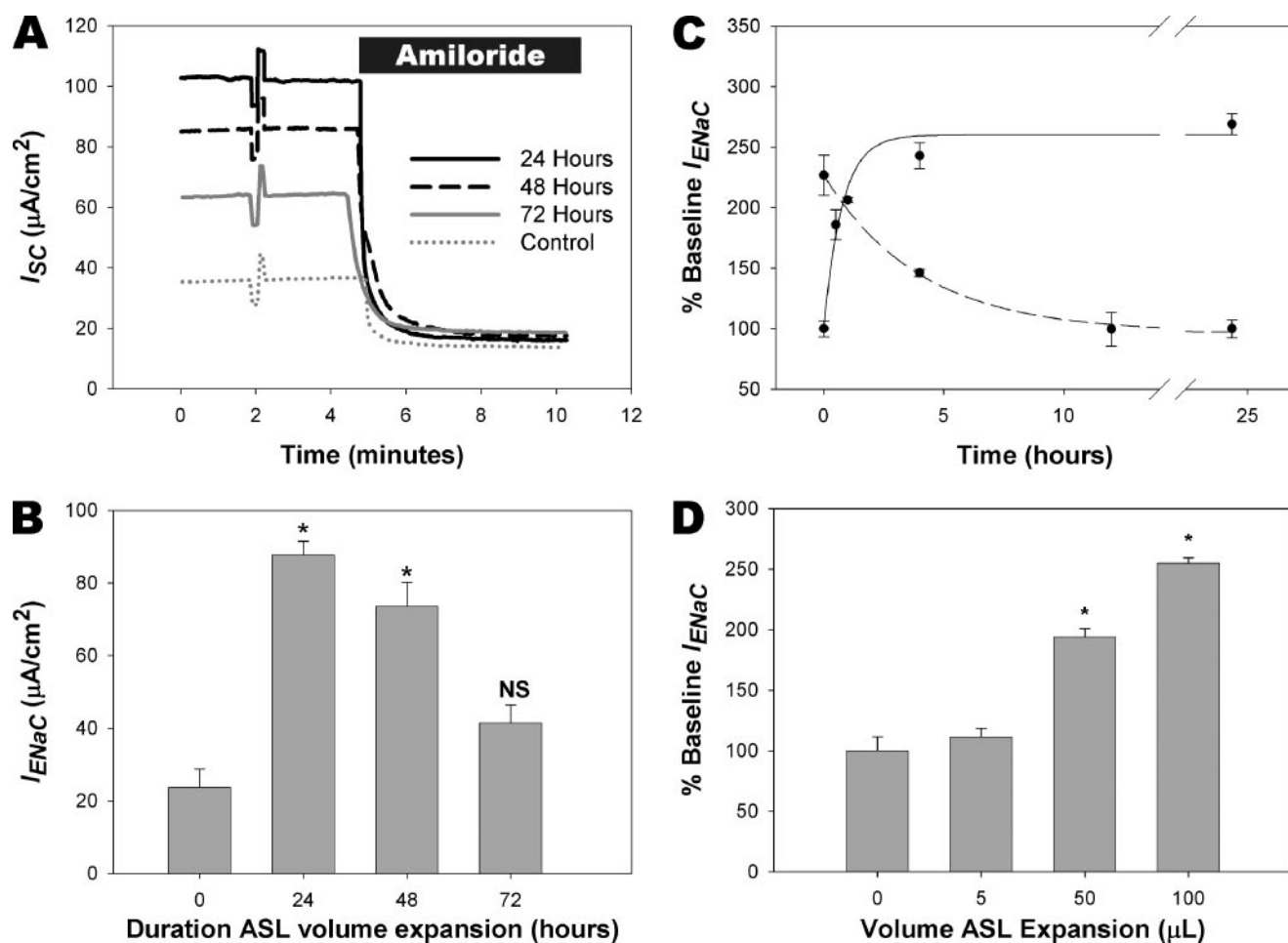


FIGURE 1. ASL volume expansion increases I_{ENaC} . A and B, matched HAEC were placed in an Ussing chamber at 0, 24, 48, and 72 h following ASL volume expansion with Ringer's solution. A, representative I_{SC} tracings demonstrate the increase in I_{ENaC} following ASL expansion. Following a 2.5-mV bipolar pulse, 10 μM amiloride was added to the apical bath. B, mean I_{ENaC} following ASL volume expansion for 0, 24, 48, and 72 h. *, 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 h following ASL expansion with Ringer's solution (solid line) and an exponential decay in I_{ENaC} following aspiration of expanded ASL volume (dashed line). Matched HAEC were submerged for 24 h and the excess apical fluid was aspirated at 4, 12, and 24 h prior to measurement of amiloride sensitive I_{SC} . D, mean percent of air-liquid base-line I_{ENaC} following ASL volume expansion with 0, 5, 50, or 100 μL of Ringer's solution for 24 h. *, significantly different from all other conditions. Data shown are mean \pm S.E., $n = 3$.

assess the kinetics, the ASL volume of matched HAEC was expanded with 100 μL of Ringer's for 0, 24, 48, and 72 h prior to measurement of the amiloride-sensitive I_{SC} . Representative I_{SC} tracings and mean I_{ENaC} are shown in Fig. 1, A and B. In this series of experiments, the air-liquid base-line I_{ENaC} was $23.7 \pm 5 \mu A/cm^2$, and increased to $87.7 \pm 3.8 \mu A/cm^2$ at 24 h ($p = 0.0005$). Following the initial rise at 24 h, I_{ENaC} declined to $41.4 \pm 5 \mu A/cm^2$ at 72 h ($p = 0.26$ compared with base line). The I_{SC} of matched control filters at air-liquid interface increased by $2.6 \pm 3.3 \mu A/cm^2$ over the 72-h interval ($p > 0.05$, $n = 3$). Thus, the increase in I_{ENaC} due to ASL volume expansion is maximal at 24 h, after which time I_{ENaC} declines to near base line.

To further define the kinetics of the I_{ENaC} response to ASL expansion, the ASL volume was increased with 100 μL of Ringer's solution 0.5, 1, 4, and 24 h prior to measurement of amiloride-sensitive I_{SC} . The I_{ENaC} was normalized to that of matched air-liquid HAEC and fitted to a first order exponential equation (% base line = $y_0 + ae^{-kx}$). As shown in Fig. 1C, there is a rapid increase in I_{ENaC} following ASL volume expansion ($t_{1/2} = 0.5 \pm$

0.09 h). 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 h, and the excess apical fluid was subsequently aspirated at 0, 4, 12, and 24 h prior to measurement of I_{ENaC} . Following the removal of excess ASL, I_{ENaC} returned to base line over ~ 12 h ($t_{1/2} = 2.8 \pm 0.8$ h, see Fig. 1C). Because of the rapidity of these changes, all I_{SC} measurements were obtained following a brief 5-min equilibration period in the Ussing chamber.

To assess the effect of increasing ASL volume, 0, 5, 50, or 100 μL of Ringer's solution was added to the apical surface of HAEC for 24 h. As shown in Fig. 1D, I_{ENaC} increased proportionately with increasing ASL volume. Volume increases above 100 μL had no further effect (data 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

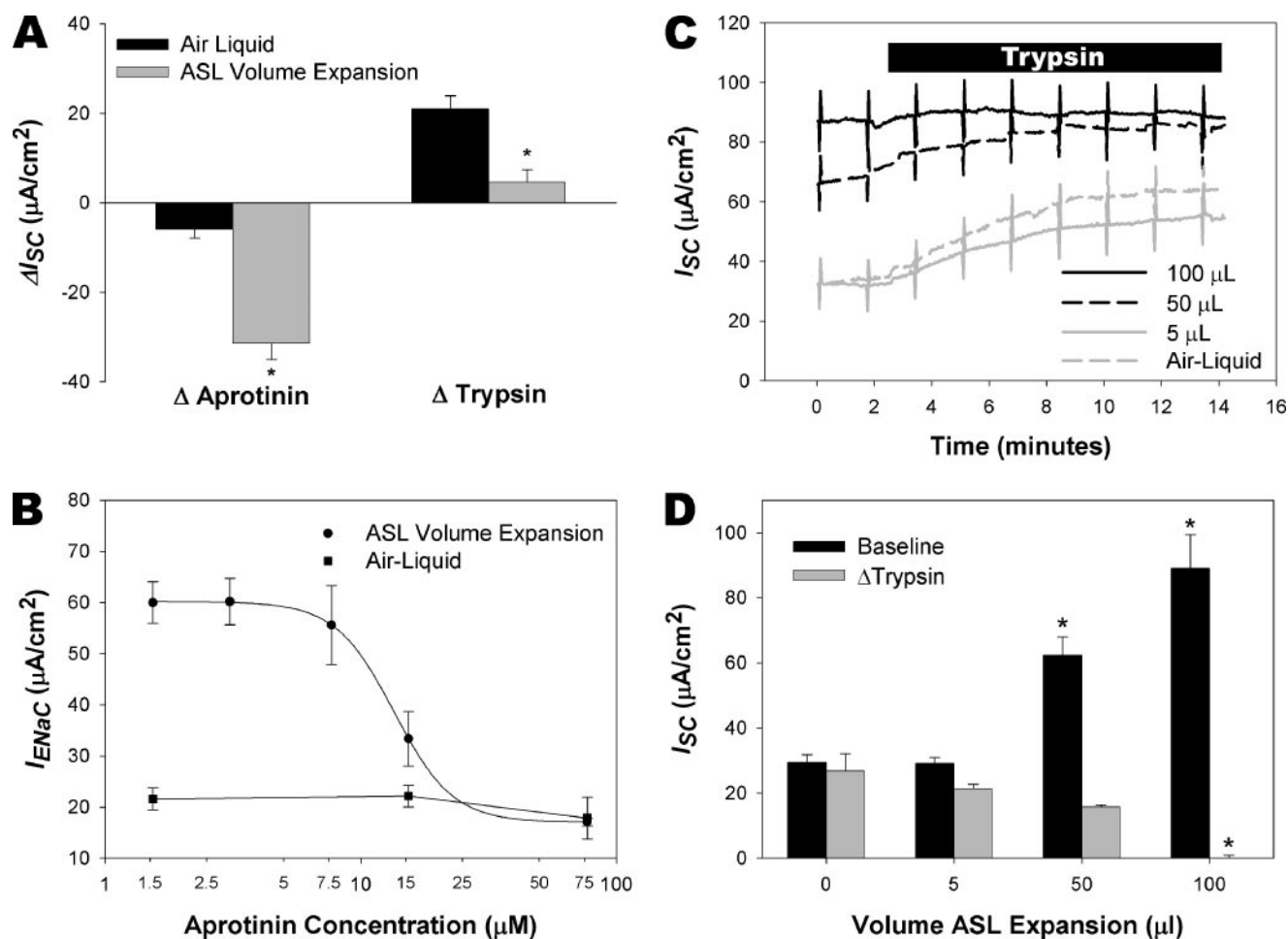


FIGURE 2. ASL volume expansion activates ENaC by altering the protease-protease inhibitor balance. *A*, comparison of the ΔI_{SC} induced by aprotinin and trypsin in HAEC in air-liquid versus ASL volume expansion conditions. Matched HAEC were exposed to either 5 μ l (air-liquid) or 100 μ l (ASL volume expansion) of Ringer's solution \pm 30 μ M aprotinin for 24 h. Subsequently, basal I_{SC} and change in I_{SC} following exposure to 1 μ M trypsin were measured. Data shown are the mean \pm S.E. change in I_{SC} attributable to aprotinin (Δ aprotinin) or trypsin (Δ 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 versus ASL volume expansion conditions. Increasing concentrations of aprotinin in either 5 or 100 μ l was apically applied to matched HAEC for 24 h. Data shown are mean $I_{ENaC} \pm$ 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 μ l of Ringer's solution for 24 h. Following stabilization of the I_{SC} , 1 μ M trypsin was added to the apical chamber. *D*, mean I_{SC} and Δ trypsin following increasing ASL volume expansion. Data shown are mean $I_{SC} \pm$ S.E., $n = 3$. *, significantly different from air-liquid condition.

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 Fig. 2A). 30 μ M aprotinin was administered in either 5 or 100 μ l of PBS to the apical surface of HAEC for 24 h prior to I_{SC} measurement. These apical volumes were selected because 5 μ l did not alter I_{ENaC} at 24 h (see Fig. 1D), and 100 μ l caused the maximal ASL volume expansion response (data not shown). In air-liquid conditions (5 μ l added), exposure to aprotinin for 24 h did not significantly alter base-line I_{SC} , suggesting that an aprotinin-like protease inhibitor is endogenously present in the ASL. However, under ASL volume expansion, aprotinin had a profound effect, decreasing base-line I_{SC} from 84.8 ± 8.9 to 41.4 ± 8.6 μ A/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 h of ASL volume expansion. To evaluate this, the ΔI_{SC} induced by 1 μ M trypsin (Δ trypsin) was compared in HAEC maintained at air-liquid and after ASL volume expansion (see Fig. 2A). The addition of trypsin increased the I_{SC} in air-liquid conditions from 34.9 ± 7.5 to 56.9 ± 8.4 μ A/cm² reflecting the presence of protease susceptible channels ($p < 0.001$). Conversely, the mucosal addition of trypsin did not affect the I_{SC} of cultures under ASL volume expansion (95.9 ± 4.5 versus 101.7 ± 5.2 μ A/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 Fig. 2B, aprotinin had virtually no effect on I_{ENaC} in HAEC under air-liquid conditions at all concentra-

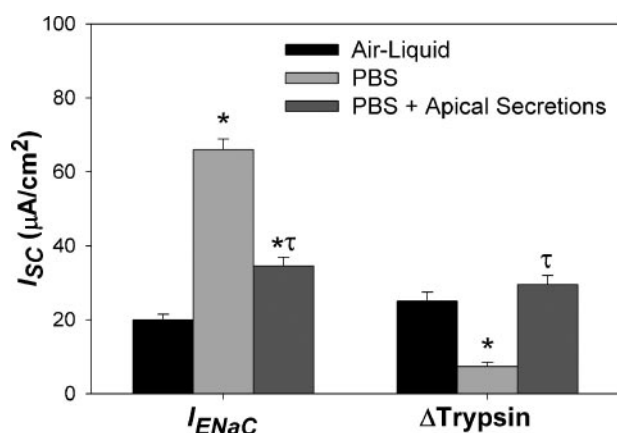


FIGURE 3. Endogenous protease inhibitors are present in the ASL that inhibit ENaC. Mean amiloride-sensitive I_{SC} (I_{ENaC}) and ΔI_{SC} induced by 1 μM trypsin (Δ Trypsin) following ASL volume expansion with 100 μl of PBS \pm reconstituted apical secretions. Data shown are the mean \pm S.E., $n = 6-14$.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

tions 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 IC_{50} was $13.5 \pm 2.1 \mu M$ ($R^2 = 0.85$, $p < 0.0001$) and I_{ENaC} decreased to the level of air-liquid cultures with 75 μM aprotinin (17.9 ± 4.1 versus $17.1 \pm 0.79 \mu A/cm^2$, $p = 0.857$, $n = 3$). While the data in Fig. 2B are representative of the relationship between ASL volume expansion and aprotinin sensitivity, the aprotinin IC_{50} varied among the different cell lines that we tested (mean IC_{50} $9.1 \pm 6.8 \mu M$ aprotinin, $n = 54$, from nine tissue donors).

To determine whether protease susceptible ENaC activity exhibits a dose response relationship to increasing ASL volume, the ΔI_{SC} following mucosal trypsin exposure was measured in HAEC across a range of ASL volumes. As shown in Fig. 2, C and D, increasing apical volume induced a stepwise increase in I_{SC} . In parallel, the ΔI_{SC} induced by trypsin decreased stepwise from $26.8 \pm 5.4 \mu A/cm^2$ at basal ASL depth to $-0.4 \pm 1.31 \mu A/cm^2$ 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 h with 100 μl of PBS \pm the apical secretions collected from parallel filters prior to measurement of I_{ENaC} and trypsin stimulated I_{SC} (Fig. 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 ($p < 0.001$, $n =$

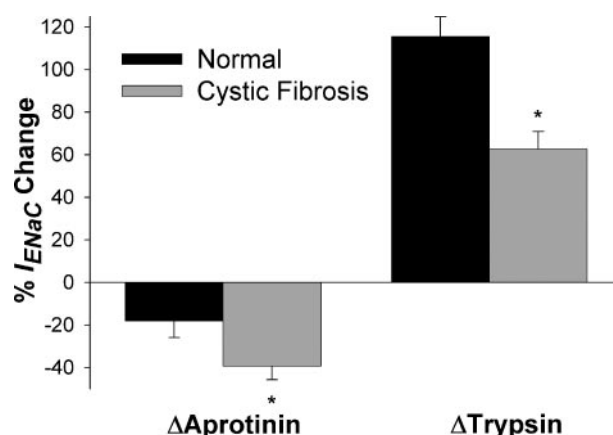


FIGURE 4. The protease-protease inhibitor balance is altered in cystic fibrosis epithelium. Matched normal and CF HAEC cultures were exposed to 5 μl of PBS \pm 10 μM aprotinin for 24 h. Subsequently, basal I_{SC} and change in I_{SC} following exposure to 1 μM trypsin were measured. Data shown are the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

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 (Fig. 4). Overnight exposure to 10 μM aprotinin in 5 μl of PBS caused a $39.4 \pm 6.3\%$ decrease in I_{ENaC} in CF HAEC, whereas Na^+ absorption was only inhibited by $18.2 \pm 7.7\%$ 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 ($62.6 \pm 8.3\%$ versus $115.5 \pm 9.2\%$ 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 h ($238.2 \pm 16.1\%$ increase in normal versus $77.9 \pm 25.7\%$ 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 α ENaC (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 (Fig. 5A). To date the following α ENaC 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 Fig. 5, B–D, there is a substantial increase in

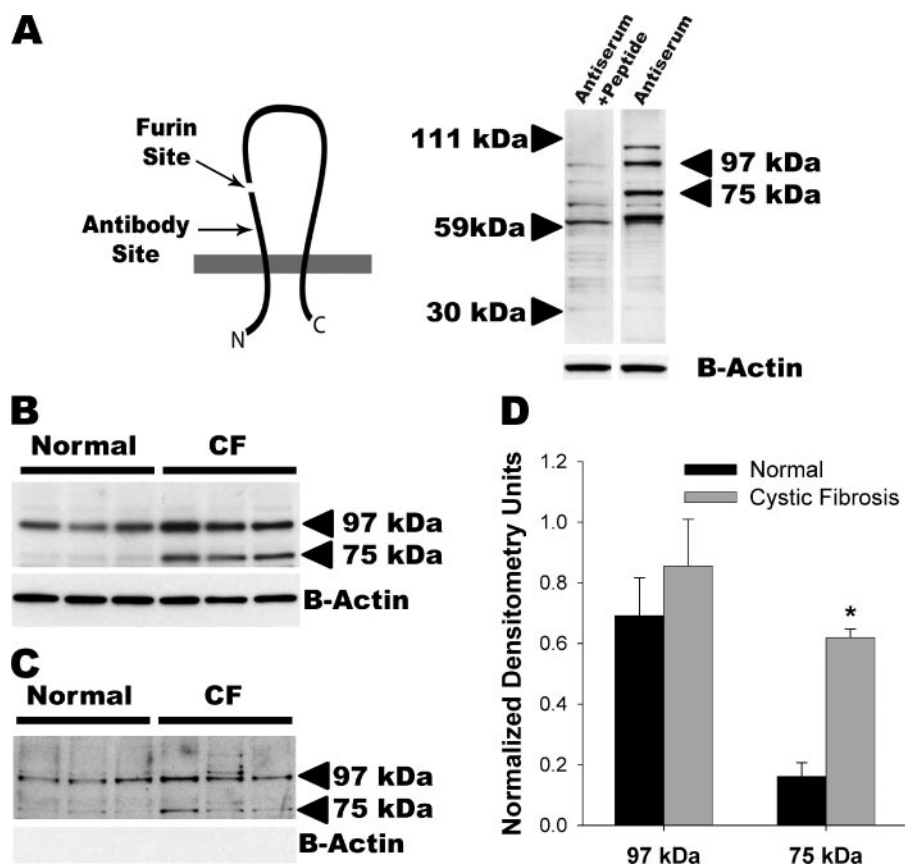


FIGURE 5. Excessive proteolysis of α ENaC in cystic fibrosis HAEC. *A*, immunoblot of CF HAEC lysate demonstrates peptide specificity of the α ENaC antisera. The accompanying schematic demonstrates the furin cleavage site and the region of the immunizing peptide. When the antisera was preincubated with a 10 μ g/ml concentration of the immunizing peptide, the intensity of the 97- and 75-kDa bands decreases demonstrating antigen competition. *B*, Western blot demonstrates an increase in a 75-kDa band of α ENaC in CF HAEC lysate, compared with normal, consistent with increased proteolysis in CF. Accompanying β -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 α ENaC molecular species are present on the cell surface. Accompanying β -actin blot demonstrates that the cellular proteins were not biotinylated. *D*, mean normalized densitometry of the 97- and 75-kDa α ENaC bands in normal and CF HAEC. Data shown are mean \pm S.E., $n = 8$. *, significantly different from normal HAEC.

the amount of cleaved ENaC in CF HAEC compared with normal. Furthermore, surface biotinylation demonstrates that both the full-length and the 75-kDa fragment exist on the cell surface (Fig. 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 α ENaC in CF cells. However, as shown in Fig. 5D, densitometric quantification of the normalized band intensity from three independent experiments using HAEC 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 α ENaC subunit in CF human airway epithelium.

DISCUSSION

Significant progress has been made toward 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 findings indicate that, in normal airways, a balance between CAPs and endogenous protease inhibitors regulates ENaC activity and provides a plausible mechanistic explanation for the ability of HAEC 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 min as an equilibration period and the prolonged voltage clamping used in previous studies. In these experiments, the effect of aprotinin was measured following 90 min in an Ussing chamber, during which time the cells are submerged. Additionally, the effects of trypsin were assessed following 20–90 min of voltage clamping. Based on our kinetic data (Fig. 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-min equilibration period, in an effort to minimize these potential obscuring effects. Furthermore, Tarran *et al.* (28) recently reported 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 (Fig. 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 activity of the channel. 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 (Fig. 3).

The significance of the 75-kDa N-terminal α ENaC cleavage product found in abundance in CF HAEC remains to be determined. Cleavage of α ENaC by furin near the first membrane-spanning domain increases the activity of the channel (15, 33, 48). Others have also observed long N-terminal fragments indicating that the channel is cleaved in a region closer to the second 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 α ENaC proteolysis following ASL volume expansion (data not shown). This may be the result of the inability of our antibody to detect the known 30-kDa N-terminal product of proteolytic processing or because the proteolytic regulation of the activity of ENaC 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 α ENaC near the second membrane-spanning region.

The extent to which altered regulation of CAP activity by protease inhibitors in cystic fibrosis contributes to the Na^+ hyperabsorption *in vivo* remains to be determined. While our data demonstrate 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 to excessive Na^+ absorption in the airways of CF patients. As aprotinin sig-

nificantly 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.

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