Acute Regulation of the Epithelial Sodium Channel in Airway Epithelia by Proteases and Trafficking

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Effective clearance of inhaled pathogens is the primary innate defense mechanism in the lung, and requires the maintenance of a proper airway surface liquid (ASL) volume to facilitate ciliary beat and optimize mucociliary clearance. Na⁺ absorption via the epithelial sodium channel (ENaC) is tightly regulated and, together with chloride movement, provides the optimal osmotic gradients to absorb excessive fluid in the airway lumen while preventing excessive ASL dehydration, which would compromise mucus clearance from the lung. To absorb excessive fluid from the luminal surface, a local mechanism of ENaC activation allows for an increase in Na⁺ absorption at times when the ASL volume is expanded. To help define these regulatory mechanisms, we examined the effects of ASL volume expansion on ENaC activity in primary human bronchial epithelial (HBE) cell cultures. We found that ENaC activity increases dramatically after rapid dilution of endogenous ASL. Approximately 35% of the increase in Na⁺ absorption was attributable to activation of ENaC by proteases. The remainder of the increase in Na⁺ current was prevented when membrane trafficking was disrupted with brefeldin A, nocodazole, or myosin light chain kinase inhibitors, demonstrating that trafficking is involved with ENaC regulation in the airway. These findings demonstrate that Na⁺ absorption in the airway is acutely modulated by the coordinated trafficking of channels to the luminal surface and by the proteolytic activation of ENaC in response to ASL volume expansion.

Keywords: ENaC; HBE; airway epithelium; airway surface liquid

The luminal surfaces of the conducting airways are lined with a thin layer of fluid known as the airway surface liquid (ASL), which facilitates mucus clearance from the lung. The height of the ASL is determined by the net osmotic gradient established by Na⁺ absorption and Cl⁻ secretion through apically located ion channels (1–4). The epithelial sodium channel (ENaC), in conjunction with basolaterally located Na⁺/K⁺ ATPase, is thought to be the predominant means of Na⁺ absorption across the airway epithelium. ENaC activity in renal and colonic epithelia is dictated by both local and systemic stimuli, whereas ENaC activity in airway epithelium appears to be regulated primarily by local factors in the luminal environment (5). This localized regulation facilitates ENaC autoregulation to maintain an optimal ASL volume. Evidence to date indicates that proteolytic activation (6–8), flow activation (9, 10), and cyclic

nucleotides/purino-receptor regulation (11–13) appear to be the predominant mechanisms that regulate airway ENaC under physiologic conditions, rather than responses to hormonal stimuli.

Previous work from our group and others indicates that a balance between the protease activity of membrane-tethered, channel-activating proteases (CAPs) and soluble protease inhibitors in the ASL modulates ENaC activity and therefore Na⁺ absorption across human bronchial epithelial (HBE) cells (6, 7). When the ASL volume is low, during steady-state and non-pathologic conditions, the concentration of soluble protease inhibitor is sufficient to minimize the constitutive activation of ENaC by CAPs. Conversely, when the ASL volume is expanded, the soluble protease inhibitors are diluted, allowing for CAP-mediated activation of ENaC. In previous work, Na⁺ absorption in HBE cells under ASL volume expansion conditions was recognized to be greater than that found in control cultures with basal ASL volumes that were exposed to exogenous channel activating proteases (6). This suggested that alternative mechanisms, in addition to proteolytic activation, are present that significantly augment Na⁺ absorption in the airway in response to ASL expansion.

Na⁺ absorption via the ENaC is enhanced through either an increase in the open probability (P_o) of the channel or through an increase in channel number (n) (14). Although proteolytic processing, flow activation, and cyclic nucleotides/purino-receptor regulation are some mechanisms that alter the P_o of the channel, the contribution of ENaC trafficking on the regulation of Na⁺ absorption in the airway epithelium is unknown. Therefore, this study sought to determine the relative contributions of proteolytic processing and trafficking of ENaC in response to increases in the ASL volume in primary HBE cells. To measure acute changes in ENaC activity in response to ASL volume expansion, we continuously measured the short circuit current (I_{SC}) of HBE cultures after the transition from an air-liquid interface to a liquid-liquid environment in modified Ussing chambers. The rate of increase in ENaC activity after apical submersion was examined in HBE cells cultured in the presence and absence of a variety of steroids, protease inhibitors, and proteases, to determine the degree to which proteolytic regulation modulates channel activity. Because only a portion of the increase in the amiloride-sensitive I_{SC} (I_{Na}) could be attributed to protease-mediated activation, we next determined the effects of various blockers of membrane trafficking on the activation of ENaC. In a final series of experiments, we investigated whether manipulation of adenosine receptor signaling, cyclic adenosine monophosphate (cAMP), protein kinase A/C (PKA/PKC), intracellular Ca²⁺, new protein synthesis, or luminal osmolarity altered the acute stimulation of I_{Na} after the washout of endogenous ASL. Our findings demonstrate that ENaC regulation in the airway occurs via multiple mechanisms, and suggest that after ASL expansion, a reserve pool of channels is trafficked to the luminal surface where channels can be activated further via proteolytic processing.

(Received in original form September 22, 2009 and in final form December 11, 2009)

This work was supported by grants from the National Institutes of Health (K08 HL087932 to M.M.M., P30 DK072506 to J.M.P., and K99-DK07891 to M.B.B.) and from the Cystic Fibrosis Foundation (MYERBU07Q0 to M.M.M., R883 to J.M.P., and BUTTER06GO to M.B.B.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 43, pp 712–719, 2010

Originally Published in Press as DOI: 10.1165/rncmb.2009-0348OC on January 22, 2010

Internet address: www.atsjournals.org

MATERIALS AND METHODS

Primary Human Airway Epithelial Cell Culture

HBE cells were cultured from excess pathologic tissue after lung transplantation and organ donation, under a protocol approved by the University of Pittsburgh Investigational Review Board, as previously described (15). Bronchi from the second to sixth generations were dissected, thoroughly rinsed, and incubated overnight at 4°C in Eagle's minimum essential media (EMEM). The bronchi were then digested overnight at 4°C in MEM containing 0.1% protease XIV and 0.01% DNase. The epithelial cells were removed from the underlying stroma by blunt dissection, isolated by centrifugation, and washed in MEM containing 5% FBS. After centrifugation, cells were resuspended in bronchial epithelial growth medium (BEGM; Lonza, Basel, Switzerland) and plated onto Type VI human placental collagen-treated tissue culture flasks. On reaching 80–90% confluence, the passage 0 cells were trypsinized (0.1%), resuspended in BEGM, and seeded onto human placental collagen-coated Costar Transwell filters (0.33 cm² 0.4 µm pore, catalogue number 3470, Corning, Lowell, MA) at a density of approximately $2 \times 10^5/\text{cm}^2$. Upon reaching confluence, the apical media was aspirated, and HBE cultures were maintained at an air-liquid interface (ALI) and fed basolaterally with differentiation media twice weekly. The differentiation media (BEGM/Ultrosor G; Pall Corporation, Crescent Chemical Company, Islandia, NY) contained 5 µg/ml insulin, 10 µg/ml transferrin, 0.07 µg/ml hydrocortisone, 0.6 µg/ml epinephrine, 0.8% vol/vol bovine hypothalamus extract, 0.5 mg/mL BSA, 0.5 µM ethanolamine, 15 ng/ml retinoic acid, 0.5 ng/ml human epidermal growth factor, 10 nM triiodothyronine, 0.5 µM phosphoethanolamine, and 0.5% vol/vol Ultrosor G (USG) in Dulbecco's MEM (DMEM)/F12. Cells were studied after 3 to 6 weeks of culture, and considered differentiated when a mucociliary phenotype was apparent on phase contrast microscopy.

I_{SC} Recordings

Differentiated HBE cultures were mounted in Ussing chambers (P2300; Physiological Instruments, San Diego, CA), with custom sliders modified to fit the Transwell inserts, and cultures were continuously short-circuited with an automatic voltage clamp (VCC MC8, Physiological Instruments). The Ringer's solution bath consisted of 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, and 10 mM glucose. Chambers were constantly gassed with a mixture of 95% O₂/5% CO₂ at 37°C, which maintained the pH at 7.4 and established a circulating perfusion bath within the Ussing chamber. The apical and basolateral chambers each contained 3 ml of Ringer's solution. Simultaneous transepithelial resistance was recorded by applying a 10-mV pulse per second via an automated pulse generator. Acquire and Analyze 2.3 (Physiological Instruments) was used to control the voltage clamp and analyze I_{SC} data. A typical I_{SC} recording included a 30-minute equilibration period, followed by stimulation with 300 nM porcine pancreatic elastase (catalogue number EC134; EPC, Owensville, MO) or 1 µM trypsin. The I_{Na} was determined by the addition of 10 µM amiloride to the apical cell chamber at the end of each recording. Additional substances included 50 nM dexamethasone, 50 nM aldosterone, 10 µM forskolin, 100 µM bumetanide, 10 µM aprotinin, 50 µM furin convertase inhibitor (FCI, dec-RVKR-cmk; Axxora, San Diego, CA), A2a-receptor antagonist (4-(2-[7-amino-2-(2-furyl)][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; Tocris Bioscience, Ellisville, MO), 50 µM H-7, 10 µM bis(2-aminophenoxy)ethane tetraacetic acid-cetoxymethyl ester (BAPTA-AM) (Invitrogen, Carlsbad, CA), 200 µg/mL cycloheximide (CHX), 5 µg/mL brefeldin A (BFA), 10 µM nocodazole, 100 µM ML-7, 100 µM ML-9, 1 µM wortmannin (Calbiochem, Gibbstown, NJ), nystatin, 200 µM ouabain, and 50 µg/ml vinblastine. Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Determination of I_{SC} Kinetics and ENaC Functional Half-Life

To determine the kinetics of I_{SC} changes, the I_{SC} values for each individual trace over the indicated time interval was fit to the exponential equation $y = y_0 + a*(1 - e^{-t/\tau})$, where $y = I_{SC}/I_{SC}$ at time 0 and $t =$ minutes. The time to reach half of the maximal I_{SC} ($t_{1/2}$) was derived as $t_{1/2} = \tau \ln 2$. To determine the functional half-life ($t_{1/2}$) of ENaC, differentiated HBE cultures were pretreated for 30 minutes

with 200 µg/mL CHX or DMSO (vehicle control) and subsequently used for I_{SC} recording. The percentage of maximal I_{Na} was plotted over time at 10-minute intervals, and fit to a linear equation ($y = y_0 + mx$). The slope (m) of individual decay plots was corrected to the average slope of untreated control filters, to correct for baseline I_{SC} rundown. The $t_{1/2}$ was derived as $t_{1/2} = y_0/2$ m.

Data Analyses and Statistics

Results are expressed as mean $\pm$ SE. Significance was determined by unpaired Student's *t* tests unless otherwise indicated. $P < 0.05$ was considered statistically different.

RESULTS

Sodium Absorption Increases after ASL Washout in an Ussing Chamber

We and others previously noted that when the ASL volume of airway epithelium is expanded, the transepithelial Na⁺ absorption increases dramatically (6, 7). This increase was attributed to the washout of substances that inhibit ENaC, such as protease inhibitors or ATP. Na⁺ and water absorption must increase in response to increased luminal volume, to maintain optimal mucociliary clearance and prevent bronchorrhea. To study this increase in ENaC activity, primary HBE cells were mounted in a well-mixed Ussing chamber, to increase the apical volume dramatically (apical chamber volume, 3 mL), effectively dilute the endogenous inhibitory factors present in the ASL, and perform transepithelial short-circuit current measurements.

As shown in Figure 1, the short circuit increases after apical washout from 12.8 ± 1.5 µA/cm² to 40.8 ± 2.3 µA/cm² over a 30-minute interval, when HBE cells are cultured in differentiation media (BEGM/USG). To assess whether growth factors or steroids increase the expression of ENaC and are necessary for the increase in I_{Na} after mounting in an Ussing chamber, HBE cells were incubated overnight in base medium (control) or DMEM containing either 50 nM aldosterone or 50 nM dexamethasone. Without steroids or growth factors, a reduced amiloride-sensitive current was evident, and the increase in I_{Na} after the 30-minute equilibration period was modest (initial current, 6.2 ± 0.8 µA/cm²; at 30 minutes, 12.0 ± 1.5 µA/cm²). In the presence of aldosterone or dexamethasone, significant increases in I_{Na} occurred during the 30-minute equilibration period (15.7 ± 3.7 to 58.3 ± 4.9 µA/cm² and 18.5 ± 4.4 to 55.6 ± 4.2 µA/cm², respectively).

In an attempt to elucidate the mechanism behind the increase in Na⁺ conductance associated with ASL washout, we calculated the kinetics of the rise in current during the initial 30 minutes. The $t_{1/2}$ of the increase in I_{Na} varied between cultures derived from different donors, and therefore these kinetics measurements were performed in parallel, using matched cultures on each individual day of the experiment. In the experiments presented in Figure 1C, the $t_{1/2}$ of the I_{Na} increase during the 30-minute equilibration period under the control, dexamethasone-stimulated, and aldosterone-stimulated conditions was relatively similar. However, the rate of I_{Na} increase was significantly slower under BEGM/USG conditions. The expansion of ASL was associated with an increase in I_{Na} that occurs over a period of minutes, suggesting that the regulation is not a result of new channel synthesis.

Next, a series of experiments was performed to confirm that the increase in I_{Na} observed during ASL washout is attributable to an increase in conductive sodium absorption through ENaC. To ensure that the increase in I_{Na} did not reflect an artifact introduced by voltage clamping, HBE cultures were placed into the Ussing chamber, and the transepithelial electric potential difference (V_T) was recorded under open-circuit conditions (as shown in Figure E1 in the online supplement). Because the

Figure 1. Na^+ absorption increases after airway surface liquid (ASL) washout in an Ussing chamber. Human bronchial epithelial (HBE) cell cultures were cultured in DMEM/F12 $\pm$ bronchial epithelial growth medium (BEGM), 50 nM dexamethasone (Dex), or 50 nM aldosterone (Aldo) for 24 hours before short-circuit current (I_{sc}) recording. After a 30-minute equilibration, 10 μM of amiloride were added to the apical chamber to determine the amiloride-sensitive I_{sc} (I_{Na}). Subsequently, Cl^- secretion was assessed by adding 10 μM forskolin and then 100 μM bumetanide to the basolateral chamber. Data shown are (A) representative I_{sc} recordings, (B) mean amiloride-sensitive current (I_{Na}) $\pm$ SE, and (C) and mean $t_{1/2}$ $\pm$ SE of the I_{Na} increase from 0 to 30 minutes ($n = 12$ cultures from three tissue donors). USG, Ultrosor G. * $P < 0.001$, different from control culture as determined by ANOVA with Bonferroni *post hoc* analysis.

increase in V_T is similar to the increase in I_{sc} over the 30-minute interval, the increase in current after placement in the Ussing chamber was unlikely to be the result of voltage clamping. To determine whether the increase in I_{Na} after ASL washout was attributable to a change in apical membrane conductance or changes in Na^+/K^+ ATPase activity, we performed apical and basolateral permeabilization experiments (Figure E2). Interestingly, when the basolateral membrane was permeabilized with nystatin and an apical to basolateral sodium gradient was established, the increase in I_{Na} during ASL washout was diminished, suggesting that either an intact plasma membrane is required for the response, or that a change occurred in Na^+/K^+ ATPase activity during the experiment. Therefore, we directly determined whether Na^+/K^+ ATPase activity changes during the course of the ASL washout. After apical permeabilization with nystatin, a large increase in I_{sc} was observed, demonstrating that apical membrane conductance constitutes the rate-limiting step in transepithelial Na^+ absorption. Because the I_{sc} was abolished with the addition of basolateral ouabain, the peak I_{sc} observed after apical permeabilization was assumed to reflect ATPase activity. No change in ATPase activity was evident during the course of I_{sc} recording and ASL washout. Therefore, the increase in I_{sc} associated with ASL washout is

likely attributable to a change in ENaC activity, and not an artifact of voltage clamping or changes in Na^+/K^+ ATPase activity.

Proteolytic Activation of ENaC in Response to ASL Washout

Previous work suggested that the increase in Na^+ absorption after ASL volume expansion is attributable to the dilution of soluble protease inhibitors and a subsequent proteolytic activation of ENaC's by CAPs (6, 7). Therefore, we investigated the degree to which the proteolytic activation of ENaC contributes to the observed increase in I_{Na} after ASL washout in an Ussing chamber. Matched HBE cultures were mounted in the Ussing chamber containing Ringer's solution, with or without 300 nM elastase or 10 μM aprotinin. This combination of CAP and protease inhibitor was selected because aprotinin was previously shown to inhibit Na^+ currents, and elastase is not inhibited by aprotinin (16–18), allowing for the sequential addition of elastase without concerns about protease inhibition. As shown in Figure 2, the I_{sc} of cultures that were exposed to elastase increased at a rate similar to that in control cultures. After the initial equilibration period, elastase treatment had no

Figure 2. Effects of proteases and protease inhibitors on acute activation of epithelial sodium channel (ENaC) after ASL washout in an Ussing chamber. HBE cultures were mounted in an Ussing chamber containing apical Ringer's solution $\pm$ 300 nM elastase (Elast) or 10 μM aprotinin (Apro). After a 30-minute equilibration, 300 nM of elastase were added to the apical chamber. Data shown are (A) representative I_{sc} recordings, (B) mean amiloride-sensitive current normalized to the post-elastase amiloride-sensitive current ($I_{Na} / I_{Na(elastase)}$) $\pm$ SE, and (C) mean $t_{1/2}$ $\pm$ SE of the I_{Na} increase from 0 to 30 minutes ($n = 8$ cultures from two tissue donors). * $P < 0.01$, different from control culture as determined by ANOVA with Bonferroni *post hoc* analysis.

effect on I_{Na} in the control HBE cultures. Under conditions where the serine protease inhibitor aprotinin was included in the apical Ringer's solution, the I_{SC} initially increased at the same rate as in HBE cultures with control Ringer's solution or Ringer's solution containing elastase. However, the steady-state I_{SC} was lower than that under control conditions, resulting in a faster $t_{1/2}$. In the aprotinin-treated HBE cultures, elastase elicited a rapid increase in I_{SC} , as previously reported (17). Thus, approximately 35% of the increase in I_{Na} associated with ASL washout is attributable to the proteolytic activation of inactive channels. Furthermore, the kinetics profile of the I_{Na} increase suggests that the proteolytic activation of ENaC is rapid in HBE, and not rate-limiting, during the increase in Na^+ absorption after ASL dilution.

We next investigated whether processing by multiple proteases is required for full ENaC activity in airway epithelia. The mechanism of channel activation by proteases is thought to occur via the removal of inhibitory peptide tracks after double cleavage of the α and γ subunits (18–23). We reasoned that we could manipulate the degree to which the channel is processed by applying various protease inhibitors, and then sequentially adding proteases to activate ENaC fully. To inhibit different protease families, HBE cultures were incubated with apical Ringer's solution, with or without aprotinin to inhibit serine proteases, including those in the CAP family, or a furin convertase inhibitor (FCI) to inhibit pro-protein convertases, such as furin, for 16 hours before I_{SC} recording. After the plateau I_{SC} was achieved, the HBE cultures were sequentially exposed to elastase, which cleaves a single site within the γ subunit (18, 24), and trypsin, which was shown to process the α and γ subunits fully (25). The I_{Na} was normalized to the maximal I_{Na} observed after trypsin addition ($I_{Na}/I_{Na(trypsin)}$) or the maximal ENaC-dependent current. As demonstrated in Figures 3A and 3B, when serine proteases were inhibited by aprotinin, the I_{SC} reached a plateau that was $69.4\% \pm 1.7\%$ of maximal ($P = 0.004$, different from control Ringer's solution-treated HBE cultures, $n = 12$ cultures from three tissue donors). In aprotinin-treated cultures, a single cleavage of the γ subunit was sufficient to restore maximal channel activity, and the subsequent addition of trypsin exerted no further effect. The inhibition of furin and related pro-protein convertases with FCI caused a more pronounced reduction in I_{Na} . In contrast to the aprotinin-treated HBE cultures, treatment with elastase only partly restored channel activity in the FCI-treated cultures, and when the other cleavage sites were processed by trypsin, the channel was fully activated. Therefore, multiple cleavage events are required to activate the channel fully when the endogenous pro-protein convertases are inhibited.

We and others previously reported that cystic fibrosis (CF) HBE cultures exhibit an imbalance between endogenous CAPs and protease inhibitors, leading to increased levels of proteolytic activation of ENaC in the CF airway (6, 7). Therefore, we tested the susceptibility of HBE cultures from CF tissue donors to protease inhibitors and exogenous CAPs. As shown in Figures 3B and 3C, no differences were evident between CF and control HBE cultures in the response to protease inhibition or activation by aprotinin or FCI. Because these experiments were performed after overnight ASL washout, the lack of difference between CF and control HBE cultures suggests that the difference in proteolytic processing between CF and non-CF HBE cultures is only evident when cells are maintained on an air-liquid interface.

ENaC Half-Life Is Similar between Non-CF and CF HBE Cultures

Knight and colleagues previously showed that channels containing the Little's mutations undergo increased proteolytic

Figure 3. Effect of aprotinin and a furin convertase inhibitor on the acute activation of ENaC in control and cystic fibrosis (CF) HBE cultures. HBE cells from control and CF tissue donors were cultured with apical Ringer's solution and basolateral media, with or without 10 μM aprotinin or 50 μM furin convertase inhibitor (FCI) for 16 hours before I_{SC} recording. After a 30-minute equilibration, 300 nM elastase and 10 μM trypsin were sequentially added apically. Data shown are (A) representative I_{SC} recordings, and (B and C) mean amiloride-sensitive current normalized to the post-trypsin amiloride-sensitive current ($I_{Na}/I_{Na(trypsin)}$) $\pm$ SE at 30 minutes, after elastase, and after trypsin for (B) control and (C) CF cultures ($n = 12$ cultures from three tissue donors). * $P < 0.004$, different from control culture as determined by ANOVA with Bonferroni post hoc analysis.

processing as the result of a prolonged membrane residency time (26). Therefore, we questioned whether mutant cystic fibrosis transmembrane conductance regulator (CFTR) prolongs the functional $t_{1/2}$ of ENaC as a potential means by which CF HBE cells undergo excessive ENaC processing. To determine the functional channel $t_{1/2}$, CF and non-CF HBE cells were treated with CHX, and the I_{SC} was monitored to determine the decay rate in channel activity, as shown in Figure 4. Interestingly, the increase in I_{Na} was not affected by treatment with CHX (Figure 5B), suggesting that new channel synthesis is not required for channel activation with ASL volume expansion. The I_{SC} decreased after treatment with CHX, and the functional $t_{1/2}$ was determined as described in MATERIALS AND METHODS. Although variability was observed between tissue donors, no significant differences in ENaC $t_{1/2}$ were observed between non-CF and CF HBE cultures (74.366 ± 8.539 minutes versus 87.442 ± 13.88 minutes, $P = 0.82$ according to Mann-Whitney rank sum test, $n = 19$ –22 cultures from five tissue donors). Based on these

functional half-life decays, the CFTR genotype appears unlikely to affect the rate at which ENaC is degraded, and no differences in ENaC activity were consistently observed between CF and non-CF HBE cultures when the ASL was expanded.

ENaC Traffics to the Apical Membrane in Response to ASL Washout

The results of protease inhibitor experiments suggest that ~60% of channels are in an active state. Because the increase in I_{Na} associated with ASL washout is larger than could be accounted for by protease activation, we investigated whether a trafficking event of ENaCs to the cell surface occurs in response to ASL washout. HBE cultures were preincubated with a variety of trafficking inhibitors for 30 minutes before I_{SC} recording. The amiloride-sensitive currents at 0 and 30 minutes of treated HBE cultures were normalized to the I_{Na} of untreated control cultures ($I_{Na}/I_{Na(control)}$). As shown in Figure 5A, the recorded I_{SC} from HBE cultures that were pretreated with BFA, which disrupts ENaC trafficking from the trans-Golgi network to the apical membrane, was significantly smaller than in control cells, and increased to $40.1\% \pm 5.6\%$ of the amiloride-sensitive current of control cultures after the 30-minute equilibration period. Similar results were evident after microtubule disruption with nocodazole and with myosin light chain kinase inhibition (ML-7 and ML-9), yielding $40.4\% \pm 4.4\%$, $42.7\% \pm 8.1\%$, and $34.5\% \pm 7.4\%$ of the amiloride-sensitive I_{SC} of control HBE cultures at 30 minutes, respectively. Therefore, when trafficking is disrupted through a variety of methods, the ability of HBE cultures to increase sodium absorption in response to ASL volume expansion is impaired. These results suggest that a trafficking step is likely involved with the increase in I_{Na} in response to ASL washout.

To support the electrophysiologic results suggesting that ENaC is trafficked in response to ASL expansion, we sought to obtain corroborating biochemical evidence. To identify a suitable ENaC antibody, we tested the ability of numerous commercially available antibodies to immunoblot (IB) and immunoprecipitate (IP) human ENaCs derived from cells transiently transfected with epitope-tagged ENaCs (data not shown). Although some ENaC antibodies targeting the N-terminal region of α ENaC were capable of IP or IB, we were unable to obtain consistent biochemistry in the biotinylated materials. We speculate that this difficulty was a result of limited access of biotin to the short N-extracellular segment of cleaved α ENaC.

Figure 5. Effect of trafficking inhibition, A2a receptor blockade, BAPTA-AM, cyclic adenosine monophosphate (cAMP), and cycloheximide (CHX) on acute activation of ENaC after ASL washout. Differentiated HBE cultures were exposed basolaterally to the indicated drug for 30 minutes before I_{SC} recording and in Ringer's solution bath during I_{SC} recording. (A) To determine the contribution of membrane trafficking during I_{SC} recording, 5 μ g/ml brefeldin A (BFA), 10 μ M nocodazole (Nocod), 100 μ M ML-7, or 100 μ M ML-9 were added to inhibit trafficking. (B) The effects of A2a (A2a), 10 μ M forskolin (Forsk), 50 μ M H-7, or 10 μ M BAPTA-AM were also examined. After a 30-minute equilibration, 10 μ M of amiloride were added to the apical chamber. Data shown are the mean amiloride-sensitive current normalized to the mean amiloride-sensitive current of matched control HBE cultures after the 30-minute equilibration ($I_{Na}/I_{Na(control)}$) $\pm$ SE at 0 minutes (black) and 30 minutes (gray) ($n \geq 6$ cultures from ≥ 2 tissue donors). * $P < 0.001$, different from control culture as determined by ANOVA with Bonferroni *post hoc* analysis.

Figure 4. Functional ENaC half-life in control and CF HBE cultures. (A) Differentiated non-CF and CF HBE cultures were treated with 200 μ g/ml cycloheximide (as indicated by +), and I_{SC} was measured at 10-minute intervals. (B) Functional $t_{1/2}$ was calculated from the I_{SC} decay for each individual culture. Data shown are mean $\pm$ SE, $n = 19$ –22 cultures from five tissue donors.

Next, to identify the stimulus that initiates trafficking in HBE cultures and other potential mechanisms of ENaC regulation involved in the increase of I_{Na} in response to ASL expansion, we tested the effects of a panel of chemical inhibitors that were shown to affect ENaC activity. These compounds were added to the basolateral media for 30 minutes before I_{SC} recording, and were included in the Ringer's solution bath when cells were mounted in Ussing chambers, specifically to inhibit membrane receptors or block secondary messenger cascades. The amiloride-sensitive current was measured at 0 and 30 minutes, and normalized to that of matched, untreated control cultures ($I_{Na}/I_{Na(control)}$). As shown in Figure 5B, I_{Na} was not altered by the A2a receptor blockade, cAMP activation by forskolin, PKA/PKC inhibition by H-7, Ca^{2+} chelation by BAPTA-AM, or the prevention of new channel synthesis by CHX. Therefore, although the A2a receptor, cAMP, PKA/PKC, and Ca^{2+} signaling may be important for ENaC regulation under some conditions, they do not play a significant role in the acute regulation of ENaC in HBE cells after ASL expansion.

Willumsen and colleagues demonstrated that luminal hyperosmolarity decreases Na^+ absorption across cultured airway epithelia (27). The effects of osmolarity on ENaC activity were investigated to determine whether the I_{Na} change after osmolarity shifts is consistent with a trafficking event. As demonstrated in Figure 6, the I_{SC} of differentiated HBE cultures was measured during the sequential osmolarity shifts induced by the addition and removal of 100 mM or 300 mM mannitol to the Ringer's solution bath. After the addition of 100 mM mannitol, the I_{SC} decreased rapidly, and this trend reversed when the mannitol was removed. Similar responses were observed with 300 mM mannitol, although the magnitude of response was larger, and the I_{SC} recovery was slower and incomplete. Because the majority of recorded I_{SC} in HBE is amiloride-sensitive (Figure 1), these I_{SC} changes in response to osmolarity shifts likely reflect changes in ENaC activity. Therefore, luminal osmolarity reversibly affects the I_{Na} with kinetics that are consistent with trafficking events, suggesting that ASL osmolarity regulates the number of ENaCs at the cell surface.

We next investigated whether the trafficking event responsible for the increase in I_{Na} during ASL washout could be modulated by the osmolarity of apical Ringer's solution. Differentiated HBE cultures, with and without pretreatment with 50 $\mu\text{g}/\text{mL}$ vinblastine to disrupt tubulin, were mounted in an Ussing chamber in which the apical Ringer's solution bath contained 100 mM mannitol. As demonstrated in Figure 7A, the bath solution was replaced with symmetric, isotonic Ringer's solution after the initial 30-minute equilibration. The amiloride-sensitive current at 0 minutes, at 30 minutes, and after the isotonic wash was normalized to that of matched, untreated control cultures ($I_{\text{Na}}/I_{\text{Na}(\text{control})}$). After the initial 30-minute equilibration, a large increase in I_{SC} was evident under control conditions. This I_{SC} increase was significantly smaller when mannitol was included in the apical Ringer's solution ($46.8\% \pm$

7.7% and $45.1\% \pm 5.6\%$ of the control I_{Na} for mannitol and mannitol + vinblastine conditions, respectively). After replacement of the hypertonic bath solution with isotonic Ringer's solution, the I_{Na} increased and matched that of control cultures. The observed increase in I_{Na} after the isotonic wash was prevented when HBE cells were pretreated with vinblastine, suggesting that trafficking and not changes in ENaC P_{O} are responsible for the I_{Na} increase after the osmolarity shift. These results suggest that the increase in I_{Na} after ASL washout is partly attributable to a change in osmolarity of the apical fluid and a resultant trafficking event.

DISCUSSION

To allow for increased fluid absorption when the ASL volume is expanded, Na^+ absorption via ENaC increases in two ways, though trafficking of channels to the cell surface to increase channel density, and by proteolytic activation of incompletely processed channels to augment Na^+ conduction through membrane-resident channels. After ASL expansion, ENaC trafficking to the luminal surface accounts for approximately 60% of the acute increase in I_{Na} . Proteolytic activation occurs rapidly, and accounts for the remainder of the increase in I_{Na} . Previous work demonstrated that this proteolytic activation occurs as a result of dilution of soluble protease inhibitors, allowing for endogenous CAPs to process the channel rapidly (6, 7). The activation kinetics suggest that ENaC activity in the airway is modulated by both proteases and trafficking events.

The proteolytic regulation of ENaC involves multiple proteases and multiple cleavage sites within the subunits' extracellular domains. As predicted by work conducted in oocyte expression systems, more than one cleavage event is required to activate ENaC fully (18–23). During the channel's biogenesis, the pro-protein convertase furin cleaves and activates the

Figure 6. Effect of apical osmolarity on I_{Na} . Representative I_{SC} tracings of differentiated HBE cultures during the addition (light gray bar) and removal of 100 mM (A) and 300 mM (B) mannitol. Amiloride was applied (black bar) at the end of the experiment to determine the I_{Na} . (C) I_{Na} relative to I_{Na} at 30 minutes during the various timeouts during the experiments with 100 mM mannitol (black bar) and 300 mM mannitol (gray bar). Data shown are mean $\pm$ SE amiloride-sensitive current normalized to the I_{Na} at 30 minutes ($n = 5$ –11 HBE cultures). (D) Kinetics of I_{Na} changes associated with osmolarity shift induced by 100 mM mannitol (black bar) and 300 mM mannitol (gray bar). Data shown are mean $\pm$ SE $t_{1/2}$, as defined in MATERIALS AND METHODS ($n = 5$ –11 HBE cultures).

Figure 7. Effect of hyperosmolarity and trafficking inhibitors on I_{Na} during ASL washout. Differentiated HBE cultures were incubated basolaterally, with and without 50 $\mu g/mL$ vinblastine for 30 minutes before I_{SC} recording. The apical Ringer's solution during the initial portion of the I_{SC} trace was isotonic or contained an additional 100 μM mannitol. After the initial equilibration period, bathing solutions were exchanged with several washes of isotonic Ringer's solution. Amiloride was applied (black bar) at the end of the experiment to determine the I_{Na} . Data shown are (A) representative I_{SC} recordings, and (B) mean amiloride-sensitive current normalized to the amiloride-sensitive current of control HBE cells at 30 minutes ($I_{Na}/I_{Na(control)} \pm SE$ at 0 minutes, at 30 minutes, and after the isotonic wash ($n > 12$ cultures from two tissue donors). * $P < 0.01$, different from control culture as determined by ANOVA with Bonferroni *post hoc* analysis.

channel through a limited proteolysis of α ENaC at two sites within the extracellular domain and one site on γ ENaC (19). Channels that are processed by furin display a moderate P_O , whereas uncleaved channels have a low P_O (28). The γ subunit undergoes additional processing on the cell surface by prostatic (21), elastase (18, 24), and plasmin (22, 29), which leads to maximal channel activation. Based on work in heterologous systems, proteolytic processing is postulated to activate ENaC via the removal of inhibitory peptide domains that span the two cleavage sites within the extracellular domain (23). Our results suggest that multiple cleavage events are also required for full channel activation in HBE, which endogenously expresses ENaCs.

Because of the paucity of reliable antibodies that detect the cleaved forms of endogenously expressed ENaC, it is difficult to determine which subunits are processed, and to what extent cleavage occurs when the ASL is expanded. However, aprotinin

appears to inhibit CAPs that activate ENaC via cleavage of the γ subunit, because elastase, which only cleaves the γ subunit (18, 24), is capable of fully activating the channel in aprotinin-treated cultures. When additional proteases were inhibited with FCI, the single cleavage event caused by elastase was insufficient to restore full channel activity. Restoration of normal channel activity in FCI-treated cultures required the addition of trypsin, and it is unclear if this additional activation is attributable to further processing of the α or γ subunit. Recent work from Carattino and colleagues suggests that the relevant activating proteolysis occurs with double cleavage of the γ subunit and removal of the intervening inhibitory peptide, regardless of the degree to which α is processed (30). It may be possible to address these questions biochemically in the future, when validated high-quality ENaC antibodies become available.

Several lines of evidence suggest that ENaC trafficking contributes to the increased I_{Na} with ASL expansion. First, the addition of elastase to the apical bathing solution from the beginning of recording in the Ussing chamber demonstrates that the rate of I_{Na} activation after ASL expansion is unaltered by the presence of exogenous protease or protease inhibitor. Because the rate of channel activation by elastase is more rapid than the rate of I_{Na} increase with ASL expansion, the addition of elastase to the apical surface would be expected to accelerate the I_{SC} increase, if unprocessed channels were present in the apical membrane. We observed a lack of effect, suggesting that channels residing in the apical membrane under basal conditions are fully processed, and that activation by CAPs is not rate-limiting. Furthermore, when endogenous CAPs were inhibited by aprotinin and the HBE cultures were subsequently exposed to elastase, a pool of protease-susceptible channels is evident after 30 minutes. This finding suggests that these elastase-activated channels represent uncleaved ENaCs that were delivered from an elastase-inaccessible subapical pool. Otherwise, they would have been activated when elastase was included at the beginning of the trace. Second, blocking trafficking through a variety of different means significantly attenuated the increase in I_{Na} after ASL expansion, suggesting the either ENaC or an accessory protein is trafficked with ASL expansion. Therefore, our findings suggest for the first time, to the best of our knowledge, that regulated channel trafficking is an additional means by which airway epithelia regulate the ASL volume.

The signaling cascade responsible for initiating the trafficking response was not completely resolved in these studies. Although an increase in apical osmolarity clearly and significantly inhibited the increase in I_{Na} observed with ASL expansion, pharmacologic inhibitors of a number of potential pathways had no effect on the I_{Na} increase. The localized sensing of changes to ASL osmolarity and the ability to direct ENaC to the apical surface in response to a hyposmotic challenge would represent a cellular solution for maintaining ASL homeostasis, and requires further investigation.

In this study, no difference in ENaC susceptibility to protease inhibitors and functional channel half-life between non-CF and CF HBE cultures was observed. In our previous report (6), we observed an increased inhibition of ENaC by aprotinin and decreased activation by trypsin in CF HBE cells that were cultured at basal ASL volumes, demonstrating an excessive proteolytic activation of ENaC in CF epithelia. In the present study, when the ASL volume was expanded, the difference in the protease-antiprotease balance between CF and non-CF was not apparent, suggesting that these differences are only apparent under thin film conditions. Furthermore, in our previous report, a large increase in I_{Na} was evident after the addition of trypsin, whereas here, the addition of elastase had no effect on

I_{Na} . One potential source for this difference involves the use of a different Ussing chamber, whose contents were well-mixed by the flow established by gassing the chamber. This increased flow likely prevents the retention of an unstirred layer at the apical surface, whereas the apical surface was relatively undisturbed in the chamber used in the previous study. Our results suggest that the I_{Na} results obtained from airway epithelia in a well-mixed Ussing chamber are dramatically altered by the removal of endogenous ASL, and may not accurately reflect the physiology of the *in vivo* airway under physiologic conditions. Therefore, studying the physiologic regulation of airway ENaCs in standard Ussing chamber systems is difficult, and alternative approaches, such as the "thin film" approach used by Tarran and Boucher (31), may be more suitable for studying airway epithelial electrophysiology.

In conclusion, sodium absorption across airway epithelia increases when the ASL volume is expanded by the coordinated trafficking of a reserve pool of channels to the cell surface and subsequent proteolytic activation. This reserve pool of channels consists of partly processed channels that, when trafficked to the cell surface, increase I_{Na} to approximately two thirds of the maximal current. For maximal activation, the channel must be processed by CAPs that are present in abundance on the cell surface, and that are highly active when endogenous inhibitors in the ASL are diluted or washed out.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank the Lung Transplantation Program at the University of Pittsburgh Medical Center for facilitating tissue acquisition, and Joseph Latoche for technical assistance.

References

- Matsui H, Grubb BR, Tarran R, Randell SH, Gatzky JT, Davis CW, Boucher RC. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 1998;95:1005–1015.
- Matsui H, Randell SH, Peretti SW, Davis CW, Boucher RC. Co-ordinated clearance of periciliary liquid and mucus from airway surfaces. *J Clin Invest* 1998;102:1125–1131.
- Tarran R, Grubb BR, Gatzky JT, Davis CW, Boucher RC. The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. *J Gen Physiol* 2001;118:223–236.
- Matsui H, Davis CW, Tarran R, Boucher RC. Osmotic water permeabilities of cultured, well-differentiated normal and cystic fibrosis airway epithelia. *J Clin Invest* 2000;105:1419–1427.
- Donaldson SH, Boucher RC. Sodium channels and cystic fibrosis. *Chest* 2007;132:1631–1636.
- Myerburg MM, Butterworth MB, McKenna EE, Peters KW, Frizzell RA, Kleyman TR, Pilewski JM. Airway surface liquid volume regulates ENaC by altering the serine protease-protease inhibitor balance: a mechanism for sodium hypersorption in cystic fibrosis. *J Biol Chem* 2006;281:27942–27949.
- Tarran R, Trout L, Donaldson SH, Boucher RC. Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia. *J Gen Physiol* 2006;127:591–604.
- Tong Z, Illek B, Bhagwandin VJ, Verghese GM, Caughey GH. Prostatic, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L928–L935.
- Satlin LM, Sheng S, Woda CB, Kleyman TR. Epithelial Na⁺ channels are regulated by flow. *Am J Physiol Renal Physiol* 2001;280:F1010–F1018.
- Fronius M, Clauss WG. Mechano-sensitivity of ENaC: may the (shear) force be with you. *Pflugers Arch* 2008;455:775–785.
- Devor DC, Pilewski JM. UTP inhibits Na⁺ absorption in wild-type and DeltaF508 CFTR-expressing human bronchial epithelia. *Am J Physiol* 1999;276:C827–C837.
- Mall M, Wissner A, Gonska T, Calenborn D, Kuehr J, Brandis M, Kunzelmann K. Inhibition of amiloride-sensitive epithelial Na⁺ absorption by extracellular nucleotides in human normal and cystic fibrosis airways. *Am J Respir Cell Mol Biol* 2000;23:755–761.
- Tarran R, Button B, Picher M, Paradiso AM, Ribeiro CM, Lazarowski ER, Zhang L, Collins PL, Pickles RJ, Fredburg JJ, *et al.* Normal and cystic fibrosis airway surface liquid homeostasis: the effects of phasic shear stress and viral infections. *J Biol Chem* 2005;280:35751–35759.
- Garty H, Palmer LG. Epithelial sodium channels: function, structure, and regulation. *Physiol Rev* 1997;77:359–396.
- Devor DC, Bridges RJ, Pilewski JM. Pharmacological modulation of ion transport across wild-type and DeltaF508 CFTR-expressing human bronchial epithelia. *Am J Physiol Cell Physiol* 2000;279:C461–C479.
- Vallet V, Chraïbi A, Gaeggeler HP, Horisberger JD, Rossier BC. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature* 1997;389:607–610.
- Caldwell RA, Boucher RC, Stutts MJ. Neutrophil elastase activates near-silent epithelial Na⁺ channels and increases airway epithelial Na⁺ transport. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L813–L819.
- Adebamiro A, Cheng Y, Rao US, Danahay H, Bridges RJ. A segment of {gamma} ENaC mediates elastase activation of Na⁺ transport. *J Gen Physiol* 2007;130:611–629.
- Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD, Kleyman TR. Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem* 2004;279:18111–18114.
- Carattino MD, Sheng S, Bruns JB, Pilewski JM, Hughey RP, Kleyman TR. The epithelial Na⁺ channel is inhibited by a peptide derived from proteolytic processing of its alpha subunit. *J Biol Chem* 2006;281:18901–18907.
- Bruns JB, Carattino MD, Sheng S, Maarouf AB, Weisz OA, Pilewski JM, Hughey RP, Kleyman TR. Epithelial Na⁺ channels are fully activated by furin- and prostaticin-dependent release of an inhibitory peptide from the gamma subunit. *J Biol Chem* 2007;282:6153–6160.
- Svenningsen P, Bistrup C, Friis UG, Bertog M, Haerteis S, Krueger B, Stubbe J, Jensen ON, Thiesson HC, Uhrenholt TR, *et al.* Plasmin in nephrotic urine activates the epithelial sodium channel. *J Am Soc Nephrol* 2009;20:299–310.
- Kleyman TR, Carattino MD, Hughey RP. ENaC at the cutting edge: regulation of epithelial sodium channels by proteases. *J Biol Chem* 2009;284:20447–20451.
- Harris M, Firsov D, Vuagniaux G, Stutts MJ, Rossier BC. A novel neutrophil elastase inhibitor prevents elastase activation and surface cleavage of the epithelial sodium channel expressed in *Xenopus laevis* oocytes. *J Biol Chem* 2007;282:58–64.
- Knight KK, Wentzlaff DM, Snyder PM. Intracellular sodium regulates proteolytic activation of the epithelial sodium channel. *J Biol Chem* 2008;283:27477–27482.
- Knight KK, Olson DR, Zhou R, Snyder PM. Liddle's syndrome mutations increase Na⁺ transport through dual effects on epithelial Na⁺ channel surface expression and proteolytic cleavage. *Proc Natl Acad Sci USA* 2006;103:2805–2808.
- Willumsen NJ, Davis CW, Boucher RC. Selective response of human airway epithelia to luminal but not serosal solution hypertonicity. Possible role for proximal airway epithelia as an osmolality transducer. *J Clin Invest* 1994;94:779–787.
- Sheng S, Carattino MD, Bruns JB, Hughey RP, Kleyman TR. Furin cleavage activates the epithelial Na⁺ channel by relieving Na⁺ self-inhibition. *Am J Physiol Renal Physiol* 2006;290:F1488–F1496.
- Passero CJ, Mueller GM, Rondon-Berrios H, Tofovic SP, Hughey RP, Kleyman TR. Plasmin activates epithelial Na⁺ channels by cleaving the gamma subunit. *J Biol Chem* 2008;283:36586–36591.
- Carattino MD, Hughey RP, Kleyman TR. Proteolytic processing of the epithelial sodium channel gamma subunit has a dominant role in channel activation. *J Biol Chem* 2008;283:25290–25295.
- Tarran R, Boucher RC. Thin-film measurements of airway surface liquid volume/composition and mucus transport rates in vitro. *Methods Mol Med* 2002;70:479–492.