Apical trypsin increases ion transport and resistance by a phospholipase C-dependent rise of Ca\textsuperscript{2+}

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Swystun, Veronica, Lan Chen, Phillip Factor, Brian Siroky, P. Darwin Bell, and Sadis Matalon. Apical trypsin increases ion transport and resistance by a phospholipase C-dependent rise of Ca\textsuperscript{2+}. Am J Physiol Lung Cell Mol Physiol 288: L820–L830, 2005. First published December 30, 2004; doi:10.1152/ajplung.00396.2004.—We investigated the mechanisms by which serine proteases alter lung fluid clearance in rat lungs and vectorial ion transport in airway and alveolar epithelial cells. Inhibition of endogenous protease activity by intratracheal instillation of soybean trypsin inhibitor (SBTI) or \alpha\textsubscript{-}antitrypsin decreased amiloride-sensitive lung fluid clearance across rat fluid-filled lungs; instillation of trypsin partially restored this effect. Gelatin zymography demonstrated SBTI-inhibitable trypsin-like activity in rat lung lavage fluid. Apical trypsin and human neutrophil elastase, but not agonists of protease activated receptors, increased Na\textsuperscript{+} and Cl\textsuperscript{-} short-circuit currents (I\textsubscript{sc}) and transepithelial resistance (R\textsubscript{TE}) across human bronchial and nasal epithelial cells and rat alveolar type II cells, mounted in Ussing chambers, for at least 2 h. The increase in I\textsubscript{sc} was fully reversed by amiloride and glibenclamide. The increase in R\textsubscript{TE} was not prevented by ouabain, suggesting that trypsin decreased paracellular conductance. Apical trypsin also induced a transient increase in intracellular Ca\textsuperscript{2+} in human airway cells; treatment of these cells with BAPTA-AM mitigated the trypsin-induced increases of intracellular Ca\textsuperscript{2+} and of I\textsubscript{sc} and R\textsubscript{TE}. Increasing intracellular Ca\textsuperscript{2+} in airway cells with either ionomycin or thapsigargin reproduced the increase in I\textsubscript{sc}, whereas inhibitors of phospholipase C (PLC) prevented the increases in both Ca\textsuperscript{2+} and I\textsubscript{sc}. These data indicate trypsin-like proteases and elastase, either present in lung cells or released by inflammatory cells into the alveolar space, play an important role in the clearance of alveolar fluid by increasing ion transport and paracellular resistance via a PLC-initiated rise of intracellular Ca\textsuperscript{2+}.

DISTAL LUNG AND ALVEOLAR EPITHELIAL cells transport Na\textsuperscript{+} vectorially from the alveolar to the interstitial spaces (37). The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (adenosine triphosphatase) in the basolateral membrane actively transports Na\textsuperscript{+} out of the cells to the interstitium, creating an ion gradient that drives Na\textsuperscript{+} into the cell from the luminal surface through Na\textsuperscript{+} channels located in the apical membrane. Potassium ions, which are exchanged for Na\textsuperscript{+} in a 2:3 stoichiometry by the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, exit the cells via K\textsuperscript{+} channels located in the basolateral membranes. Chloride ions, which must follow Na\textsuperscript{+} ions to preserve electrical neutrality, enter cells through Cl\textsuperscript{-} channels or cross through paracellular junctions (23, 42). CFTR is expressed in the epithelium of both the distal airway and the alveoli (20, 34). The coordinated movement of these ions creates an osmotic gradient that favors the movement of fluid from the alveolar into the interstitial spaces (37, 38). A variety of studies have clearly established that active Na\textsuperscript{+} transport limits the degree of alveolar edema in pathological conditions (51, 55).

There has been a lot of interest in identifying how proteases affect ion transport. The original studies of Garty and Edelman (24) showed that trypsin (1 mg/ml) decreased short-circuit current (I\textsubscript{sc}) across the toad mucosa without affecting paracellular permeability. However, more recent studies have shown that serine proteases, such as trypsin and prostatin, activate Na\textsuperscript{+} transport across Xenopus oocytes, fibroblasts stably expressing epithelial Na\textsuperscript{+} channel (ENaC) subunits, and kidney epithelial cells (6, 11, 49). Chraibi et al. (11) reported that trypsin treatment greatly increased the percentage of oocyte membrane patches that contained active Na\textsuperscript{+} channels, and Caldwell et al. (6) demonstrated that patches with very low Na\textsuperscript{+} channel activity increased their NP\textsubscript{o} (channel open probability times the number of open channels) by up to 66-fold upon the addition of trypsin. These results show that proteases may either cause direct modification of ion transporters or induce signaling events resulting in increased vectorial ion transport.

A number of proteases, including prostatin (54), trypsinogen (30), and human airway trypsin-like (HAT) protease (47) have been localized in lung tissue. Inhibition of prostatin decreased Na\textsuperscript{+} I\textsubscript{sc} across cultured human bronchial (5) and nasal (18) epithelial cells, indicating a basal activating role of this protease on Na\textsuperscript{+} transport. On the basis of these observations, we hypothesized that the membrane-bound, epithelium-expressed serine proteases have a constitutive activating role in Na\textsuperscript{+} transport in vivo and that their inhibition would decrease Na\textsuperscript{+}-dependent lung fluid clearance (LFC). We investigated this possibility by measuring in vivo LFC in the presence of soybean trypsin inhibitor (SBTI), aprotinin, and \alpha\textsubscript{1}-antitrypsin in the rat lung.

Among the numerous serine proteases present in the lung are those released from immune cells, such as tryptase, released from resident mast cells during immune reactions (4), and elastase from neutrophils recruited into the lung during inflammatory responses (35). The effect of these proteases on ion transport has not been investigated. We hypothesized that neutrophil elastase and mast cell tryptase, in addition to exogenous trypsin, may increase ion transport across human lung epithelial cells, thus facilitating the absorption of alveolar and bronchial liquid.
airway edema. Because a number of proteases are released in close proximity to the apical surfaces of airway epithelial cells, we added trypsin, human elastase, and mast cell trypsin in the apical compartments of Ussing chambers containing cultured human airway and alveolar type II (ATII) cells and measured changes of Na\(^{+}\) and Cl\(^{-}\) and transepithelial resistance (R\(_{TE}\)). Our data showed that apical trypsin and elastase but not trypsin caused a sustained activation of I\(_{sc}\) and R\(_{TE}\).

Because previous studies have shown that both trypsin and the novel HAT protease increase intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{IC}\)] in human airway epithelia (15, 39) and that trypsin increased [Ca\(^{2+}\)\(_{IC}\)] in dog pancreatic duct epithelial cells (41), colonic myocytes (14), and lung tracheal and bronchial tissue (10, 15, 32, 41), we measured changes of [Ca\(^{2+}\)\(_{IC}\)] in polarized monolayers of human lung epithelial cells by imaging them with fura-2. We then examined the involvement of [Ca\(^{2+}\)\(_{IC}\)] on the trypsin induced increase of I\(_{sc}\) and R\(_{TE}\) by incubating airway cells with BAPTA-AM, thapsigargin, or ionomycin. We repeated these measurements, following inhibition of phospholipase C (PLC), to identify a possible mechanism for the Ca\(^{2+}\) mobilization. To our knowledge, this is the first demonstration of steady-state increases in Na\(^{+}\) and Cl\(^{-}\)-transport and R\(_{TE}\) produced by both apical trypsin and neutrophil elastase in human lung epithelial cell monolayers by a Ca\(^{2+}\)-dependent mechanism. We are also the first to report a role of trypsin-like proteases on LFC.

**METHODS**

Experiments using animals and human cells were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of the University of Alabama at Birmingham (UAB), respectively. Reagents were from Sigma Chemical (St. Louis, MO) unless otherwise stated.

LFC. Fluid clearance was measured in Sprague-Dawley rats (150–200 g; Harlan, Indianapolis, IN). Rats were killed with 300 mg/kg of body weight with sodium pentobarbital (60 nM), or amiloride (100 M), or thapsigargin, or ionomycin. We repeated these measurements, following inhibition of phospholipase C (PLC), to identify a possible mechanism for the Ca\(^{2+}\) mobilization. To our knowledge, this is the first demonstration of steady-state increases in Na\(^{+}\) and Cl\(^{-}\)-transport and R\(_{TE}\) produced by both apical trypsin and neutrophil elastase in human lung epithelial cell monolayers by a Ca\(^{2+}\)-dependent mechanism. We are also the first to report a role of trypsin-like proteases on LFC.

Wells of the Bio-Rad Ready Gel (0.1% gelatin) were loaded with 6.5 M\(\mu\)g of protein from each sample, and the assay was performed according to manufacturer’s instructions. Purified bovine pancreatic trypsin (Calbiochem) was used as the trypsin standard. Gels were incubated on at 37°C for 42 h.

**Cell culture.** The human bronchial epithelial cell line 16HBE140 (16HBE) was provided by Dr. C. Venglakir (Environmental Health Sciences, UAB) and cultured in MEM (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 5% FBS and 1% penicillin-streptomycin. Cells were seeded onto permeable polycarbonate cell culture filters (Corning Costar, Corning, NY) with a 0.4-µm pore size as previously described (21). Primary human nasal epithelial cells were obtained from a Cystic Fibrosis Research Center (UAB), seeded onto filters treated with vitronectin, and cultured in similar fashion to the 16HBE cells. ATII cells were isolated from pathogen-free male Sprague-Dawley rats (200–225 g) as previously described (21, 26). Cells were grown to confluence on filters (3–4 days) with 400 nM dexamethasone added to the media, and the apical fluid was removed. The cells were grown exposed to air on the apical side and media on the basolateral side for 4–6 days before experiments (air-liquid interface). Calu-3 cells (purchased from ATCC) were seeded and grown to confluence with an air-liquid interface for 4–6 days as previously described (1). Primary human nasal epithelial cells were obtained from a Cystic Fibrosis Research Center (UAB), seeded onto filters treated with vitronectin, and cultured in similar fashion to the 16HBE cells. ATII cells were isolated from pathogen-free male Sprague-Dawley rats (200–225 g) as previously described (21, 26). Cells were grown to confluence on filters (3–4 days) with 400 nM dexamethasone added to the media.

**Ussing chamber experiments.** Filters with cell monolayers were inserted into Ussing chambers (J. M. Instrom Instrumentes, San Diego, CA). Trypsin (2.5–250 M, Invitrogen), human neutrophil elastase (12–150 M; Molecular Probes, Eugene, OR), and Cl\(^{-}\)-free solutions as previously described (34). All procedures have been described previously (26). Bath solution in the basolateral chamber contained 10 mM glucose, whereas that in the apical chamber contained 10 mM mannitol in place of glucose to minimize the contribution of the Na\(^{+}\)-glucose cotransporter to the Na\(^{+}\) transport. Bath solutions were continuously bubbled with 95% O\(_2\), 5% CO\(_2\) (pH 7.4; osmolality 290–300 mosmol/kg). The monolayers were voltage clamped to 0 mV, and I\(_{sc}\) and R\(_{TE}\) were measured using 5-mV pulses every 20 s. Data was collected using the Acquire and Analyze program, version 1.45 (Physiologic Instruments, San Diego, CA). Trypsin and trypsin-activated protease-activated receptors (PARs), we measured calcium measurements. [Ca\(^{2+}\)\(_{IC}\)] measurements were made on polarized 16HBE cell monolayers grown on permeable filters in a chamber allowing separate perfusion of apical and basolateral surfaces. Cells were seeded onto clear 12-mm polyester, permeable Costar filters, grown to confluence, and cultured at an air-liquid interface for 6–8 days, identical to the culture conditions for the Ussing experiments. This culture method is important for the polarization and differentiation of lung epithelial cells. Cells on the filters were incubated at 33°C in fura-2 AM (10 M; Molecular Probes,.
Eugene, OR) for 60 min in Ringer solution containing 1 μM probenecid, an organic anion-exchange inhibitor, shown to minimize extravasation of the indicator from the cells. After thorough rinsing, the filters were placed in a temperature-controlled perfusion chamber mounted on an inverted epifluorescence microscope (Eclipse TE2000, Nikon), which was linked to a cooled charge-coupled device camera (SenSys, Photometrics) interfaced with a digital imaging system (Photon Technologies). Cells were observed with a Nikon S Fluor ×20 long-working distance objective. Fluorescence was recorded at 510 nM wavelength with excitation wavelengths of 340 and 380 nm and the ratio (R) of emitted fluorescence used to calculate [Ca$^{2+}$]_{j} using Image Master software (Photon Technologies). In situ calibration was performed with the Ca$^{2+}$ ionophore ionomycin (5 μM) in a 1.2 mM Ca$^{2+}$ solution (Ringer) to obtain R_{max}; R_{min} was obtained in Ca$^{2+}$-free solution with 10 mM EGTA (pH 8.0) and 5 μM ionomycin. [Ca$^{2+}$]_{j} was determined by: K_d(R - V × R_{min})/F_{380max}/F_{380min} = (V × R_{max} - R) where F_{380max} and F_{380min} are the maximum and minimum fluorescence values at 380-nm wavelength, V is the viscosity coefficient (0.8), and K_d is the dissociation constant for the dye, set at 224 nM (25).

**Statistical analysis.** Data are shown as means ± 1 standard error of the mean (X ± 1 SE). Statistical analysis was performed using InStat3 (GraphPad Software, San Diego, CA). Data were compared with the Kruskal-Wallis ANOVA (Dunn’s multiple-comparisons test), paired t-testing for parametric data, and the Mann-Whitney test for non-parametric data. Data are presented as means ± SE with P < 0.05 considered significant.

**RESULTS**

**LFC.** The results of LFC measurements are summarized in Fig. 1A. Our mean values of LFC in rat fluid-filled lung (7.7 ± 1.0% of instilled volume/30 min) are in agreement with previous reports on ex vivo rat lungs (45). LFC was significantly attenuated by the addition of SBTI (1.2 ± 0.40, P < 0.01) and α$_1$-antitrypsin (2.45 ± 1.1%) but not aprotinin (3.3 ± 1.8) to the instillate. Inhibition of trypsin before SBTI partly restored clearance to a level not significantly different from control (P > 0.05). Additional of the Na$^+$ channel inhibitor amiloride (100 μM) into the instillate totally inhibited LFC (0.1 ± 1.1, P < 0.01). In previous studies, amiloride inhibited 50–90% of LFC in mammalian lungs (16, 23, 27, 55). The degree of inhibition is increased significantly by higher instilled liquid volumes, which improve the uniform distribution of amiloride in the alveolar space (27).

**Trypsin activity assay.** SBTI-inhibitable trypsin activity was detected in the lung lavage samples, as shown by the presence of clear bands in the zymogram at 20–28 kDa molecular mass range (Fig. 1B). Bands for the metalloprotease matrix metalloprotease-2 were consistently detected in the 68- to 75-kDa range, and these could be inhibited by the addition of EGF to the buffer solution (data not shown).

**Bioelectric measurements of I$_{sc}$ and R$_{TE}$.** Addition of trypsin into the apical compartments of Ussing chambers containing 16HBE, Calu-3, and human nasal epithelial cells (25 μM) or rat ATII cell monolayers (50 nM) resulted in immediate and sustained increases of both I$_{sc}$ and R$_{TE}$ (Fig. 2). I$_{sc}$ increased from 8.0 ± 0.7 to 14.4 ± 0.9 μA/cm$^2$ in 16HBE cells (n = 28, P < 0.01), from 15.0 ± 3.0 to 30.0 ± 6 μA/cm$^2$ in Calu-3 cells (n = 9, P < 0.01), from 2.3 ± 0.16 to 3.8 ± 0.24 μA/cm$^2$ in human nasal epithelial cells (n = 6, P < 0.01), and from 5.2 ± 0.7 to 7.4 ± 1.1 μA/cm$^2$ in rat alveolar cells (n = 5, P < 0.05); values are X ± 1 SE, n = number of monolayers. In epithelial cells exhibiting both Na$^+$ and Cl$^{-}$ vectorial transport (human nasal, rat ATII, and 16HBE cells), the trypsin-induced increases of I$_{sc}$ and R$_{TE}$ were reversed by apical amiloride (100 μM) and to a lesser extent by glibenclamide (200 μM). In Calu-3 cells (which lack Na$^+$ transport) the trypsin effects were inhibited completely by apical glibenclamide (Fig. 2A).

**Pretreatment of 16HBE cells with H-89 (0.1–1 μM for 1–22 h), an inhibitor of protein kinase A (PKA), had no effect on trypsin-activated I$_{sc}$ [ΔI$_{sc}$ = 7.5 ± 0.7 for H-89 vs. 7.1 ± 1.1 μA/cm$^2$ for vehicle (P = 0.76, n = 3)], nor did brefeldin A (1 μg/ml for 30 min), an inhibitor of protein trafficking to the membrane [ΔI$_{sc}$ = 6.5 ± 0.7 for brefeldin vs. 6.5 ± 1.1 μA/cm$^2$ for vehicle; P = 1.0, n = 3).

All further measurements were performed on the 16HBE cells, since they exhibit both Na$^+$ and Cl$^{-}$ vectorial transport. Human neutrophil-derived elastase added to the apical compartment in the range of 12–150 μg/ml increased both I$_{sc}$ and R$_{TE}$ (Fig. 3). On average, elastase produced an increase in I$_{sc}$ of 89 ± 5.7% (ΔI$_{sc}$) (increase from baseline, X ± 1 SE), whereas the addition of human mast cell-derived tryptase to both the apical and the basolateral sides of 16HBE monolayers did not increase I$_{sc}$ (0.1 ± 3.6%, Fig. 4A). Trypsin failed to increase I$_{sc}$ or R$_{TE}$ when added into the apical chambers containing either SBTI (100 nM, Fig. 4A) or BSA (5%, data not shown), indicating that its enzymatic activity was necessary for these effects to occur. Figure 4B shows that both Na$^+$ and Cl$^{-}$ transport contribute to the trypsin-induced increase in I$_{sc}$. When trypsin was added in the apical side of
monolayers bathed in Na\(^+\)-free solutions (NMDG-Cl\(^-\)) it increased \(I_{sc}\) from 3.9 ± 1.4 to 5.3 ± 1.4 μA/cm\(^2\) \((n = 9, \ P < 0.01)\), and the entire trypsin-inducible \(I_{sc}\) \((\Delta I_{sc} = 1.24 ± 0.24)\) was inhibited with glibenclamide (200 μM). When trypsin was added in the apical membranes of monolayers pretreated with 100 μM amiloride, \(I_{sc}\) increased by 2.4 ± 0.1 μA/cm\(^2\), this being 38% what was seen in the absence of amiloride \((\Delta I_{sc} = 6.47 ± 0.7, \ n = 11; \ P < 0.01)\). In Cl\(^-\)-free (Na\(^+\) gluconate) solution, trypsin increased \(I_{sc}\) from 5.5 ± 1 to 10.0 ± 1.3 \((n = 8, \ P < 0.01)\). When Cl\(^-\) transport was inhibited with apical glibenclamide and basolateral bumetanide, trypsin increased \(I_{sc}\) by 3.10 ± 0.1 μA/cm\(^2\). With both Na\(^+\) and Cl\(^-\) transport inhibited (by the addition of amiloride in the Cl\(^-\)-free solutions), trypsin increased \(I_{sc}\) from 0.38 ± 0.6 to 0.6 ± 6 μA/cm\(^2\) \((P > 0.1)\).

Trypsin decreased the sensitivity of the \(I_{sc}\) to amiloride inhibition by 10-fold (Fig. 4C), with an IC\(_{50}\) (concentration at which 50% maximal inhibition is achieved) in trypsin-treated cells \((n = 19)\) of 3.8 vs. 0.4 μM in control cells \((n = 11)\).

\(R_{TE}\) increased 143% in the 16HBE cells following apical trypsin, from 927 ± 32 to 2,250 ± 91 Ω × cm\(^2\) \((P < 0.01, n = 14)\). The rise in \(R_{TE}\) was maintained for at least 120 min. Pretreatment with 2 mM ouabain had no effect on the trypsin increase in \(R_{TE}\) \((\Delta R_{TE} = 480 ± 60 vs. \ control 540 ± 33 Ω × cm^2; \ n = 6, \ P = 0.82)\) although it completely blocked the increase in \(I_{sc}\) (data not shown).

Trypsin-induced \(I_{sc}\) and \(R_{TE}\) are not mediated by PAR-1,2.

To determine the involvement of the PARs in the trypsin-mediated effects, we measured changes in \(I_{sc}\) and \(R_{TE}\) follow-
ing addition of the PAR-2-activating peptide SLIGKV and of the PAR-1 activator thrombin (Fig. 5). Addition into the basolateral bath solutions of either SLIGKV (100 μM) or trypsin (25 μM) stimulated rapid, transient rises in $I_{sc}$ ($\Delta I_{sc}$: 18.7 ± 2.81 vs. 18.8 ± 2 μA/cm$^2$, X ± 1 SE, n = 3 for each, P = 0.964), confirming the presence of a basolateral PAR-2 (15). In contrast, apical addition of trypsin induced a rapid and sustained rise in $I_{sc}$ ($\Delta I_{sc}$ 8.9 ± 0.8 μA/cm$^2$, n = 3), whereas SLIGKV did not alter $I_{sc}$. Trypsin added to the apical bath solution of cells after SLIGKV increased $I_{sc}$ to the same extent as when SLIGKV was not present, whereas the peptide did not alter $I_{sc}$ when added to the apical side of monolayers treated with trypsin. However, basolateral addition of SLIGKV in monolayers treated with apical trypsin caused the same transient rise in $I_{sc}$ as in the absence of trypsin ($\Delta I_{sc}$ 16.7 ± 8.1 μA/cm$^2$, n = 3). Apical SLIGKV also failed to increase $R_{TE}$ (Fig. 5). Thrombin (5–10 U/ml), a known activator of PAR-1, had no effect on $I_{sc}$ or $R_{TE}$ when added to the apical bath solution (data not shown).

Role of $[\text{Ca}^{2+}]_{IC}$. Perfusion of the apical surface of 16HBE polarized cell monolayers with trypsin (25 μM) produced a rapid transient rise in $[\text{Ca}^{2+}]_{IC}$ from a baseline of 81.9 ± 2.98 to 536 ± 63.0 nM (Fig. 6A). When cells were preincubated with the BAPTA-AM for 60 min, trypsin increased $[\text{Ca}^{2+}]_{IC}$ from 48.6 ± 4.3 to 206 ± 18.1 nM (n = 19, P = 0.0002 as compared in the absence of BAPTA-AM). Trypsin also resulted in a steady-state increase of $[\text{Ca}^{2+}]_{IC}$ (121 ± 3 nM, P < 0.001 compared with control), even in the BAPTA-treated cells (125 ± 7 nM) with no significant difference between them (P < 0.663). Apical application of the PAR-2-activating peptide SLIGKV increased $[\text{Ca}^{2+}]_{IC}$ from 98.4 ± 22.0 to 228 ± 35.1 nM, but this increase was significantly lower than that induced by the subsequent addition of trypsin ($\Delta [\text{Ca}^{2+}]_{IC}$ =606 ± 27 nM, n = 12, P < 0.01) (Fig. 6B). Perfusion with thapsigargin (1 μM) increased $[\text{Ca}^{2+}]_{IC}$ from 82 ± 3 to 1,530 ± 113 nM (n = 19), and subsequent addition of trypsin had no additional effect (Fig. 6B).

To assess the role of $[\text{Ca}^{2+}]_{IC}$ in the trypsin-induced increases of $I_{sc}$ and $R_{TE}$, BAPTA-AM was added into the apical bath solution of Ussing chambers containing confluent monolayers of 16HBE cells for 50 min before addition of trypsin.

Fig. 3. $I_{sc}$ (top) and $R_{TE}$ (bottom) following addition of 25 μM human neutrophil-derived elastase in the apical compartment of Ussing chambers containing 16HBE cells. Results are means from 2 typical experiments (see Fig. 4 for mean changes of the elastase-induced $I_{sc}$).

Fig. 4. A: $I_{sc}$ values (expressed as % change from the corresponding control value) in 16HBE cells induced by addition of trypsin (25 μM, n = 28), human neutrophil elastase (12.5–150 μg/ml, n = 8), mast cell tryptase (12.5–25 μg/ml, n = 4), and trypsin (25 μM) in the presence of 240 nM SBTI (n = 4). All chemicals were added into the apical Ussing chamber at the times indicated by the arrows. *Significant differences from the trypsin treatment. B: $I_{sc}$ induced by 25 μM in 16HBE cells in control (Ringer) solution (n = 28), Na"-free solution (NMDG-Cl"), n = 9), Cl"-free (Na" gluconate) solution (n = 8), and in Cl"-free solution with Na" channel inhibitor amiloride (n = 5). *Data that are statistically different from the control. All values are X ± 1 SE of the mean; n = number of monolayers. C: Dose-response curves for amiloride in 16HBE cells after trypsin treatment (dashed line, n = 15) and without trypsin (solid line, n = 11). Changes in $I_{sc}$ after addition of amiloride were expressed as % of the maximum change in $I_{sc}$, achieved with addition of 200 μM amiloride.
Fig. 5. *I* \(_{sc}\) (top) and *R* \(_{TE}\) (bottom) following addition of 25 μM trypsin or of the protease-activated receptor (PAR)-2 activating peptide SLIGKV (100 μM). Two different tracings, representing mean values of 3 different experiments, are shown in each panel; in the 1st experiment (solid black line) trypsin was added into the apical compartment of an Ussing chamber containing confluent monolayers of 16HBE cells, eliciting increases in *I* \(_{sc}\) and *R* \(_{TE}\). Subsequent addition of SLIGKV into the apical compartment had no additional effect. However, basolateral addition of SLIGKV or trypsin, at the times indicated by the arrows, elicited a transient increase of *I* \(_{sc}\) and a transient decrease of *R* \(_{TE}\). Glibenclamide (200 μM) and amiloride (100 μM) were then added in the apical compartment followed by basolateral ouabain (2 mM). The 2nd experiment (solid gray line), SLIGKV was added into the apical compartment (causing no response) followed by trypsin first into the apical and then into the basolateral compartment. Glibenclamide (200 μM) and amiloride (100 μM) were then added in the apical compartment followed by basolateral ouabain (2 mM). Values for each line are means from 3 different experiments.

BAPTA-AM significantly attenuated both the *I* \(_{sc}\) and the *R* \(_{TE}\) response to apical trypsin (Fig. 6C). *I* \(_{sc}\) and *R* \(_{TE}\) at 50 min after the addition of trypsin increased by 3.5 ± 0.50 μA/cm\(^2\) and 401 ± 55.4 Ω × cm\(^2\) in the BAPTA-AM-treated monolayers vs. 7.6 ± 0.8 μA/cm\(^2\) and 817 ± 120 Ω × cm\(^2\) in the vehicle-treated controls (P = 0.0036). Increasing BAPTA concentration had no additional inhibitory effect. Apical addition of either thapsigargin or ioneomycin (in 1 μM Ca\(^{2+}\) bath solution) elicited sustained increases in *I* \(_{sc}\) (Fig. 7, A and B) from 10.7 ± 1.3 to 22 ± 2.6 (n = 3, P = 0.0296) and 13.3 ± 4.8 to 21.3 ± 6.6 μA/cm\(^2\) (n = 3, P = 0.028) μA/cm\(^2\) (n = 3, P = 0.0296), respectively. These increases in *I* \(_{sc}\) were totally inhibited by addition of amiloride and glibenclamide, as was the case with trypsin. However, neither thapsigargin nor ioneomycin increased *R* \(_{TE}\) (Fig. 7, A and B).

**Involvement of PLC in trypsin-induced changes.** Pretreatment of 16HBE monolayers with the PLC inhibitor U-73122 (100 μM) for 10 min inhibited both the trypsin-induced increases in *I* \(_{sc}\) and *R* \(_{TE}\) (Fig. 8). The trypsin-induced increase of *I* \(_{sc}\) (∆*I* \(_{sc}\)) in U-73122-treated cells was −2.03 ± 0.33 vs. 15.4 ± 1.41 μA/cm\(^2\) in control cells (n = 4, P = 0.0286); the increase in *R* \(_{TE}\) (∆*R* \(_{TE}\)) was 8 ± 30 in U-73122-treated cells and 570 ± 170 Ω × cm\(^2\) in controls (n = 4, P = 0.0286). Pretreatment with the phosphatidylinositol-specific PLC inhibitor D-609 (100 μM) also inhibited the trypsin-induced increases in *I* \(_{sc}\) (Fig. 9) but not the increase in *R* \(_{TE}\). In the presence of D-609, ∆*I* \(_{sc}\) was −0.60 ± 0.42 μA/cm\(^2\) (n = 3) compared with 13 ± 1.65 (n = 3) in control cells (P = 0.0014), and ∆*R* \(_{TE}\) was 401 ± 147 (n = 3) vs. 351 ± 111 Ω × cm\(^2\) in controls (n = 3, P = 0.8). Both inhibitors prevented the trypsin-induced increase of [Ca\(^{2+}\)]\(_{IC}\) (Fig. 10). Pretreatment of 16HBE monolayers with the phosphatidylinositol-specific PLC inhibitor ET-18-OCH\(_3\) (100 μM) inhibited the trypsin-induced increase in *R* \(_{TE}\) (∆*R* \(_{TE}\) = 9.7 ± 6.0 in the ET-18-treated group, n = 3, vs. 324 ± 63.2 Ω × cm\(^2\) in controls n = 5; P = 0.0357) and of [Ca\(^{2+}\)]\(_{IC}\) but did not prevent the increase in *I* \(_{sc}\) (∆*I* \(_{sc}\) = 13.3 ± 1.67 in ET-18-treated cells vs. 13.4 ± 0.99 μA/cm\(^2\) in controls, P = 0.95).

**DISCUSSION**

The major novel findings of this study are: 1) inhibition of endogenous protease activity by SBTI and α\(_1\)-antitrypsin decreases amiloride-sensitive fluid clearance in rat lungs; 2) addition of trypsin and human elastase into apical compartments of Ussing chambers containing confluent monolayers of airway and alveolar cells results in an immediate and sustained increase of active Na\(^{+}\) and Cl\(^{−}\) transport and paracellular resistance; and 3) the effects of trypsin are mediated, at least in part, by a rise in intracellular Ca\(^{2+}\), following activation of PLC. The concomitant increase of active Na\(^{+}\) transport and paracellular resistance is of great significance: active Na\(^{+}\) transport plays an important role in the clearance of alveolar fluid under normal and pathological conditions, and increases of Na\(^{+}\) transport, either by β-agonists or via intratracheal instillation of adenoviral vectors containing Na\(^{+}\)-K\(^{+}\)-ATPase, increase survival of animals with acute respiratory distress syndrome-type injury (21, 40). At the same time, an increase in paracellular resistance will prevent the basolateral to apical movement of Na ions across the paracellular junctions, thus helping to maintain the generated osmotic driving force for the absorption of alveolar fluid.

A number of proteases have been localized in lung cells and may contribute to the basal activation of Na\(^{+}\) transport. Prostasoin and a trypsin-like protease have been found in lung epithelium as transmembrane proteins with extracellular catalytic sites (15, 33, 47, 52, 54). Prostasoin, which is inhibited by aprotinin (bovine) and BAY 39-9437 (humanized analog) and not by other serine protease inhibitors such as SBTI and α\(_1\)-antitrypsin (5), constitutively activates Na\(^{+}\) channels in human bronchial and nasal epithelial cells by an as yet unknown mechanism, and its inhibition decreases baseline *I* \(_{sc}\) by ~30% (5, 18). In human airway epithelial cell lines, HAT protease activates PAR-2, leading to the generation of prostaglandin E\(_2\) and to an increase in mucin gene expression (9, 39). Soluble forms of trypsin-like proteases are found in sputum of patients with chronic bronchitis (53). HAT is inhibited by SBTI, α\(_1\)-antitrypsin, aprotinin, and several other serine protease inhibitors (15, 53). Circulating neutrophils are recruited to the lung during inflammation, trauma, and injury, where they release elastase and other proteases. Mast cells reside in airway parenchyma or are interspersed among the epithelial cells and degranulate in response to inflammatory stimuli, releasing trypsin from stored granules. Both cell types and...
their respective proteases are found in bronchoalveolar lavage fluid sampled from patients during inflammation and injury.

Because SBTI resulted in the largest inhibition of LFC we speculate that a trypsin-like protease may be responsible for the tonic activation of Na\(^+\)/H\(^+\) transport in rat lungs, by most likely increasing the activity of epithelial Na\(^+\)/H\(^+\) channels. The presence of an epithelium-expressed trypsin-like protease in the rat is supported by the results of the zymography, in which a 20- to 28-kDa protease can be detected, which is inhibited by SBTI and migrates on the gel to the same level as purified trypsin. Because the animals were normal and without lung disease, there should not have been serine proteases derived from inflammatory cells present in the lung lavage fluid. Additionally, inflammatory cell-derived proteases such as elastase are often unstable and are unlikely to maintain their enzymatic activity long enough to cause an effect in this assay. Together, we have presented evidence for a facilitating role of a serine protease in the clearance of fluid from the lung luminal space. To our knowledge, this is the first report of a physiological role for the epithelium-expressed serine proteases that was tested in the whole animal. Further investigations are required to identify the exact nature and expression of the protease indicated in our studies.

Fig. 6. Recordings of intracellular calcium concentration ([Ca\(^{2+}\)]\(_{ic}\)) in polarized 16HBE cell monolayers incubated with 10 \(\mu\)M of fura-2 AM. Values were recorded every 10 s. A: changes in [Ca\(^{2+}\)]\(_{ic}\) following perfusion of apical surfaces with trypsin (25 \(\mu\)M) as indicated. Monolayers shown at right were preincubated with 50 \(\mu\)M BAPTA-AM for 60 min before perfusions with trypsin. B: changes [Ca\(^{2+}\)]\(_{ic}\) following perfusion of apical surfaces with SLIGKV (100 \(\mu\)M, left) or perfusion of both apical and basolateral surfaces with thapsigargin (1 \(\mu\)M, right). Once steady-state values were reached, the apical surfaces were perfused with trypsin. C: mean data for trypsin-induced \(I_{sc}\) and \(R_{TE}\) in BAPTA-AM-treated (50 \(\mu\)M for 50 min, \(n = 14\)) vs. control (DMSO, \(n = 13\)) in 16HBE cells in Ussing experiments; representative experiments (each tracing represents mean values for 3 experiments) are shown at right.
mean values for 3 monolayers. Mean values for steady-state changes are shown after apical additions of 1.

It is well accepted that the effects of trypsin are due, at least in part, to an activation of PLC. Instead our findings clearly point against a role for PARs is that the rise in Ca^2+ initiated by apical trypsin could not be reproduced by the PAR-2 activating peptide SLIGKV. Furthermore, application of basolateral trypsin produced only a transient increase of I_{sc} (Fig. 5). Thus the possibility that trypsin crossed through paracellular junctions and stimulated the basolateral membrane can be discarded. However, it is possible that apical trypsin may stimulate another type of PAR receptor. Although earlier reports have not shown significant expression of PAR-4 in human airway epithelial cells (15, 39), it is possible that PAR-4 expression could be upregulated by dexamethasone, which we have used in our experiments.

The vectorial transport of Na^+ and Cl^- ions requires the coordinate action of both apical and basolateral transporters. Thus the trypsin-induced increase of Na^+ and Cl^- currents may be due to stimulation of either basolateral or apical transporters. Shin et al. (46) reported that activation of purinergic receptors of normal human epithelial cells with ATP resulted in transient elevation of intracellular Ca^2+, which was responsible for the activation of Na^+-K^+-Cl^- cotransporter. Increases in intracellular Ca^2+ have been shown to downregulate the activity of ENaC (43) in rat cortical collecting tubes but to stimulate nonselective, cation channels with low affinity to amiloride in adult and fetal ATII cells (8, 36). The fact that in our experiments trypsin increased the IC_{50} for amiloride by 10.2 ± 0.33 is consistent with this possibility. Ca^2+-induced activation of an actin-severing protein such as gelsolin may account for the stimulation of both CFTR (7) and ENaC (2). In addition, an increase in intracellular Ca^2+ may phosphorylate and activate CFTR via a Ca^2+-dependent PKC pathway (3).

Hughey et al. (28) have made the important finding that furin, a serine protease that is localized to the Golgi and can also be found in the cell membrane, cleaves the α- and γ-subunits of ENaC and this cleavage is essential for normal, robust channel activity. When the sites for furin cleavage are mutated or in the presence of furin-specific inhibitors, channel activity is greatly suppressed. However, Jovov et al. (29) were unable to locate extracellular trypsin cleavage sites in ENaC subunits. Vallet et al. (49) also found no evidence for proteolytic cleavage of the ENaC proteins. Direct proteolysis of the Na^+ channel by trypsin or elastase is yet to be demonstrated in intact, polarized epithelial cells.

We show that the protease activation of current was accompanied by a two- to threefold increase in R_{TE}, which confers a potential protective effect to the epithelium. Inhibition of the Na^+/K^+-ATPase with ouabain, which should ablate all active transcellular ion transport, does not prevent this increase. The
increase in $R_{TE}$ is contrary to that expected in accordance with Ohm’s law. From these results, we hypothesize that the protease decreases paracellular ion conductance by unknown mechanisms. The mechanism that confers the trypsin-induced increase in $R_{TE}$ differs from that which increases $I_{sc}$. The increase in $R_{TE}$ is inhibited by ET-18 and not by D-609, the reverse being so for $I_{sc}$, indicating that different PLC enzymes are involved. Increased $[Ca^{2+}]_{IC}$ increases $I_{sc}$ in a sustained fashion but does not increase the $R_{TE}$, yet $Ca^{2+}$ chelation with BAPTA attenuates the trypsin-induced $R_{TE}$, implying that $Ca^{2+}$ is required but not sufficient for this increase. Tang and Goodenough (48) have recently characterized the paracellular conductances of four major ions, including $Na^+$ and $Cl^-$, in kidney and colonic cell lines, describing fluxes that appear to be dependent only on concentration gradient. It is not known how these paracellular pathways are regulated. It is possible that similar ion paracellular pathways exist in lung epithelial cells and that trypsin or neutrophil elastase can initiate changes at the paracellular junctions, which would limit the paracellular flow of ions. Increasing active transcellular ion transport, while decreasing paracellular conductance, could improve the vectorial transport of ions. Because fluid follows the ion transport,
preventing the paracellular backflow of ions across the epithelium would increase efficiency in the removal of fluid from the alveolar space. Recently, Kawkitinarong et al. (31) have shown that thrombin increases $R_{TE}$ in an alveolar cell line. The inability of an agent to increase $R_{TE}$ is a rare and important physiological finding. Defining a mechanism will require further investigations.

In summary, these data support a physiological role in lung fluid management for serine proteases derived from both lung epithelium and immune cells. We demonstrate trypsin-like protease activity in rat lung lavage fluid and show that SBTI and α1-antitrypsin inhibit fluid clearance, with clearance being partly restored by exogenous trypsin. Neutrophil-derived elastase, as well as trypsin, increases ion transport and $R_{TE}$ in human bronchial and nasal epithelial cells and rat alveolar cells. The increased $R_{TE}$ indicates decreased paracellular ion conductance, which would enhance the vectorial transport of ions and water across the lung epithelium. Our data support a conductance, which would enhance the vectorial transport of serine proteases, as well as trypsin, increases ion transport and cells. The increased human bronchial and nasal epithelial cells and rat alveolar space. Recently, Kawkitinarong et al. (31) have shown preventing the paracellular backflow of ions across the epithelium.

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REFERENCES

27. Kon W, McConalogue K, Khitin LM, Hollenberg MD, Payan DG, Bohm SK, and Bunnett NW. Luminal trypsin may regulate enterocytes...


