In vitro and in vivo regulation of transepithelial lung alveolar sodium transport by serine proteases

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Planès, Carole, Céline Leyvraz, Tokujiro Uchida, Milena Apostolova Angelova, Grégoire Vuagniaux, Edith Hummler, Michael Matthay, Christine Clerici, and Bernard Rossier. In vitro and in vivo regulation of transepithelial lung alveolar sodium transport by serine proteases. Am J Physiol Lung Cell Mol Physiol 288: L1099–L1109, 2005. First published January 28, 2005; doi:10.1152/ajplung.00332.2004.—The amiloride-sensitive epithelial sodium channel (ENaC) constitutes a rate-limiting step for sodium (Na+) and water absorption across lung alveolar epithelium. Recent reports suggested that ENaC is regulated by membrane-bound extracellular serine proteases, such as channel-activating proteases (CAPs). The objectives of this study were to examine the role of serine proteases in the regulation of transepithelial alveolar Na+ and water transport in vitro and in vivo and the expression of CAPs in rodent distal lung. In vitro experiments showed that inhibition of endogenous serine proteases by apical aprotinin 1) decreased ENaC-mediated currents in primary cultures of rat and mouse alveolar epithelial cells without affecting the abundance nor the electrophoretic migration pattern of biotinylated α- and β-ENaC expressed at the cell surface and 2) suppressed the increase in amiloride-sensitive short-circuit current induced by the β2-agonist terbutaline. RT-PCR experiments indicated that CAP1, CAP2, and CAP3 mRNAs were expressed in mouse alveolar epithelial cells, whereas CAP1 was also expressed in alveolar macrophages recovered by bronchoalveolar lavage. CAP1 protein was detected by Western blotting in rat and mouse alveolar epithelial cells, alveolar macrophages and bronchoalveolar lavage fluid. Finally, in vivo experiments revealed that intra-alveolar treatment with aprotinin abolished the increase in Na+-driven alveolar fluid clearance (AFC) induced by terbutaline in an in situ mouse lung model, whereas trypsin potentiated it. These results show that endogenous membrane-bound and/or secreted serine proteases such as CAPs regulate alveolar Na+ and fluid transport in vitro and in vivo in rodent lung.

channel-activating protease; epithelial sodium channel; β-adrenergic agonist; trypsin; lung fluid balance

ACTIVE TRANSEPITHELIAL SODIUM (Na+) transport by alveolar epithelial cells provides a major driving force for the reabsorption of fluid from the alveolar space, a function that accounts for the ability of the lung to remove alveolar fluid at the time of birth (18) and represents the main mechanism for alveolar edema resolution (24). The amiloride-sensitive epithelial Na+ channel (ENaC) (5, 6) located in the apical membrane of alveolar epithelial cells constitutes a rate-limiting step for Na+ absorption across alveolar epithelium (14). Despite its physiological importance in lung fluid homeostasis, the tissue-specific regulation of ENaC in the distal lung is still poorly understood. Most studies have focused on the systemic regulation of ENaC by hormones (24), but the role of extracellular luminal factors present in the immediate vicinity of the channel, i.e., in the alveolar epithelial lining fluid, has been scarcely investigated.

In recent years, the concept of an autocrine regulation of ENaC by epithelium-derived extracellular serine proteases has emerged from several observations. In 1997, using functional complementation assays to detect increases in ENaC activity in the Xenopus kidney A6 cell line, Vallet et al. (34) cloned a trypsin-like serine protease, the channel-activating protease 1 (CAP1). This glycosylphosphatidylinositol-anchored protease increased amiloride-sensitive Na+ current when coexpressed with ENaC in Xenopus oocytes (34, 35). ENaC activation was fully prevented by extracellular addition of the serine protease inhibitor aprotinin and mimicked by external trypsin. Mammalian homologs of Xenopus CAP1, such as mouse mCAP1 or human and rat prostatins, were also shown to activate ENaC in the Xenopus oocyte expression system (1, 11, 38, 39). More recently, additional transmembrane serine proteases activating ENaC have been identified in mammals, including channel-activating protease 2 (CAP2) and channel-activating protease 3 (CAP3) cloned from the mpkCDD47 mouse kidney cell line (37), TMPRSS3 from human inner ear (15), or TMSP-1 from rat kidney (27). The precise mechanism for protease-mediated activation of ENaC has not been elucidated. It seems likely from studies in Xenopus oocytes (9, 37, 38) or transfected mammalian cells (4) that trypsin-like serine proteases increase Na+ transport by activating a population of near-silent channels rather than by promoting plasma membrane insertion of new channels. The membrane-bound serine proteases may cleave ENaC subunits directly (16, 17) or act via intermediary proteins. In mammals, the channel-activating proteases (CAP1, -2, and -3) are coexpressed with ENaC in epithelial tissues transporting Na+, like renal cortical collecting duct, lung, and colon (36, 37). Indeed, apical treatment with aprotinin was shown to decrease amiloride-sensitive Na+ transport in kidney cell lines in vitro (21, 38). Together, these data suggest that endogenous CAPs may act as regulators of Na+ transport in vivo, at least in the kidney, although this remains to be clearly...
established. Concerning the lung, functional studies recently reported that Na\(^+\) absorption across bronchial or nasal epithelial cells was regulated in vitro by endogenous aprotinin-sensitive serine protease(s) (3, 11, 33). Prostasins, the homolog of CAP1 expressed in human proximal airways, was proposed as a likely candidate for this regulation (11, 33). Whether such a mechanism for autocrine regulation of ENaC also takes place in distal lung epithelial cells in vitro and what is its physiological importance in vivo regarding alveolar Na\(^+\) and fluid clearance have not been investigated.

The objectives of this study were to evaluate the potential role of endogenous/exogenous serine proteases in the regulation of ENaC-mediated alveolar Na\(^+\) transport and to characterize the cell-specific expression of CAP1, CAP2, and CAP3 in rodent distal lung. We first studied in vitro the effects of aprotinin and trypsin on amiloride-sensitive Na\(^+\) transport in primary cultures of rat and mouse alveolar epithelial cells under baseline and β-adrenergic agonist-stimulated conditions and examined whether aprotinin would change either the abundance or the electrophoretic migration pattern of ENaC subunits expressed at the cell surface. The expression of CAPs was also characterized at the mRNA or at the protein level in mouse bronchiolar-alveolar epithelial cells (BAEC), in nonepithelial alveolar cells recovered by bronchoalveolar lavage (BAL), and in BAL fluid. Finally, we evaluated in vivo the effects of aprotinin and trypsin on Na\(^+\)-driven alveolar fluid clearance (AFC) in an in situ model of mouse/rat lung under baseline and β-adrenergic agonist-stimulated conditions. Our data strongly suggest that serine proteases regulate transepithelial alveolar Na\(^+\) transport and that endogenously expressed CAPs could be involved in this process.

**MATERIALS AND METHODS**

**Isolation and Culture of Rat and Mouse Alveolar Epithelial Cells**

The procedure of mouse BAEC and rat alveolar epithelial cell (AEC) isolation accorded with legislation currently in force in France and Switzerland and was approved by French Animal Care and Use Committee (Ministèr Francais de la Pêche et de l’Agriculture, agreement 5669). AEC were isolated from adult male pathogen-free Sprague-Dawley rats (200–250 g) by elastase digestion of lung followed by sequential filtration and differential adherence on bacteriological dishes as previously described (29). Cells (purity >80%, viability >95%) were seeded onto Transwell-Snapwell (polycarbonate membrane with a pore size of 0.4 μm; Costar, Cambridge, MA) filters and cultured in DMEM containing 25 mM d-glucose, 10 mM HEPES, 23.8 mM NaHCO\(_3\), 2 mM L-glutamine, 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml gentamicin. On day 3 after isolation, 1 μM dexamethasone was added to the culture medium, and FBS concentration was reduced to 5%. Experiments were performed between day 4 and 6 following isolation.

Mouse BAEC were isolated from pathogen-free C57B/6J mice aged 2 mo by dispase digestion of lung tissue, followed by sequential filtration and differential adherence on culture dishes coated with rat anti-mouse CD45 and rat anti-mouse CD16/32 (BD Pharmingen), as described by Corti et al. (10) and Rice et al. (30). The yield was 4–5 × 10\(^5\) BAEC/mouse, with a percentage of alveolar type II cells (as assessed by Phosphine 3R staining) ≥78% and a cell viability >95%. BAEC were either used immediately for RNA or protein extraction or resuspended in defined culture medium [DMEM/Ham’s F-12 medium (1:1 vol/vol)] containing 60 nM sodium selenite, 20 mM HEPES, 2 mM L-glutamine, 0.25% BSA, 5 μg/ml transferrin, 5 μg/ml insulin, 1 mM triiodothyronine, 10^−7 M dexamethasone, 10 ng/ml epidermal growth factor, 10 ng/ml keratinocyte growth factor, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2% decomplemented mouse serum (Sigma) and seeded on type IV collagen (Sigma)-coated Transwell (0.33 or 1 cm\(^2\)) filters (1 × 10\(^6\) cells/cm\(^2\)). Bronchiolar-alveolar cells on filters were cocultured with irradiated mouse embryonic fibroblasts grown in the same medium on 24- or 12-well plastic dishes in a 5% CO\(_2\)-95% air atmosphere. Cell medium (basolateral and apical) was changed on day 1 after BAEC isolation and then on alternate days. Experiments were performed at day 5 following isolation.

**Isolation of Cells and Proteins from BAL Fluid**

Mice were anesthetized with pentobarbital intraperitoneally, and a 20-gauge catheter was inserted in the trachea. The lungs and heart were removed in block from the thorax, and two successive BAL were performed with intratracheal instillations of 1.5 ml of PBS. BAL fluids from four or five animals were pooled together, and alveolar nonepithelial cells were collected by centrifugation (130 g for 10 min at 4°C) and immediately processed for RNA or protein extraction as described below. Total protein in BAL fluid supernatant was precipitated with TCA (final concentration: 15%). The samples were centrifuged at 12,000 g for 10 min, and the pellets were washed three times with ice-cold 80% acetone. The precipitated proteins were solubilized at 100°C in a solution containing 200 mM Tris, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol before sonication.

**Electrophysiological Studies in Primary Cultures of AEC**

**Short-circuit current measurements.** Measurements of short-circuit current (I\(_{sc}\)), transepithelial potential difference (PD), and transepithelial resistance (R\(_{te}\)) were performed in rat AEC grown on Snapwell filters on day 5 or 6 using a voltage-clamp system (Costar) with the apical and basolateral surfaces bathed in DMEM thermostated at 37°C. I\(_{sc}\) was measured every 5 or 10 min by clamping PD to 0 mV for 1s, and R\(_{te}\) was calculated from I\(_{sc}\) and PD with Ohm’s law. Snapwell filters with unstable I\(_{sc}\) or with R\(_{te}\) <400 Ω cm\(^2\) were discarded. To test the effect of the serine protease inhibitor aprotinin, we added aprotinin or vehicle after establishing a stable I\(_{sc}\) into the apical or basolateral compartment (final concentration of aprotinin: 1–100 μg/ml). I\(_{sc}\) was monitored for up to 75 min until a plateau was reached. Then, the serine protease trypsin (final concentration 200 μg/ml) was directly added to the apical bath of some filters treated with either vehicle or aprotinin. Five minutes later, amiloride (final concentration: 10 μM) was added to the apical bath of all filters to calculate the amiloride-sensitive I\(_{sc}\) defined as the difference between the I\(_{sc}\) value just before amiloride addition and the I\(_{sc}\) value 5 min after amiloride addition. In another set of experiments, the effect of aprotinin on I\(_{sc}\) stimulation induced by the β2-agonist terbutaline was tested. After a 15-min equilibration period, aprotinin (final concentration: 50 μg/ml) or vehicle was added to the apical side, followed 3 min later by basolateral addition of terbutaline (final concentration: 100 μM) or vehicle. I\(_{sc}\) was monitored for 45 min before apical addition of amiloride, and amiloride-sensitive I\(_{sc}\) was calculated in the four groups: control cells (having received only vehicle), terbutaline alone, aprotinin + terbutaline, and aprotinin alone.

**Measurements under open-circuit conditions.** R\(_{te}\) and voltage (PD) were measured in mouse BAEC grown on semipermeable Transwell filters for 5 days under open-circuit conditions via dual silver/silver chloride electrodes connected to the Millicell electrical resistance clamp apparatus. The development of high R\(_{te}\) was carefully monitored using dual silver/silver chloride electrodes. Equivalent short-circuit current (I\(_{sc}\)) was calculated with Ohm’s law from R\(_{te}\) and PD. By convention, positive I\(_{sc}\) corresponded to a flow of positive charges from the apical to the basal solution. Measurements of R\(_{te}\) and PD were performed before and after apical addition of 50 μg/ml aprotinin for 1 h at 37°C, followed by apical addition of 10 μM amiloride.
Detection of ENaC Subunits Expressed at the Apical Surface of Rat AEC

Biotinylation and recovery of apical membrane proteins from cultured rat AEC were performed as previously described (28). In brief, AEC grown on Transwell filters were placed on ice and washed three times with ice-cold PBS-Ca2+/Mg2+ (PBS with 0.1 mM CaCl2 and 1 mM MgCl2). Apical membrane proteins were then biotinylated by a 15-min incubation at 4°C with NHS-ss-biotin 1.25 mg/ml (Pierce, Rockford, IL) freshly diluted into biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, 150 mM NaCl, pH 7.5) with gentle agitation. Alveolar type II cells were rinsed with PBS-Ca2+/Mg2+, scraped in cold PBS, and pelleted at 2,000 rpm at 4°C. Pellets were solubilized for 45 min at 20 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5] containing protease inhibitors. The lysates were clarified by centrifugation at 14,000 g for 10 min at 4°C, and supernatants were incubated overnight with packed streptavidin-agarose beads (Pierce, Rockford, IL) to recover biotinylated proteins. The beads were then pelleted by centrifugation, and aliquots of supernatants were taken to represent the unbound, intracellular pool of proteins. Biotinylated proteins were eluted from the beads by heating to 100°C for 5 min in SDS-PAGE sample buffer [containing 10% glycerol, 12.5% 0.5 M Tris-HCl (pH 6.8), 10% of 20% SDS, 5% 2-mercaptoethanol, and 2.5% of 0.05% (wt/vol) bromphenol blue]. Samples of biotinylated and nonbiotinylated proteins were resolved through 10% acrylamide gels, electroblotted, electrically transferred to nitrocellulose paper, and subsequently probed for the α- or β- or γ-ENaC subunits. To ensure the absence of leakage of biotin into the cells, we systematically verified the absence of the intracellular protein actin in biotinylated extracts. Rabbit polyclonal anti-α-ENaC and anti β- and γ-ENaC antibodies (12) were used at the dilution 1:2,000, and mouse monoclonal antiactin at the dilution 1:5,000. Quantification of αENaC and actin levels was obtained using NIH Image software.

RT-PCR Analysis

Total RNA was prepared from freshly isolated mouse BAEC, BAL nonepithelial cells or whole mouse kidney using the RNeasy extraction kit (Qiagen, Hilden, Germany). The RNAs (2.5 μg/sample) were reverse-transcribed at 37°C for 1 h using the Superscript II RNase H reverse transcriptase (Invitrogen, Basel, Switzerland). One hundred twenty-five nanograms of BAEC and BAL cells and whole kidney cDNA were amplified for 30 or 40 cycles in 25 μl of total volume containing 50 nM KCl, 20 mM Tris-HCl (pH 8.4), 40 μM dNTP, 1.5 mM MgCl2, 1 unit Taq polymerase, and 4 ng of either mCAP1, mCAP2, mCAP3, or GPDH specific primers. The mCAP1 primers were mCAP1-mouse prostate 116 (5′-TGG CTT CAA AAC CAG CCT CT-3′) and mCAP1–4 antisense (5′-TCA CCC CAA CTC ACA ATG CC-3′). The thermal cycling program for mCAP1 was as follows: 95°C for 45 s; 70°C for 45 s (with a decrease in annealing temperature of −0.5°C/cycle during the first 20 cycles), and 72°C for 45 s. The mCAP2 primers were mCAP2–7 sense (5′-GAT TAC AAC GCA AGC CTC AA-3′) and mCAP2–2 antisense (5′-GAG CAC TCT CAA AGC AAC AGC AC-3′), the mCAP3 primers were mCAP3–5 sense (5′-CGT GTA TGA TAG CCT CAG GCC CC-3′), and mCAP3–6 antisense (5′-GCA GTT GTT GGG CGG GTT-3′), and GPDH primers were GAPDH37 sense (5′-GCT CTT CAC CAT GGA GA-3′) and GAPDH38 antisense (5′- CGG CCA TCA CGC CAG GTT-3′). The thermal program for mCAP2, mCAP3, and GPDH was as follows: 95°C for 1 min, 66°C for 1 min (with a decrease in annealing temperature of −0.5°C/cycle during the first 20 cycles), and 72°C for 1 min. Amplification products were run on 2% agarose gel, stained with ethidium bromide, and photographed. The identity of the mCAP1 amplified products was controlled by digestion with KpnI (Invitrogen).

Detection of CAP1 Protein by Western Blot Analysis

Preparation of antibodies. Rabbit polyclonal antibodies were raised against the NH2 terminus (amino acids F7–A118) or the COOH terminus (amino acids A298–F312) of mCAP1. The fusion proteins were generated in the pGEX-4T vector (Pharmacia, Uppsala, Sweden), isolated from Escherichia coli bacterial lysate (GST-agarose beads, Sigma), and eluted with reduced glutathione according to the manufacturer’s instruction. The GST-fusion proteins in Freund adjuvant were injected subcutaneously into rabbits. The antibodies in immune sera were then affinity-purified with the Actigel ALD system (Sterogene Bio separations). Preimmune and immune sera were tested for Western blotting of mCAP1 protein using injected or noninjected Xenopus laevis oocyte extracts.

Preparation of protein extracts and Western blotting procedure. Freshly isolated mouse BAEC and BAL cells, as well as cultured rat AEC scraped off the filters in ice-cold PBS and centrifuged at 1,500 rpm for 10 min at 4°C, were resuspended in 30 μl of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1% Triton X-100, 0.1% SDS, and protease inhibitors and kept on ice for 1 h. Cell lysates were then centrifuged (12,000 rpm, 15 min) at 4°C, and samples of the supernatants were immediately frozen before use. X. laevis oocytes injected or not with mCAP1 cRNA (4 ng) were homogenized by being vortexed for 45 s in ice-cold Triton homogenization buffer (0.1 M NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.6, and protease inhibitors), centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were immediately frozen before use. For Western blotting, samples of protein extracts (30–40 μg protein for cell or oocyte extracts, 300 μg protein for BAL fluid) in one volume of sample buffer (containing 13.8% sucrose, 9.6% SDS, 4.2% 2-mercaptoethanol, and 0.0126% bromphenol blue in H2O) were resolved through 10% acrylamide gels, electroblotted, electrically transferred to nitrocellulose paper, and subsequently probed for mCAP1 protein detection. Rabbit polyclonal anti-mCAP1 NH2-terminal and anti-mCAP1 COOH-terminal antibodies were used at the dilution 1:1,000.

The anti-rabbit IgG secondary antibody (Amersham Pharmacia Bio tech, UK) was used at dilution 1:10,000, and the signal was developed with the Supersignal West Dura system (Pierce, Rockford, IL). For deglycosylation experiments, protein extracts from Xenopus oocytes injected with mCAP1 cRNA (100 μg of protein/sample), from freshly isolated mouse BAEC or BAL cells (200 μg of protein/sample) and protein from BAL fluid (300 μg/sample), were acetone-precipitated, resuspended in 0.14% sodium deoxycholate by sonication, incubated overnight with or without peptide N-glycosidase F (PNGase F) according to the manufacturer’s instructions (New England BioLabs, Beverly, MA), and processed for Western blotting as described above.

Measurement of AFC in Mouse and Rat

AFC was measured in vivo using an in situ model of mouse lung as previously described (13). This model has been shown to give AFC values similar to those obtained with the ventilated mouse model over a 15-min period. In brief, male or female C57Bl/6j mice aged 2–3 mo were killed with intraperitoneal pentobarbital (250 mg/kg) and maintained at 37–38°C with a heating pad, an infrared lamp, and an intraabdominal monitoring thermistor. A 20-gauge venous catheter was inserted in the trachea through a tracheotomy and tightly fixed. The lungs were inflated with 100% O2 at 7 cmH2O continuous positive airway pressure throughout the experiment. Then, 10 ml/kg of labeled alveolar fluid volume tracer. An alveolar fluid sample (50–100 μl of protein/sample), from freshly isolated mouse BAEC or BAL cells (200 μg of protein/sample) and protein from BAL fluid (300 μg/sample), were acetone-precipitated, resuspended in 0.14% sodium deoxycholate by sonication, incubated overnight with or without peptide N-glycosidase F (PNGase F) according to the manufacturer’s instructions (New England BioLabs, Beverly, MA), and processed for Western blotting as described above.
(15 or 30 min later). The aspirates were centrifuged at 3,000 g for 10 min, and the radioactivity in supernatants was counted in duplicate. AFC (percentage fluid absorption at 15 or 30 min) was calculated from the increase in alveolar fluid albumin as follows: AFC (%) = (Ci − C0)/C0 × 100, where Ci and C0 represent the initial and final concentrations of 125I-albumin in the aspirate at 1 and 15 (or 30) min, respectively, as assessed by radioactivity measurements. AFC in rat was measured over a 30-min period by the same method, except that the instillate consisted of 10 ml/kg normal saline containing 5% BSA and 0.1 μCi/ml 125I-albumin. To test the effect of serum protease inhibitors on mouse and rat AFC, we added aprotinin (100 μg/ml) to the instillate and measured AFC at 30 min. In another set of experiments, the combined effects of aprotinin (100 μg/ml), terbutaline (100 μM), trypsin (100 μg/ml), and amiloride (1 mM) were studied in mice over a 15-min period. In this series of experiments, BSA was omitted in the instillate because a high concentration of BSA (5%) may inhibit the activity of trypsin.

Statistical Analysis

Results are presented as means ± SE. For functional data, one-way or two-way variance analyses were performed, and, when allowed by the F-value, results were compared by the modified least significant difference (Statview software). For Western blot experiments, differences between groups were evaluated with paired t-test. P < 0.05 was considered significant.

RESULTS

Effect of Aprotinin and Trypsin on Na⁺ Transport Across Primary AEC Cultures

The effects of aprotinin and of the exogenous serine protease trypsin were first evaluated in cultured rat AEC. Addition of aprotinin (50 μg/ml) to the apical bath (but not to the basolateral bath) of rat AEC monolayers mounted in an Ussing chamber induced a marked decrease in Isc, which reached a plateau within 70–75 min. As shown in Fig. 1A, the amiloride-sensitive Isc was decreased by almost 70% in cells treated with aprotinin compared with control cells treated with vehicle, whereas the amiloride-resistant component of Isc remained unchanged [1.1 ± 0.24 vs. 1.2 ± 0.10 μA/cm² in aprotinin-treated and vehicle, respectively; not significant (NS); n = 5]. In cell monolayers treated with aprotinin for 75 min, apical addition of trypsin (200 μg/ml) completely reversed within 3–5 min the Isc inhibition induced by aprotinin, whereas trypsin was without significant effect in vehicle controls. Therefore, amiloride-sensitive Isc was not significantly different from control in cells treated with trypsin alone or treated with aprotinin followed by trypsin (Fig. 1A).

The aprotinin-induced Isc decrease was concentration dependent: 1 and 2.5 μg/ml aprotinin significantly decreased Isc, but the maximal inhibition was obtained with concentrations ≥20 μg/ml (Fig. 1B). The effect of aprotinin was also studied in well-differentiated primary mouse BAEC monolayers that had developed high Rsc (around day 5 following isolation) and expressed mRNA transcripts encoding for α-, β-, and γ-mENaC subunits as assessed by RT-PCR analysis (data not shown). Addition of aprotinin (50 μg/ml) to the apical (but not basolateral) compartment of mouse BAEC monolayers decreased by >30% the transepithelial PD measured under open-circuit conditions and paradoxically decreased Rsc (Table 1). The Ieq calculated from PD and Rsc was significantly reduced by 20%. This decrease represented ~35% of the amiloride-sensitive component of Ieq.

Together, these results suggest that endogenous serine proteases activate ENaC in native rodent AEC.

To study the time course of ENaC activation by endogenous serine proteases, we investigated the effect of aprotinin removal following long-term exposure (20 μg/ml aprotinin overnight) in rat AEC monolayers mounted in an Ussing chamber. As expected, overnight exposure to aprotinin significantly reduced amiloride-sensitive Isc (6 ± 0.34 vs. 3.87 ± 0.09 μA/cm² in the control untreated group and aprotinin group, respectively; P < 0.01; n = 4–5). In aprotinin-treated cells, aprotinin removal induced a rapid and marked increase in Isc over the first 10 min followed by a plateau, together with a marked increase in PD and a smaller increase in Rsc (data not shown). The amiloride-sensitive component of Isc was increased by more than two times 10 min after aprotinin removal compared with filters maintained under aprotinin (8.18 ± 0.31

Fig. 1. Effect of aprotinin and trypsin on short-circuit current (Isc) in primary rat alveolar epithelial cells (AEC). Rat AEC cultured on semipermeable filters for 5 days were mounted in Ussing chambers for measurement of Isc. A: rat AEC monolayers were exposed after a 15-min equilibration period to either vehicle or aprotinin (Apro, 50 μg/ml) for 75 min. Then, trypsin (200 μg/ml) was added for 5 min to the apical side of some filters previously exposed to vehicle or aprotinin. Amiloride (10 μM apical) was added for 5 min to all filters at the end of the experiment. Amiloride-sensitive Isc, representing the difference between Isc value just before amiloride addition and 5 min after amiloride addition was calculated in the four groups: vehicle alone (control), aprotinin alone, trypsin alone, and aprotinin followed by trypsin. Results represent means ± SE of 4–7 filters in each condition. **Significantly different from control (P < 0.01). B: changes in Isc induced by a 75-min exposure to increasing concentrations of aprotinin (1–100 μg/ml). ∆Isc represents the difference between the value of Isc at the end of exposure to aprotinin (1, 2.5, 5, 20, 50 or 100 μg/ml, solid bars) or vehicle (0 μg/ml aprotinin, open bar) and the baseline Isc at the moment of aprotinin or vehicle addition. Results represent means ± SE of 4–6 filters in each condition. ***Significantly different from vehicle (P < 0.001); §significantly different from 1 μg/ml aprotinin (P < 0.05); #significantly different from the 2.5 μg/ml aprotinin group (P < 0.05); NS, not significant.
Table 1. Effect of aprotinin on electrophysiological parameters of cultured mouse bronchiolar-alveolar epithelial cells

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<tr>
<th></th>
<th>Control</th>
<th>Aprotinin</th>
<th>Amiloride</th>
<th>Aprotinin + Amiloride</th>
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<tr>
<td>PD, mV</td>
<td>-11 ± 3.1</td>
<td>-7.5 ± 2.27*</td>
<td>-4.9 ± 1.06*</td>
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<tr>
<td>(R_c), Ω/cm²</td>
<td>814 ± 215</td>
<td>688 ± 176*</td>
<td>1,059 ± 275</td>
<td>856 ± 227†</td>
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<td>(I_{sc}), µA/cm²</td>
<td>12.3 ± 0.99</td>
<td>9.8 ± 1.01†</td>
<td>3.8 ± 0.26†</td>
<td>4.0 ± 0.57§</td>
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vs. 3.87 ± 0.09 µA/cm², respectively; \(P < 0.001; n = 4–5\), overshooting the value of control untreated filters.

We also evaluated whether aprotinin would modify the \(I_{sc}\) response induced in rat AEC monolayers by the β2-agonist terbutaline. As shown in Fig. 2A, basolateral addition of terbutaline (final concentration: 100 µM) induced a transient decrease in \(I_{sc}\) in cells pretreated for 3 min with either vehicle or aprotinin (50 µg/ml). This initial decrease was followed in cells pretreated with vehicle by a gradual increase in \(I_{sc}\) to steady-state levels surpassing the baseline. By contrast, no gradual \(I_{sc}\) increase could be seen in aprotinin-pretreated cells (Fig. 2A). As shown in Fig. 2B, preincubation with aprotinin completely abolished the increase in amiloride-sensitive \(I_{sc}\) induced in rat AEC by terbutaline. Of note, amiloride-sensitive \(I_{sc}\) value was still significantly greater in cells treated with aprotinin + terbutaline than in cells treated with aprotinin only.

Effect of Aprotinin on \(\alpha\) - and \(\beta\)-rENaC Subunit Surface Expression In Primary AEC

To examine whether the inhibitory effect of aprotinin on transepithelial Na⁺ transport was associated with a change either in the abundance or in the electrophoretic migration pattern of \(\alpha\)- and \(\beta\)-ENaC subunits expressed at the apical membrane, we treated rat AEC monolayers for 60 min with apical aprotinin (20 µg/ml) or vehicle before apical cell surface biotinylation and subsequent separation of intracellular (non-biotinylated) and surface (biotinylated) proteins were performed. As shown in Fig. 3A, top, immunoblotting with rabbit polyclonal anti-\(\alpha\)-rENaC antibody revealed a major band with molecular mass of 85–90 kDa, a minor band with molecular mass of 65 kDa in intracellular extracts from control cells, and only one band at 65 kDa in biotinylated extracts, as previously described (28). Aprotinin treatment did not modify the migration pattern of \(\alpha\)-rENaC in intracellular or surface extracts or its abundance at the apical cell surface (Fig. 3A, bottom). Immunoblotting with rabbit polyclonal anti-\(\beta\)-rENaC showed one single band migrating at 95–100 kDa in both intracellular and surface extracts from control cells, and no change in the migration pattern of \(\beta\)-rENaC protein was detected in aprotinin-treated cells (Fig. 3B, top). Aprotinin did not significantly modify the abundance of \(\beta\)-rENaC expressed at the apical cell surface (Fig. 3B, bottom). The effect of aprotinin on \(\gamma\)-ENaC surface expression could not be investigated in this study, due to the very faint signal of biotinylated \(\gamma\)-ENaC in native AEC.

Expression of CAP mRNAs in Mouse Distal Lung Cells

Expression of mCAP1, mCAP2, and mCAP3 mRNAs was studied by RT-PCR in freshly isolated mouse BAEC and in nonepithelial alveolar cells (mostly macrophages) recovered by BAL (BAL cells). As shown in Fig. 4A, the set of mCAP1 primers permitted detection of amplified products of the expected size (851 bp) in BAEC (lane 3) and in nonepithelial BAL cells (lane 1), as well as in whole kidney (lane 5) and wild-type mCAP1 cDNAs (lane 9). In addition, a second amplified product (690 bp) was detected in BAEC only (lane 3). The specificity of these two amplified products was confirmed by their digestion with the KpnI restriction enzyme, yielding two bands in BAL cells (lane 2), whole kidney (lane 6), and wild-type mCAP1 cDNAs (lane 10), and three bands in BAEC (lane 4). Sequence of the 690-bp product obtained in BAEC confirmed mCAP1 sequence wherein the exon 3 was

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Fig. 2. Effect of aprotinin on transepithelial (Terb)-induced \(I_{sc}\) response in rat AEC monolayers. Rat AEC monolayers mounted in Ussing chambers were preincubated with apical aprotinin (final concentration: 50 µg/ml) or vehicle for 3 min before basolateral addition of terbutaline (final concentration: 100 µM) or vehicle. \(I_{sc}\) was monitored for 45 min before apical addition of amiloride (final concentration: 10 µM). A: representative traces of AEC monolayers preincubated with vehicle (○) or aprotinin (□) before treatment with terbutaline. B: effect of aprotinin on terbutaline-induced increase in amiloride-sensitive \(I_{sc}\). Control, cells treated with vehicle only; Terb, cells treated with terbutaline only; Terb + Apro, cells treated with aprotinin + terbutaline; Apro, cells treated with aprotinin only. Results represent means ± SE of 4–6 filters in each condition. **Significantly different from control (\(P < 0.01\)); ***significantly different from control (\(P < 0.001\)); §§§significantly different from terbutaline alone (\(P < 0.001\)); #§§significantly different from terbutaline + aprotinin (\(P < 0.01\)).

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Fig. 3. Effect of aprotinin on α- and β-rat epithelial sodium channel (rENaC) subunit cell surface expression in primary rat AEC. Rat AEC grown on Transwell filters were exposed to aprotinin (50 μg/ml, apical) or vehicle for 1 h, and apical cell surface biotinylation experiments were then performed as described in MATERIALS AND METHODS to assess cell surface expression of α-rENaC (A) and β-rENaC (B) subunits. Representative immunoblots showing the expression of α-rENaC subunit (A, top) and β-rENaC subunit (B, top) in nonbiotinylated (intracellular) and biotinylated extracts (surface). Actin, an abundant intracellular protein, was not detected in biotinylated (surface) extracts. Quantification of biotinylated α-rENaC (A, bottom) and β-rENaC (B, bottom) signals in aprotinin (solid bars)- or vehicle (open bars)-treated cells was obtained using NIH Image software, and data were normalized for the actin signal in corresponding intracellular extracts. Results are expressed as the ratio of α- or β-rENaC/actin (% of the value in vehicle-treated cells) and represent means ± SE of 3–4 separate experiments. Statistical significance was calculated from the raw data by paired t-test.

Fig. 4. Expression of mouse channel-activated protease (mCAP) mRNAs in mouse distal lung cells. Total RNA was extracted from freshly isolated bronchiolar-alveolar epithelial cells (BAEC) and from nonepithelial cells recovered by bronchoalveolar lavage (BAL cells) and reverse transcribed. Each sample was amplified by RT-PCR with sets of primers specific for mCAP1 (40 cycles) (A) and mCAP2 and mCAP3 (30 cycles) (B). A: mCAP1 amplified products for of the expected size (851 bp) were obtained in BAL cells (lane 1) and in BAEC (lane 3), as well as in whole kidney (lane 5) and wild-type mCAP1 cDNA (lane 9) used as positive controls. A second amplified product (690 bp) was detected in BAEC only (lane 3). Digestion of the amplified products with KpnI yielded two bands (652 and 199 bp) in BAL cells (lane 2), whole kidney (lane 6), and wild-type mCAP1 cDNA (lane 10), and 3 bands (652, 491, and 199 bp) in BAEC (lane 4). No amplified products were detected in mCAP1 stop cDNA wherein exons 3–6 were absent (lane 8) or when cDNA was omitted (lane 7). B: amplified products for mCAP2 (158 bp) (top) and mCAP3 (267 bp) (middle) were detected in BAEC (lanes 3 and 4) and in whole kidney (lane 5), but not in BAL cells (lanes 1 and 2) or when cDNA was omitted (lane 6). Amplified products for GAPDH (310 bp) used as an internal standard were detected in BAL cells (lanes 1 and 2), BAEC (lanes 3 and 4) and whole kidney (lane 5), but not when cDNA was omitted (lane 6, bottom).
Expression of mCAP1 Protein in Mouse and Rat Distal Lung

Expression of mCAP1 protein was evaluated by Western blotting in mouse and rat distal lung cell extracts, as well as in extracts from *Xenopus* oocytes injected or not with mCAP1 cRNA, using two rabbit polyclonal affinity-purified antibodies raised against either the COOH (C terminus ab) or the NH₂ (N terminus ab) fragment of mCAP1 (Fig. 5). As shown in Fig. 5A, immunoblotting with the C terminus ab revealed in oocytes injected with mCAP1 cRNA the presence of a major band with molecular mass of 36–38 kDa. A single band was detected in mouse BAEC and rat AEC at 36 kDa. The specificity of the signal was confirmed by the fact that no signal was obtained with the preimmune serum or when the C terminus ab was incubated with the corresponding fusion protein (Fig. 5A). In oocytes injected with mCAP1 cRNA, immunoblotting with the N terminus ab showed a signal that was similar to that obtained with the C terminus ab, whereas in mouse BAEC and in rat AEC it revealed a major band ~45 kDa (Fig. 5B). Immunoblotting with the preimmune serum or competition experiments with the corresponding fusion protein confirmed the specificity of the signals.

The protein mCAP1 possesses two potential sites of N-glycosylation (N110 and N159). To investigate whether the difference in molecular mass of mCAP1 signal obtained with the C and N terminus ab (36 and 45kDa, respectively) in mouse BAEC could arise from different glycosylation patterns of mCAP1 that could favor the recognition by one or the other antibody, we performed experiments of deglycosylation with PNGase F. As shown in Fig. 6A, treatment of oocytes expressing mCAP1 with PNGase F before immunoblotting with the C terminus ab induced a shift of the major band from 36–38 to 32–36 kDa and the disappearance of the minor 40- to 45-kDa band. In BAEC, PNGase F treatment induced a shift of both the 36-kDa band recognized by the C terminus ab and the 45-kDa band recognized by the N terminus ab, yielding one single band migrating at 32 kDa for both antibodies (Fig. 6A).

Finally, expression of mCAP1 with or without PNGase F treatment was also studied in mouse BAL cell extracts and BAL fluid (Fig. 6B). A single band ~42–45 kDa was detected in nonepithelial mouse BAL cells and proteins TCA precipitated from mouse BAL fluid using both the N terminus ab (Fig. 6B) and the C terminus ab (not shown). A ~42- to 45-kD band was also seen in BAL fluid from rats and in the culture medium of rat AEC in primary culture (data not shown). Treatment of mouse BAL fluid proteins with PNGase F induced a complete shift of the signal from 42–45 to 32–34 kDa, whereas treatment of BAL cell extracts yielded two additional bands at 36 and 32–34 kDa, probably corresponding to partial and complete deglycosylation of mCAP1 (Fig. 6B). Together, these results suggest that mCAP1 protein is expressed and detectable under different forms in mouse BAEC, one partially (36 kDa) and the other fully N-glycosylated (45 kDa), while it is secreted in the epithelial lining fluid in a fully N-glycosylated form.
Effect of Aprotinin and Trypsin on Active AFC in Mice

To evaluate the potential role of serine proteases on alveolar Na\(^+\) and water transport in vivo, we measured AFC in mice and rats using an in situ (non ventilated) lung model in which the air space was instilled with an isoosmolar solution containing \(^{125}\)I-albumin as a volume marker. The effects of aprotinin and trypsin on AFC were studied under basal conditions and under conditions of stimulation by β\(_2\)-adrenergic agonists. Experiments were performed over a 15- or a 30-min period, since AFC values obtained with the in situ mouse model are not linear over longer time periods. Aprotinin (100 \(\mu\)g/ml of instillate for 30 min) modified neither mouse AFC (16.9 ± 2.15 vs. 15.8 ± 1.37% in aprotinin-treated and vehicle controls, respectively; \(n = 5\) in each condition; NS) nor rat AFC (17.1 ± 1.34 vs. 14.2 ± 0.79% in aprotinin-treated and vehicle controls, respectively; \(n = 5–7\) in each condition; NS) under basal conditions. As shown in Fig. 7, terbutaline alone (10\(^{-4}\) M in the instillate for 15 min) significantly increased AFC by 55% in mouse lung compared with controls. The stimulatory effect of terbutaline was completely abolished by coinubcation with aprotinin (100 \(\mu\)g/ml of instillate). Trypsin alone (100 \(\mu\)g/ml of instillate) did not significantly modify AFC, but coinubcation of terbutaline + trypsin induced a marked increase in AFC (191% of control) that overshot the value of AFC with terbutaline alone. Importantly, the changes in AFC induced by terbutaline alone, terbutaline + aprotinin, and terbutaline + trypsin were only related to changes in the amiloride-sensitive part of AFC, since the amiloride-resistant AFC values in these three groups were not significantly different from the group amiloride alone (Fig. 7).

**DISCUSSION**

Extracellular activation of ENaC by endogenous membrane-bound serine proteases such as the CAPs represents a potential mechanism for tissue-specific regulation of Na\(^+\) and water absorption in the distal lung. The present study shows that the Kunitz-type serine protease inhibitor aprotinin, when added to the apical side of cultured rat AEC, induced a marked and sustained decrease in the amiloride-sensitive \(I_{\text{sc}}\) that was rapidly and fully reversible after removal of aprotinin. Also, apical addition of trypsin, an exogenous serine protease that activates ENaC in the *Xenopus* oocyte system, completely reversed the inhibition of Na\(^+\) current in aprotinin-treated cells but was without effect in untreated rat AEC. These functional data provide the first evidence that ENaC is constitutively activated in vitro by endogenous serine proteases located at the apical side of native rat AEC and suggest that this activation was maximal under our usual culture conditions. Of note, inhibition of transepithelial Na\(^+\) transport by apical aprotinin was also obtained in cultured mouse BAEC using open-circuit studies, although to a lesser extent than in rat AEC. The difference in magnitude could be explained by different cell culture conditions or by interspecies differences in the expression of endogenous serine proteases or in their sensitivity to aprotinin, as previously shown between mouse and amphibian kidney cells (38). Together, our results are in line with previous studies.
showing that aprotinin can block ENaC-mediated Na\(^{+}\) transport in mouse cortical collecting duct mpkCCD14 (38) and M-1 (19, 21, 26) cell lines. Similar to what was obtained in cultured AEC, it was recently reported that aprotinin and BAY 39-9437, a recombinant Kunitz-type serine protease inhibitor derived from human bikunin, decreased amiloride-sensitive Na\(^{+}\) transport in upper airway epithelial cells including primary cultures of human bronchial or nasal epithelial cells (3, 11).

Another important finding of the present study is that aprotinin completely abolished the increase in amiloride-sensitive \(I_{Na}\) induced by the \(\beta_{2}\)-agonist terbutaline in rat AEC. Stimulation of \(\beta_{2}\)-adrenergic receptors, either by endogenous catecholamines or by \(\beta_{2}\)-agonist drugs, is the main mechanism for stimulation of alveolar Na\(^{+}\) transport and fluid clearance in vivo in most mammalian species (24). In vitro, AMP agonists have been shown to increase ENaC activity and transepithelial Na\(^{+}\) transport in various cell types including AEC, mostly by increasing the turnover and promoting the insertion of ENaC subunits at the cell surface (8, 25, 28, 32). Our data suggest that endogenous membrane-bound serine proteases and \(\beta_{2}\)-agonists could have a synergistic effect on Na\(^{+}\) transport, the serine proteases activating Na\(^{+}\) channels newly addressed to the apical membrane by the \(\beta_{2}\)-agonist (3, 37). Such a synergism on ENaC-mediated Na\(^{+}\) transport has been previously reported between membrane-bound CAPs and serum- and glucocorticoid-regulated kinase (Sgk-1) in Xenopus oocytes (37).

The mechanism(s) whereby endogenous or exogenous serine proteases increase ENaC activity is not fully elucidated. In the present study, apical biotinylation experiments were used to determine whether inhibition of endogenous serine proteases by aprotinin would change the cell surface expression of \(\alpha\)- and \(\beta\)-ENaC subunits in native rat AEC. The abundance of biotinylated \(\alpha\)- and \(\beta\)-ENaC subunits was not modified by aprotinin, suggesting that protease-mediated activation of Na\(^{+}\) transport was not related to a change in ENaC density at the cell surface. This finding is consistent with previous experiments in the Xenopus oocyte expression system showing that expression of CAP1, CAP2, or CAP3 or treatment with exogenous trypsin activated ENaC without an associated increase in the number of channels expressed at the cell surface (9, 37, 38). In the same line, recent patch-clamp experiments in NIH/3T3 fibroblasts stably expressing ENaC reported that trypsin activates ENaC by increasing the open probability \(P_o\) of a near-silent population of channels expressed in the plasma membrane, rather than by promoting cell surface insertion of new channels (4). Yet, it is not known whether this change in \(P_o\) could be due to a direct cleavage of ENaC by serine proteases (20) or whether it may involve a cascade of intermediary proteins (31). Using Chinese hamster ovary (CHO) or Madin-Darby canine kidney cells transfected with tagged mENaC subunits, Hughey et al. (16, 17) recently suggested that ENaC maturation and activation could involve furin-dependent cleavages of \(\alpha\)- and \(\gamma\)-ENaC subunits. Furin, a serine protease located in the trans-Golgi network, would cleave the full-length \(\alpha\)-ENaC subunit, leading to a 65-kDa fragment containing the COOH terminus and a 30-kDa fragment containing the NH\(_2\) terminus (16). In our experiments, biotinylated \(\alpha\)-rENaC had an apparent molecular mass of 65 kDa, lower than the major intracellular band of \(\alpha\)-rENaC migrating at 85 kDa (28). However, the surface 65-kDa fragment is likely not secondary to a furin-dependent cleavage since the antibody we used was raised against the NH\(_2\) terminus of \(\alpha\)-rENaC (amino acids E10–F77) (12), located upstream of consensus motifs for furin cleavage described by Hughey et al. (16). This discrepancy suggests that ENaC maturation and activation may be different in transfected CHO cells and native rat AEC and dependent on the cell type.

Examining the effect of aprotinin on the electrotransport migration pattern of ENaC subunits in rat AEC, we found that aprotinin modified neither intracellular nor biotinylated \(\alpha\)- and \(\beta\)-ENaC migration patterns. If we assume that aprotinin did not enter the cells, this finding argues against a direct proteolytic cleavage of \(\alpha\)- and \(\beta\)-ENaC by aprotinin-sensitive serine proteases located at the cell surface. However, it does not exclude intracellular proteolytic processing of \(\alpha\)-ENaC subunit or surface proteolytic cleavage by a protease insensitive to aprotinin. Moreover, endogenous serine proteases might specifically cleave the \(\gamma\)-ENaC subunit as previously suggested by Masilamani et al. (23).

Which serine proteases could be involved in the regulation of Na\(^{+}\) transport across the alveolar epithelium? Donaldson et al. (11) first proposed that prostasin, the human homolog of mCAP1, was a likely candidate for protease-mediated regulation of Na\(^{+}\) absorption in human lung, at least in the proximal airways. Accordingly, it was recently reported that silencing the expression of prostasin with short interfering RNA led to a marked decrease in ENaC-mediated Na\(^{+}\) currents across the human immortalized nasal epithelial cell line JME/CF15 (33). In addition, the transmembrane type II serine proteases mCAP2 and mCAP3 activating ENaC in Xenopus oocytes have been shown to be expressed at the mRNA level in total mouse lung extracts (37). In this study, analysis of the distribution of CAPs in mouse distal lung cells revealed that mCAP2 and mCAP3 mRNAs were specifically expressed in BAEC, whereas mCAP1 mRNA was detected in BAEC and also in nonepithelial cells (mostly alveolar macrophages) recovered by BAL. The expression of CAP1 was also confirmed at the protein level in rat AEC as previously reported (36), as well as in mouse BAEC and mouse BAL cells. Immunoblotting and deglycosylation experiments using polyclonal rabbit antibodies raised against either the COOH or the NH\(_2\) terminus of mCAP1 suggested that mCAP1 protein underwent two N-glycosylations during its maturation process in mouse BAEC. Indeed, one interesting finding was that mCAP1 protein was also detectable in BAL fluid in a mature fully glycosylated form. The fact that CAP1 protein was also found in culture medium from rat AEC in primary culture (data not shown) strongly suggests that CAP1 in BAL fluid originates at least in part from AEC but does not exclude additional secretion by alveolar macrophages. If the secreted form is enzymatically active as previously shown for human prostasin (7), then mCAP1 may act as an autocrine but also as a paracrine modulator of Na\(^{+}\) absorption in the epithelial lining fluid, able for instance to activate ENaC in alveolar type I cells covering most of the surface of the alveolus.

So far, the role of endogenous serine proteases in the regulation of Na\(^{+}\) absorption in vivo has not been clearly established, at least in the kidney or the lung. Using an in situ mouse lung model, we examined the effect of endogenous/exogenous serine proteases on Na\(^{+}\)-driven AFC in vivo. We found that aprotinin and trypsin did not modify AFC under baseline conditions but that they clearly modulated AFC stim-
ulation by the β2-agonist terbutaline. The fact that aprotinin did not change baseline AFC could be accounted for by different explanations. It could be due to the presence in the alveolar lining fluid of endogenous serine protease inhibitors such as bikunin, a Kunitz-type inhibitor of CAP1 expressed in the lung and detected in bronchial mucus (2, 22). For example, it was recently reported that the proteolytic activity of prostasin was inhibited when bound to a prostasin-binding protein present in seminal vesicle fluid (7). However, this explanation seems unlikely since baseline AFC was not significantly modified by excess trypsin, which should circumvent potential AFC inhibition due to endogenous serine protease inhibitors. Considering the slow time course of aprotinin in vitro, it is most likely that the in situ lung model (13) measuring AFC over a short period (15 min) was not appropriate to detect a delayed inhibition by aprotinin in vivo. Thus the lack of effect of aprotinin on baseline AFC in this model should be considered with caution. By contrast, our data indicate that aprotinin abolished terbutaline-induced stimulation of AFC, whereas excess trypsin potentiated it. These in vivo data in mouse are in line with those obtained in vitro in rat AEC showing that aprotinin suppressed the L9 stimulation induced by terbutaline. Considering that β2-agonists are known to increase the turnover rate of ENaC at the cell surface (8, 25, 28, 32), they regulate transepithelial alveolar Na+ transport both in vitro and in vivo in rodent lung. Although other known or unknown serine proteases might also be involved, endogenously expressed CAPs constitute good candidates for this regulation. Of particular interest is CAP1, abundantly expressed by both epithelial and nonepithelial alveolar cells and secreted in the epithelial lining fluid, which could act as a paracrine regulator of ENaC activity in the alveolus. The involvement of CAP1, CAP2, or CAP3 in this process remains to be specifically established. Also, the regulation of endogenous serine protease activity under normal or pathological conditions needs to be further clarified. Specific conditional knockouts of CAP1, 2, or 3 targeting the distal lung epithelium should help to address these issues in the near future.

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