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Coupling of epithelial Na^+ and Cl^- channels by direct and indirect activation by serine proteases

Veronika Gondzik,[1,2] Wolf Michael Weber,[2] and Mouhamed S. Awayda[1]

[1]*Department of Physiology and Biophysics, State University of New York at Buffalo, Buffalo, New York; and* [2]*Institute of Animal Physiology, Westphalian Wilhelms University, Munster, Germany*

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Gondzik V, Weber WM, Awayda MS. Coupling of epithelial Na^+ and Cl^- channels by direct and indirect activation by serine proteases. *Am J Physiol Cell Physiol* 303: C936–C946, 2012. First published August 22, 2012; doi:10.1152/ajpcell.00395.2011.—The mammalian collecting duct (CD) is continuously exposed to urinary proteases. The CD expresses an epithelial Na^+ channel (ENaC) that is activated after cleavage by serine proteases. ENaC also exists at the plasma membrane in the uncleaved form, rendering activation by extracellular proteases an important mechanism for regulating Na^+ transport. Many exogenous and a small number of endogenous extracellular serine proteases have been shown to activate the channel. Recently, kallikrein 1 (KLK1) was shown to increase γENaC cleavage in the native CD indicating a possible direct role of this endogenous protease in Na^+ homeostasis. To explore this process, we examined the coordinated effect of this protease on Na^+ and Cl^- transport in a polarized renal epithelial cell line (Madin-Darby canine kidney). We also examined the role of native urinary proteases in this process. Short-circuit current (I_{sc}) was used to measure transport of these ions. The I_{sc} exhibited an ENaC-dependent Na^+ component that was amiloride blockable and a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl^- component that was blocked by inhibitor 172. Apical application of trypsin, an exogenous S1 serine protease, activated I_{ENaC} but was without effects on I_{CFTR}. Subtilisin an exogenous S8 protease that mimics endogenous furin-type proteases activated both currents. A similar activation was also observed with KLK1 and native rat urinary proteases. Activation with urinary proteases occurred within minutes and at protease concentrations similar to those in the CD indicating physiological significance of this process. ENaC activation was irreversible and mediated by enhanced cleavage of γENaC. The activation of CFTR was indirect and likely dependent on activation of an endogenous apical membrane protease receptor. Collectively, these data demonstrate coordinated stimulation of separate Na^+ and Cl^- transport pathways in renal epithelia by extracellular luminal proteases. They also indicate that baseline urinary proteolytic activity is sufficient to modify Na^+ and Cl^- transport in these epithelia.

chloride transport; serine protease; sodium transport; kallikrein

IT IS NOW WELL ESTABLISHED that activity of the epithelial Na^+ channel (ENaC) is proteolysis dependent (2, 19, 21, 23, 37) . Proteolysis occurs during the normal cellular biosynthetic pathway as well as at the plasma membrane and is limited to the α- and γ-subunits of ENaC (21, 24, 25). This process creates two ENaC pools at the membrane: processed and unprocessed, with the idea that extracellular processing can lead to channel activation at the plasma membrane. This has been borne out by numerous studies, the majority of which utilized either heterologous expression systems or surrogate proteases not necessarily native to ENaC expressing epithelia (1, 6, 12, 19, 21, 25, 26). With the use of this approach, many proteases have been demonstrated to activate ENaC expressed in *Xenopus* oocytes. These include trypsin, chymotrysin, prostasin, plasmin, and elastase.

Recently, a role of the native and ubiquitously expressed serine protease kallikrein 1 (KLK1) in the activation of ENaC has been described previously (38). In these experiments, KLK1 was proposed to endogenously cleave γENaC. KLK1-deficient mice exhibited only the full-length uncleaved γ as determined by Western blotting. Moreover, intracellular Na^+ concentration was elevated in KLK1-microperfused collecting ducts (CDs) indicating stimulation of Na^+ entry. These results demonstrate a role of this protease in ENaC activation. However, they do not provide a time course of this activation. They also do not address if this effect is observed with native urinary proteases and if this response is solely due to activation of ENaC with no changes to Cl^- transport, a necessity to observe coordinated changes of NaCl (salt) absorption.

The effect of ENaC activating proteases on Cl^- transport is an important process, as it is now emerging that the CD contains a cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (9, 29). In this case, activation by luminal proteases of a Na^+ pathway in the absence of effects on Cl^- would limit the overall transport of Na^+ and Cl^- in the CD to the endogenous rate of Cl^- transport, be it cellular or paracellular. As the effect of ENaC activating proteases on Cl^- channels and specifically CFTR is undetermined, we examined this process in the current work.

To better address the acute role of ENaC activation by proteases to overall epithelial Na^+ transport, we utilized polarized Madin-Darby canine kidney (MDCK) cells. We have recently described the procedures for stable and prolonged recordings from these cells (17), a necessity for examining acute effects of proteolytic activation of ENaC. Using these cells, we examined the role of urinary proteases in ENaC activation. ENaC as expressed in the CD of the kidney is routinely exposed to such proteases, and it is uncertain whether proteolytic activity in the urine exists at sufficient concentrations to alter channel function. MDCK cells also contain an endogenous apical CFTR Cl^- channel (32, 45). Therefore, we examined the extracellular proteolytic regulation of both Na^+ and Cl^- channels. This allowed us to assess the coordinated regulation of both channel pathways in response to urinary-space present proteases.

To assess the acute effects of extracellular proteases on Na^+ and Cl^- transport, we utilized polarized MDCK cells. These cells stably express tagged ENaC subunits and have been previously used by others to examine ENaC regulation (28). ENaC activity was measured as the amiloride-sensitive short-

Address for reprint requests and other correspondence: M. S. Awayda, Dept. of Physiology and Biophysics, SUNY at Buffalo, 124 Sherman Hall, 3435 Main St., Buffalo NY 14214 (e-mail: awayda@buffalo.edu).

circuit current (I_{ENaC}). These cells have also been described to express an endogenous CFTR Cl^- channel (32, 45), and CFTR activity was measured in the presence of amiloride as the current inhibited by CFTR inhibitor 172 (I_{CFTR}). These effects were examined in cells that have not been pretreated a protease inhibitor, as such treatment would modify many other cellular functions that are dependent on endoplasmic reticulum/Golgi proteolysis. Trypsin, an S1 protease, cleaved and activated ENaC but was without effects on CFTR. On the other hand, KLK1, rat urinary proteases and subtilisin activated both I_{ENaC} and I_{CFTR}. The effects on ENaC were cleavage mediated and irreversible. The effects on CFTR were indirect and mediated by an apical protease activated receptor. These results demonstrate coordinated activation of separate Na^+ and Cl^- channels in CD cells by extracellular proteases. They also demonstrate the physiological significance of this process in regulating Na^+ Cl^- transport and that urinary proteases exist in vivo at sufficiently activity to allow this stimulation. The urinary and ubiquitously expressed serine protease KLK1 was also found to stimulate both channels. All together, these data indicate that direct and indirect activation by luminal proteases can lead to coordinated activation of NaCl transport in renal epithelia and possibly in a variety of other cell types given the ubiquitous expression of kallikreins.

MATERIALS AND METHODS

MDCK cells. Canine kidney cell lines expressing ENaC were obtained from Daniela Rotin (University of Toronto) and were grown as previously described (17, 28, 45) Briefly, cells were grown in DMEM (GIBCO, Grand Island, NY) containing 10% FBS, 10% sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air. Cells were grown to confluency and subcultured by passaging after trypsinization. For experimental use, cells were seeded on 12-mm Transwell tissue culture inserts (Corning Scientific, Corning, NY). Cells were seeded at defined density (~5% confluency) and were used within 4–5 days of developing stable voltages and resistances. This particular subclone of MDCK cells (sometimes referred to as MDCK-C7) is known to form high-resistance monolayers and has been used by various other investigators (15, 28, 32–34, 45) to examine Na^+ or Cl^- transport.

Solutions and chemicals. Reagents were obtained from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific (Waltham, MA) and were of the highest purity available. Subtilisin, trypsin, and elastase were also obtained from Sigma. KLK1 was obtained from 3H Biomedical (Uppsala, Sweden). KLK1 exhibited an activity of 5 U/mg, where 1 U was defined as that which could hydrolyze 1 μM of peptide substrate/min. Amiloride was a gift of Merck Laboratories (Rahway, NJ), and CFTR inhibitor 172 was obtained from Robert Bridges (Rosalind Franklin University) through the Cystic Fibrosis Foundation (Bethesda, MD). Rat urine was obtained from rats housed in modified metabolic chambers that allowed cooling of the collected urine to 2°C (described in Ref. 2). To eliminate carryover of osmolytes and to select for the urinary protein fraction, collected urine was concentrated 100-fold in a 3-kDa cutoff Millipore centrifugal filtration unit (Millipore, Billerica, MA). The concentrated urine was then used at 1:1,000 dilution yielding a final proteolytic activity of ~10% of that found in the original rat urine. To inactivate urine, samples were heated at 85°C for 15 min. Inactivation was verified as a >80% decrease of proteolytic activity utilizing the amidolytic assay and the two γENaC peptides RKRR and RKRK.

Ussing chambers. Chambers for mounting polarized cultured tissue were obtained from Physiologic Instruments (model P2300; San Diego, CA). Chambers were modified as previously described (17). The recording solution consisted of 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 24 mM $NaCO_3$ equilibrated at 37°C with 5% CO_2-95% O_2. When mounted in chambers, tissues were short circuited using Ag/AgCl agar electrodes. Electrodes were constructed using 3 M NaCl. Agar electrodes were made in batches and were discarded after 4 wk. Tissue clamping and measurements of resistance and current were carried out using a two-channel clamping hardware and software system from EP Devices (Bertem, Belgium).

Surface biotinylation and Western blotting. Confluent monolayers were grown on 24-mm Transwell tissue culture inserts (Corning Scientific). Cells were washed in ice-cold PBS-CM (PBS with calcium and magnesium to maintain monolayer integrity) and incubated for 45 min with 1.5 mg/ml NHS-SS-biotin (Pierce Chemical, Rockford, IL), at 0–4°C. After biotinylation, cells were washed and then incubated for 10 min at 0–4°C with quenching buffer containing 192 mM glycine, 25 mM Tris in PBS-CM. After multiple wash steps, cells were scraped in lysis buffer containing 1% Triton and 150 mM NaCl, 50 mM HEPES, 5 mM EDTA, and a protease inhibitor cocktail (Pierce Chemical). Cells were homogenized by a Polytron homogenizer (Brinkmann Instruments, Rexdale, Canada), followed by sonication with a Sonic Dismembrator (Fisher Scientific). Cell debris were pelleted, and an aliquot was saved as the total fraction. The supernatants were then incubated overnight with streptavidin beads. Biotinylated proteins were then pulled down and eluted into SDS-sample buffer. This fraction constituted the plasma membrane fraction. Denaturing/reducing electrophoresis and Western blotting were carried out as previously described (2). Rabbit anti-γENaC was used at a dilution of 1:1,000 followed by the appropriate horseradish peroxidase-conjugated secondary antibody as previously described (2). Enhanced chemiluminescence was carried out using Super Signal Dura West ECL reagent (Pierce Chemical). Images were digitally acquired using BioRad (Hercules, CA) Versa Doc 4,000. All image analyses were carried out on unattenuated raw images using software supplied with the imager.

Amidolytic assay. Urinary proteases were assessed for their ability to cleave two peptides with sequences generated from the identified cleavage sites on γENaC. The assay has been previously described (2) and utilized the sequences RKRR and RKRK. Cleavage was determined by increased fluorescence after cleaving the COOH-terminal methyl coumarin molecule and was measured using a 96-well plate fluorimeter (BioTek Instruments, Winooski VT).

Statistical significance was determined using Student's *t*-test at the 95% confidence level.

RESULTS

Polarized MDCK cells were examined for effects of proteases on ENaC and CFTR activities. Cells were studied in the absence of prior treatment with protease inhibitors. The changes observed with no protease inhibitor treatment are smaller than those observed with prior protease inhibition and require a stable and reproducible amiloride-sensitive short-circuit current (I_{sc}) to be resolved. We followed our recently established methods that allow the stable and reproducible measurements of the I_{sc}. An example of this is shown in Fig. 1 examining changes of the amiloride-sensitive ENaC currents (I_{ENaC}). The stable baseline allows better resolution of the I_{sc} where we can examine the effects of acute proteolysis in the absence of overnight protease inhibition. Further, effects can be examined in the same tissue resulting in paired values before and after stimulation.

Concerted changes to epithelial salt transport require effects on both Na^+ and Cl^- transport. Emerging evidence indicates that CD cells contain an apical CFTR Cl^- channel (9, 29). Such a channel may therefore serve as a regulated pathway for the cellular movement of counter ions. To our knowledge, this has not been examined in the published literature.

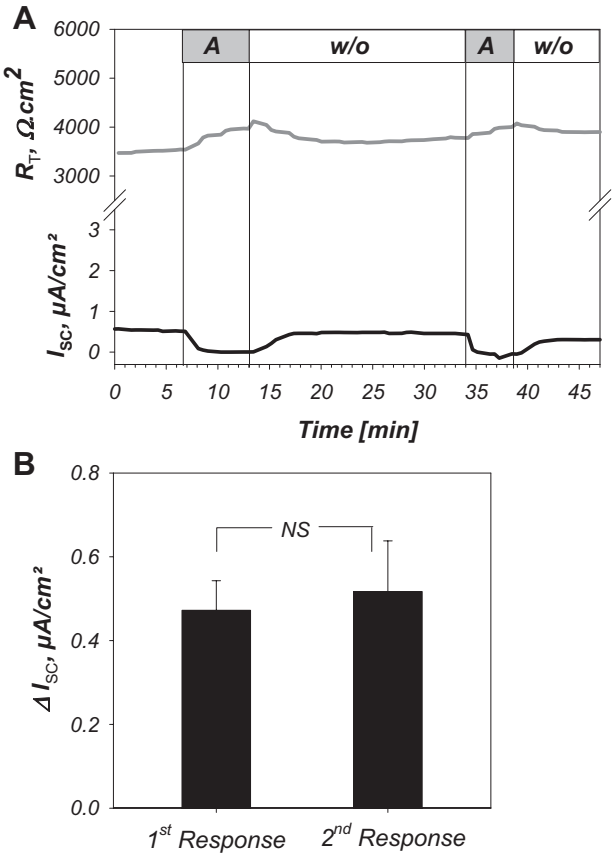

Fig. 1. Representative time-control experiment demonstrating stability and reproducibility of the current and resistance (I_{sc} and R_T). *A*: both I_{sc} and R_T were stable for over 45 min allowing us to use each tissue as its control where feasible. Note the reversible increase in R_T following addition of 10 μM amiloride demonstrating the electrical "tightness" of the tissue. *B*: summary of the amiloride (10 μM)-sensitive I_{sc} as a function of time. A, w/o, and NS indicate amiloride, washout from amiloride, and not significant as determined statistically; $n = 6$.

The effects on ENaC were examined first. In the absence of cAMP stimulation, little to no Cl^- current was observed (see below). This allowed us to examine the effects on ENaC as revealed by the amiloride-sensitive current (I_{ENaC}). In the presence of amiloride, a CFTR current was observed. I_{CFTR} was stimulated by the cAMP activator forskolin and blocked by CFTR inhibitor 172. The kinetics of this current, as well as direction (secretion of Cl^- under short-circuit current conditions), were similar to those described by others for CFTR in MDCK cells (33, 34, 45). Therefore, the effects of CFTR were examined in amiloride-treated forskolin-stimulated tissues and were verified by an effect of inhibitor 172.

Effects of the S1 protease trypsin. Trypsin is a serine protease belonging to the S1 family of proteases. Trypsin cleaves after arginines and lysines and to some extent after histidines. Therefore, it serves as a general tool for examining the effects of cleavage. Trypsin is a known activator of endogenous and heterologously expressed ENaC through mechanisms involving channel subunit cleavage. As shown in Fig. 2, 10 μg/ml trypsin stimulated the I_{sc} and increased I_{ENaC} by ~1.7-fold. This occurred in the absence of major changes to resistance (not shown) indicating the absence of changes to epithelial integrity.

The effects of trypsin on CFTR are shown in Fig. 3. In the presence of amiloride, trypsin caused a small change of the I_{sc}, which was not sensitive to inhibitor 172. This indicates that CFTR is not directly affected by extracellular proteolytic cleavage by this serine protease.

Effects of the S8 protease subtilisin. We (21) have previously used the S8 protease subtilisin to cleave and activate ENaC. The advantage of this protease is increased selectivity over trypsin and similarity of the actions of this protease to other endogenous S8 proteases such as furin and kallikreins. Subtilisin stimulated I_{ENaC} (Fig. 4). The mean increase was ~4.3-fold. This effect was irreversible, consistent with channel proteolysis, and was observed when added to the apical side. We have previously demonstrated that subtilisin can activate ENaC expressed in *Xenopus* oocytes, and the effect in MDCK confirms that this stimulation in expression system independent, as would be expected from an extracellular proteolytic enzyme.

The effect of subtilisin on CFTR is shown in Fig. 5. Subtilisin caused an initial rapid phase of stimulation accompanied by a rebound leading to a smaller but sustained increase, similar to the effect of forskolin on CFTR. These effects were predominantly blocked in the presence of inhibitor 172, demonstrating the nature of the current. On average, subtilisin caused a nine-

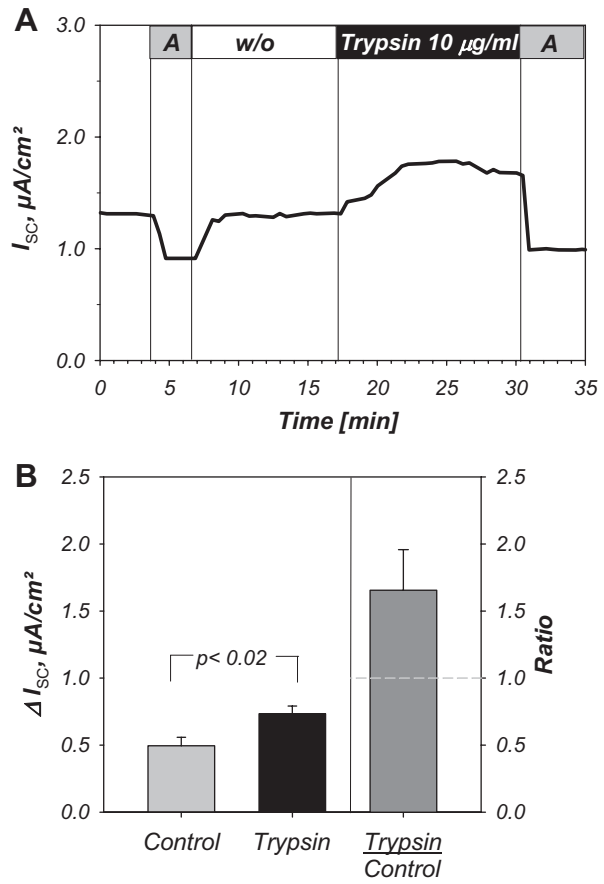

Fig. 2. Trypsin stimulates the amiloride sensitive current. *A*: representative example demonstrating acute stimulation of amiloride-sensitive short-circuit current (I_{ENaC}) by 10 μg/ml trypsin. Effects of apical trypsin on the I_{ENaC} are summarized in *B*. Ratio indicates the value of the amiloride sensitive current after trypsin to that before trypsin. NS indicates not statistically significant; A and w/o indicate apical amiloride and washout from amiloride; $n = 6$.

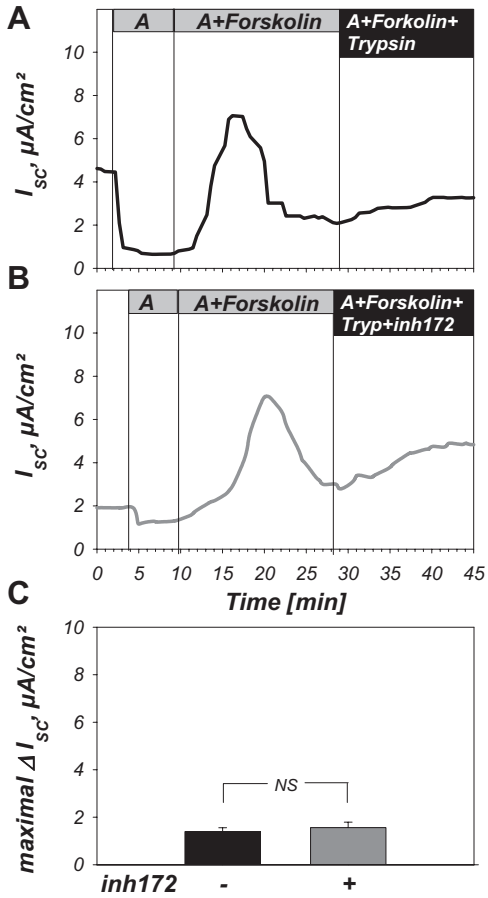

Fig. 3. Forskolin stimulates a cystic fibrosis transmembrane conductance regulator (CFTR) current that is trypsin insensitive. *A*: representative example demonstrating acute stimulation of an amiloride-insensitive current by 5 μM forskolin (I_{CFTR}). Apical trypsin was without effect on this current. *B*: representative example demonstrating the absence of an effect of 10 μM CFTR inhibitor 172 (inh_{172}) on the trypsin response. *C*: summary of the effects of 10 μg/ml trypsin on the current observed in the presence of amiloride and forskolin. No difference were observed with and without inh_{172} indicating the absence of changes to I_{CFTR} with trypsin. NS indicates not statistically significant; A indicates apical amiloride; Tryp + inh_{172} indicates apical trypsin and CFTR inhibitor 172; $n = 6$ in each group.

and twofold stimulation of the peak and sustained responses, respectively. These data demonstrate an effect of an ENaC activating protease on CFTR, providing a coupling that could lead to concerted NaCl absorption in this cell line and potentially in the CD (see DISCUSSION).

Effects of rat urinary proteases. The above data indicate activation of ENaC and CFTR by exogenous extracellular proteases in renal epithelial cells; however, it is unknown if proteases exist in vivo at sufficient levels to activate these channels. To address this issue, we examined the effect of urinary proteases. We chose urinary proteases for two reasons. First, we (2) have recently demonstrated that proteolytic activity in the rat urine is preserved and that such proteases can cleave synthetic peptides with sequences from γENaC (also see Fig. 11). Second, owing to the presence of ENaC in the luminal membrane of the CD, a final stage in the nephron, it is normally subjected to urinary proteases at concentrations similar to those found in the final urine (given the small volume absorption in the CD as a percent of the initial filtrate).

Rat urinary proteases activated ENaC similar to the effect observed with both trypsin and subtilisin (Fig. 6). A 1/10th dilution of the normal rat urinary protease concentration led to a 2.6-fold increase of I_{ENaC}. This effect was abolished by protease inactivation by heat and was absent when applied from the basolateral side. This indicates that the observed effect required catalytic activity of an enzyme, presumably a protease (see below), and rules out a stimulation by a lipid soluble molecule contained in the rat urine.

The effects of urinary protease on CFTR are shown in Fig. 7. Similar to subtilisin, native urinary proteases caused a biphasic stimulation of I_{CFTR}, which was markedly attenuated in the presence of inhibitor 172. The overall effect was smaller in magnitude at both the peak and sustained phases than that observed with subtilisin (see Fig. 5). However, ENaC was also activated to a lower extent by this concentration of urinary proteases compared with purified subtilisin. Therefore, it is likely that this dilution of urinary proteases was of lower activity than the concentration of subtilisin used to activate ENaC and CFTR.

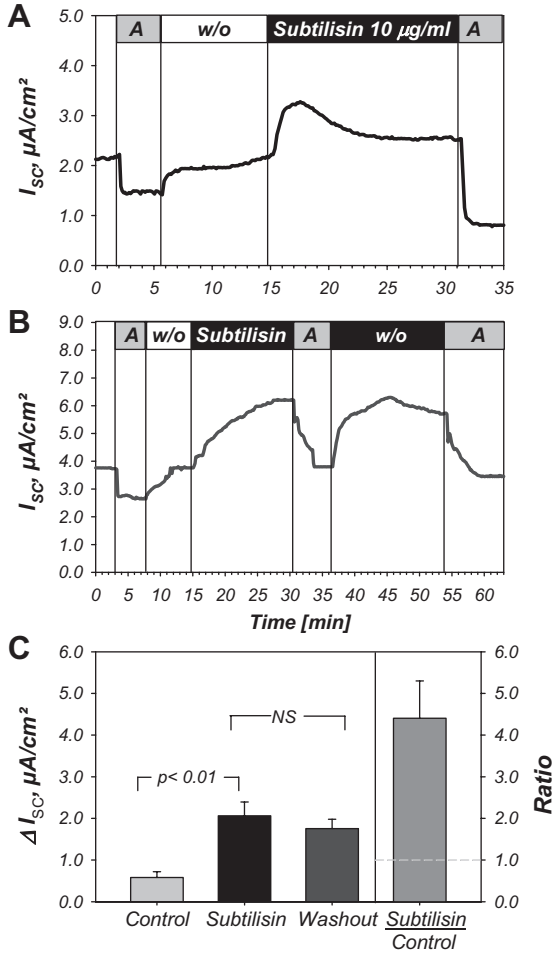

Fig. 4. Subtilisin irreversibly stimulates ENaC. *A*: S8 protease subtilisin stimulated the amiloride-sensitive I_{sc} (I_{ENaC}). *B*: this effect was irreversible consistent with channel proteolysis. *C*: summary of the effects of 10 μg/ml subtilisin indicating a 4.3-fold stimulation of ENaC by this protease. Ratio indicates the value of the amiloride sensitive current after subtilisin to that before subtilisin. A and w/o indicate apical amiloride and washout from amiloride in *A* and washout of amiloride and subtilisin in *B*; $n = 6$ in each group.

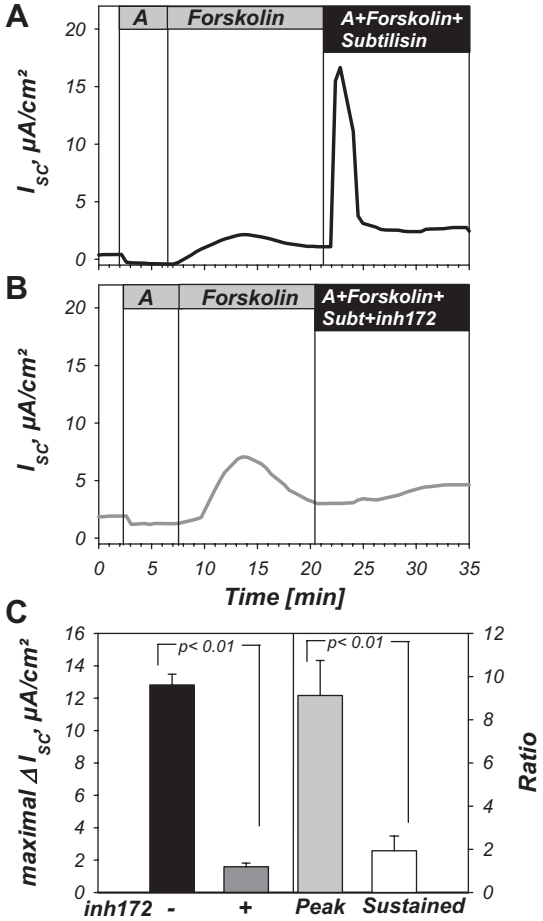

Fig. 5. Subtilisin stimulated I_{CFTR}. *A*: representative example demonstrating acute stimulation of I_{CFTR} by 10 μM subtilisin. Similar to stimulation with forskolin, this effect was biphasic with a large initial peak followed by a smaller sustained stimulation. *B*: stimulation of I_{sc} was prevented in the presence of 10 μM CFTR inh_{172} demonstrating that both the initial peak and sustained responses were due to CFTR. *C*: summary of the changes of the amiloride insensitive current indicating inhibition of the peak stimulation by inh_{172}. Ratio indicates the value of the amiloride-insensitive current before and after subtilisin demonstrating stimulation of both the peak and sustained responses by subtilisin. A indicates apical amiloride; subt + inh_{172} indicates apical subtilisin and CFTR inhibitor 172; $n = 6$ in each group.

Irrespective of the exact activities, our data demonstrate that native proteases can exhibit a significant effect on both I_{ENaC} and I_{CFTR} at ~10% of their final concentration in rat urine, and that this process of activation can lead to physiologically relevant changes of NaCl transport.

Effects of KLK1. Given the effects of KLK1 knockout on renal Na^+ handling (38) and the presence of this protease in the renal urinary proteome (41, 44), as well as our own unpublished observations confirming this finding, we examined the effect of this serine protease on Na^+ and Cl^- transport. The effects of apical KLK1 on ENaC are shown in Fig. 8. Treatment with KLK1 stimulated the I_{ENaC} by 2.6-fold and was similar to the stimulation observed with native rat urinary proteases and subtilisin. Moreover, subtilisin was without additional effects in KLK1 stimulated cells indicating a common mechanism of activation of I_{ENaC}. The effect of KLK1 was also irreversible indicating channel proteolysis (not shown).

The effects of KLK1 on CFTR are shown in Fig. 9. At a concentration of 8 μg/ml, KLK1 caused a biphasic stimulation of I_{CFTR} with a peak stimulation of sevenfold and a sustained stimulation of twofold. This effect was similar to that observed with subtilisin and absent in the presence of inhibitor 172.

The above data indicate that KLK1, which is also known as tissue kallikrein given its widespread expression, is capable of coupling a stimulation of both Na^+ and Cl^- transport. These data also indicate that the effects observed in the KLK1 knockout mice are likely due, at least in part, to the absence of the activation of both Na^+ and Cl^- transport observed in Figs. 8 and 9.

Mechanisms of activation. We and others have previously reported a critical role of γ ENaC cleavage in activating the channel in vivo. The role of γENaC in the stimulations observed above is shown in Fig. 10. The γ migrated at ~90 kDa in the uncleaved and at ~ 65–70 kDa in the cleaved form. The majority of γ at the membrane was already in the cleaved form, as expected given the lack of protease inhibitor pretreatment.

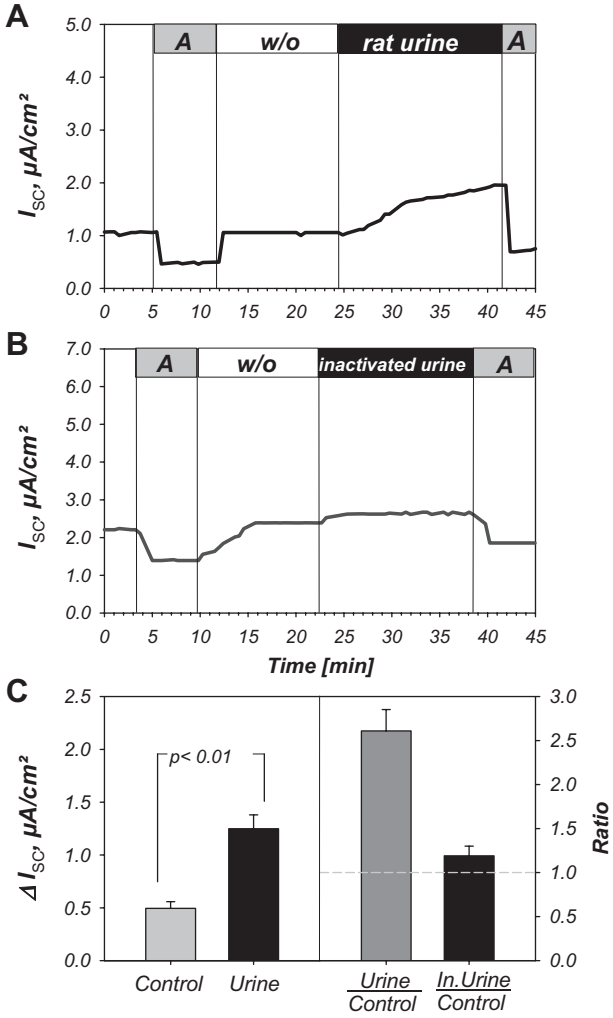

Fig. 6. Stimulation of ENaC by urinary proteases. *A*: representative example demonstrating stimulation of ENaC mediated I_{sc} by rat urinary proteases at a final concentration of 10% of that found in the urine (see MATERIALS AND METHODS). *B*: this effect was absent in heat inactivated urine (inactivated at 85°C for 15 min). *C*: summary data indicate a 2.6-fold increase of I_{ENaC} by rat urinary proteases and a marked attenuation of the response in heat-inactivated urine. A and w/o indicate apical amiloride and washout from amiloride; $n = 6$ in each group.

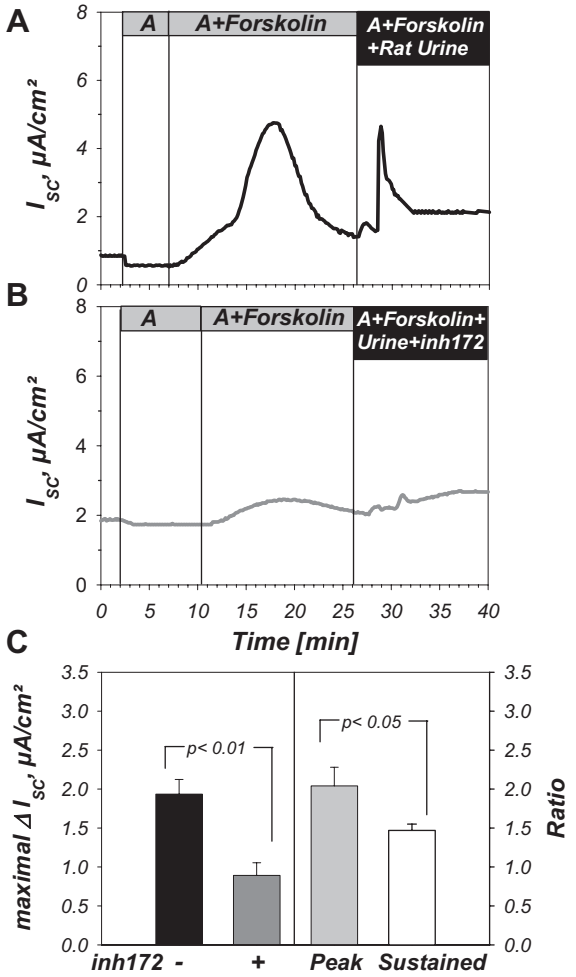

Fig. 7. Stimulation of CFTR by rat urinary proteases. *A*: representative example demonstrating stimulation of CFTR mediated I_{sc} by rat urinary proteases at the same concentration as that which stimulated I_{ENaC}. *B*: this effect was absent in the presence of CFTR inh_{172} demonstrating the nature of the current. *C*: summary of the changes of current indicating significant differences in the presence of inh_{172}. Urinary proteases stimulated both the peak and sustained phases of I_{CFTR}. A indicates apical amiloride; urine + inh_{172} indicates apical rat urinary proteases and CFTR inhibitor 172; $n = 6$ in each group.

No changes were observed in intracellular γ, an expected result as only as small pool of ENaC exist at the membrane and as the short-term treatment with a protease should not affect intracellular processing. To better resolve the changes of processed γ at the membrane, its levels were normalized to the intracellular uncleaved pool. Treatment with any of the stimulatory proteases caused an increase in the levels of cleaved γ at the membrane. All together, these data demonstrate a role of γ in activating ENaC by endogenous and exogenous proteases and more specifically by KLK1.

To further demonstrate γENaC cleavage by KLK1, we tested the ability of this protease to cleave peptides generated from the first and second identified furin/prostasin cleavage sites. These cleavage sites are represented by the peptides RKRR and RKRK, respectively, and utilized a previously described amidolytic assay (2). As shown in Fig. 11, KLK1 exhibited a much higher activity toward RKRR, the known furin cleavage site and little to no activity toward RKRK a putative prostasin site. These data demonstrate site specificity of this protease and that it stimulates Na^+ transport by cleavage of γ ENaC in epithelial cells, as previously postulated in experiments in the native mouse CD (38).

The absence of an effect of trypsin on I_{CFTR} indicates a mechanism that does not involve CFTR proteolysis. Moreover, the similarity of the biphasic stimulation of CFTR, with both forskolin and extracellular proteases, support a role of a potential second messenger system in this process. Given these two observations, a possible mediator of this effect is a class of membrane-bound G-protein-coupled receptors that are activated by cleavage by extracellular proteases [proteinase activated receptors (PARs)].

We examined the effects of two main classes of PARs; PAR1 and PAR2. These results are summarized in Fig. 12. Experiments examined if PAR1 and PAR2 agonists attenuated the subtilisin response. Both human and mouse sequences were used, as these show different degrees of homology with the predicted endogenous canine PAR2 tethered ligand. As shown in Fig. 12, *A* and *B*, pretreatment with human PAR2 tethered ligand did not affect the subsequent stimulation of I_{CFTR} with subtilisin. Similarly, treatment with thrombin or rat PAR2 tethered ligand was without effect on the subtilisin response,

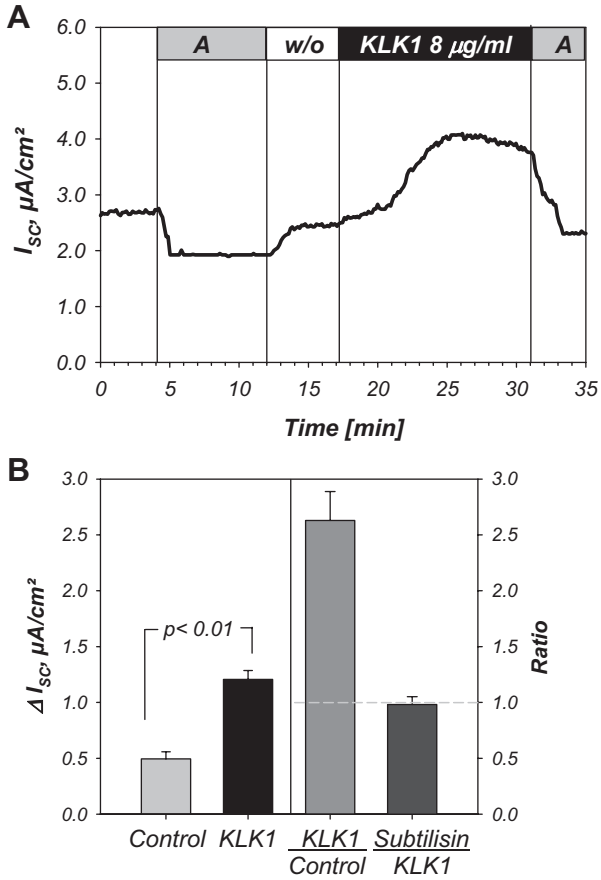

Fig. 8. Kallikrein 1 (KLK1) stimulates ENaC. *A*: time course of the effect of 8 µg/ml KLK1. *B*: summary of the increase of I_{ENaC} with KLK1 which averaged 2.6-fold. No effect of subtilisin was observed following KLK1 treatment indicating overlapping mechanism of channel stimulation by these proteases. Ratio indicates the values of I_{ENaC} in KLK1 to those in control or I_{ENaC} in subtilisin and KLK1 to those in KLK1. A and w/o indicate apical amiloride and washout from amiloride; $n = 6$ in each group.

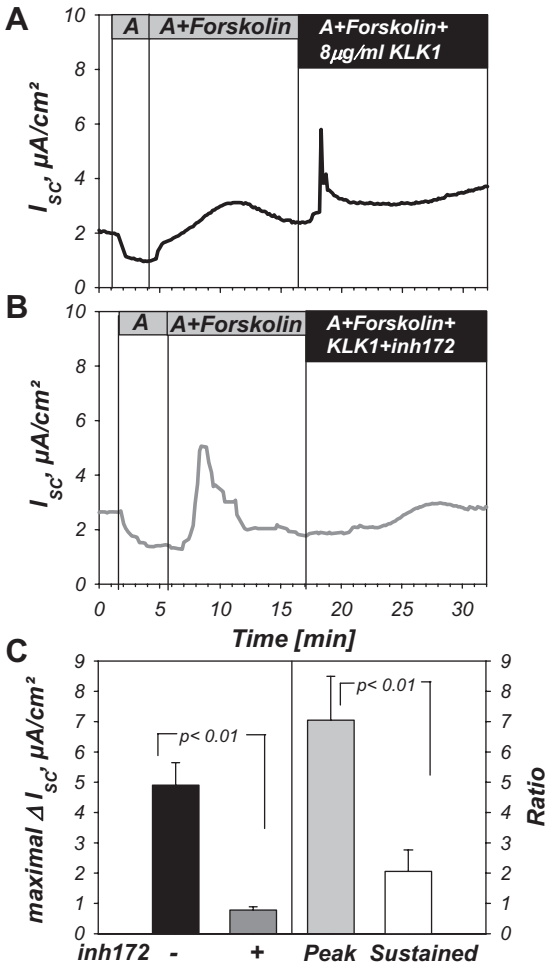

Fig. 9. Stimulation of CFTR by KLK1. *A*: representative example demonstrating biphasic stimulation of current by KLK1. *B*: these changes were absent in the presence of CFTR inh_{172}. *C*: summary of the changes of current indicating a block of this stimulation by inh_{172}, and the CFTR nature of this current. KLK1 caused a peak and sustained stimulation of 7- and 2-fold, respectively. A indicates apical amiloride; KLK1 + inh_{172} indicates apical KLK1 and CFTR inhibitor 172; $n = 6$ in each group.

indicating the absence of a role of both PAR1 and PAR2 in this process (Fig. 12*E*).

The above data do not rule out indirect activation of CFTR by other protease activated receptors. To further test this hypothesis, we examined if the antibiotic neomycin can attenuate this effect. Neomycin has many downstream targets but is an established broad spectrum inhibitor of phospholipase C (PLC), an enzyme that is downstream target of many G-protein-coupled protease activated receptors (3, 31, 42). As shown in Fig. 12, *C*, *D*, and *F*, the effect of subtilisin on CFTR is eliminated by prior treatment with neomycin demonstrating an indirect role of S8 proteases in activating CFTR and a potential role of PLC in this activation.

DISCUSSION

We examined functional effects of extracellular proteases on ENaC and CFTR in polarized MDCK epithelia. We specifically tested if extracellular proteases can provide a mechanism for coordinated activation of both transport pathways. We also tested if these processes are physiologically relevant by determining *1*) if a pool of uncleaved ENaC exists at the membrane, *2*) if fluids that normally bathe this channel (namely urine) contain sufficient proteolytic activity to cause its activation, and *3*) the mechanism of activation of CFTR by these proteases. We report that uncleaved ENaCs at the plasma membrane are amenable to cleavage and stimulation by multiple endogenous and exogenous proteases. These included the S1 and S8 proteases trypsin and subtilisin. The endogenous proteases include KLK1 and rat urinary proteases. We also report that the S8 protease subtilisin and KLK1, as well as native urinary proteases, led to indirect activation of CFTR via an apical receptor that is neomycin sensitive. These results demonstrate a novel mechanism of extracellular protease coupling of Na^+ and Cl^- transport and indicate that such activation can occur at baseline levels of native urinary proteolytic activity.

ENaC cleavage: functional vs. biochemical. It is established that the majority of cellular ENaC is intracellular and that a plasma membrane pool containing both functional and nonfunctional channels is in the range of 5–10% of the total pool (3, 4, 8, 13, 14). Therefore, it is likely that in many instances it would not be feasible to biochemically detect changes of channel cleavage by examining the total pool. The presence of

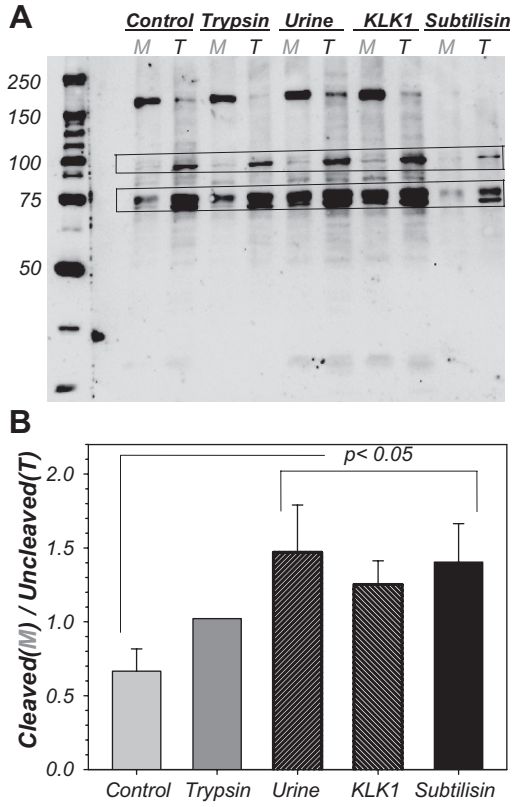

Fig. 10. Enhanced γ ENaC cleavage by activating proteases. *A*: representative blot demonstrating the presence of cleaved and uncleaved γ at both the membrane (M) and total (T) fractions. The majority of this subunit was already in the cleaved form as expected given that intracellular furin mediated cleavage was intact and unblocked. To eliminate variability, cleaved γ levels at the membrane were normalized to total γ levels in the cells and more specifically to total uncleaved γ levels as this represents the maximal pool reactive to proteolysis and activation. All channel activating proteases were accompanied by increased cleaved γ. *Top* and *bottom boxes* denote uncleaved and cleaved forms. Data represent 4 blots. *B*: summary of the changes observed to γ ENaC proteolysis; $n = 3–4$ in each group except for trypsin where $n = 2$.

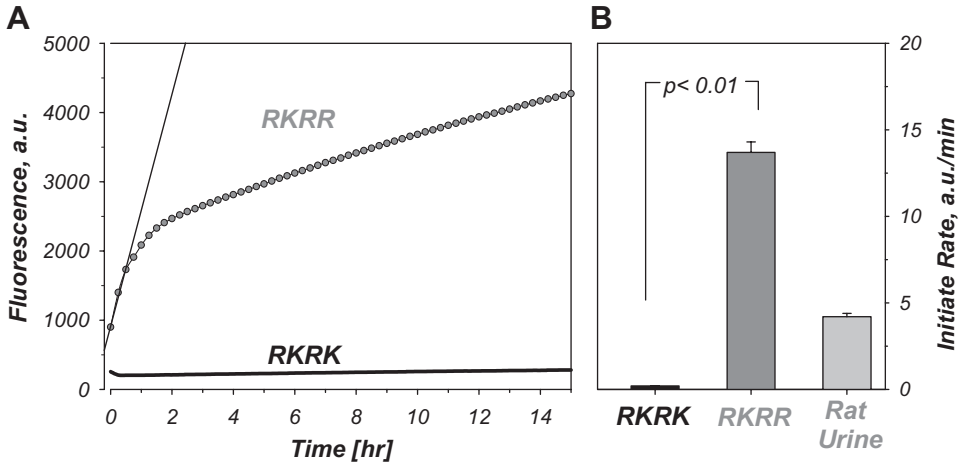

Fig. 11. KLK1 cleaves γ ENaC sequences in vitro. *A*: representative example indicating much higher cleavage activity toward the 1st RKRR site than the 2nd RKRK site. *B*: summary of the reaction rates (calculated as the initial slope shown by the line in *A*) of both peptides in response to KLK1. Final KLK1 levels in both reactions were 1 μg/ml. Rat urine indicates activity toward RKRR at its final diluted concentration as used in Figs. 6 and 7; a.u., arbitrary units; $n = 4$.

functional and nonfunctional channels at the plasma membrane also pose limitations at biochemically detecting changes of channel cleavage and directly correlating these changes with changes of activity or ion transport.

The first limitation is circumvented in systems where the plasma membrane is amenable to labeling by biotinylation. However, the second limitation affects the interpretation of these results as the major assumption is that all channel

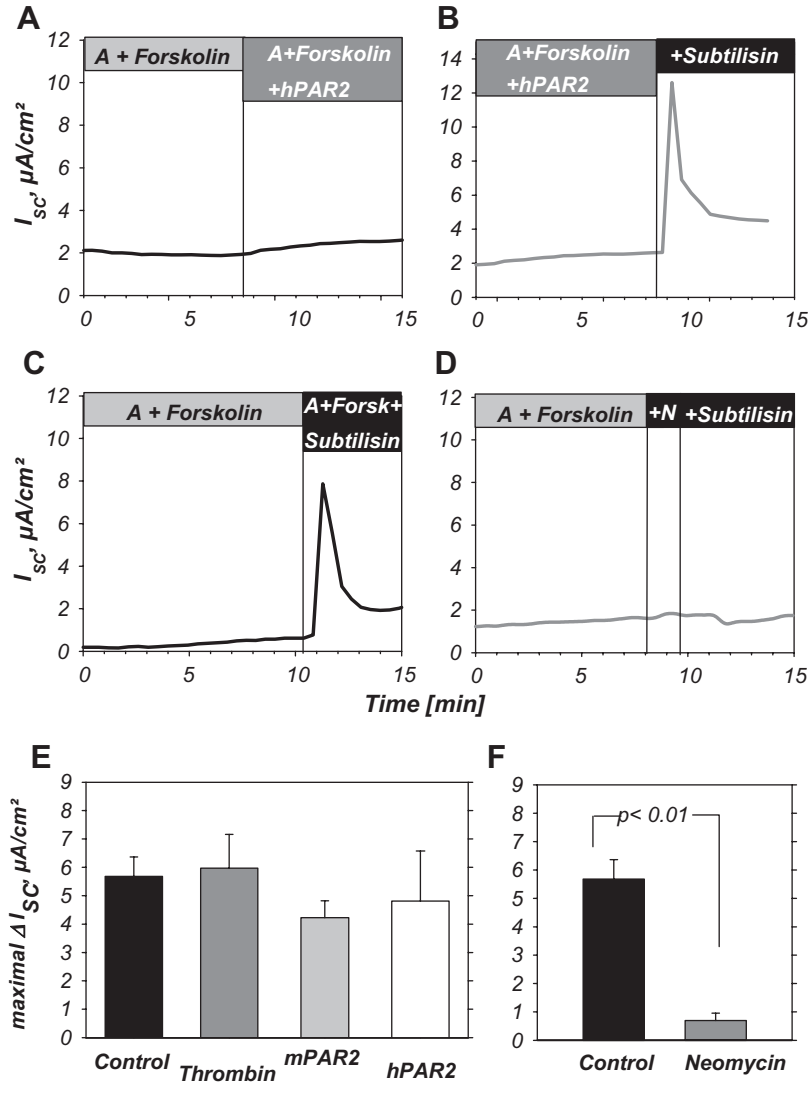

Fig. 12. PAR1 and 2 independent activation of CFTR by subtilisin. *A* and *B*: activation of PAR2 by a peptide with sequences from the human tethered ligand did not modify the subsequent response to subtilisin. A indicates apical amiloride; A+forskolin +hPAR2 indicates forskolin and apical amiloride and human PAR2 tethered ligand; +subtilisin indicates addition of apical subtilisin to A+forskolin+hPAR2. *C* and *D*: neomycin at 1 mM blocked the effects of subtilisin on I_{CFTR}. A indicates apical amiloride; A+forskolin indicates apical amiloride and forskolin; +N indicates addition of neomycin to A+forskolin; +subtilisin indicates addition of apical subtilisin to +N. *E* and *F*: summary data indicating that prior stimulation of PAR1 or PAR2 did not affect the response to subtilisin, while treatment with neomycin markedly attenuated this response; $n = 4–6$ in each group.

proteins at the plasma membrane can form high activity channels after cleavage. This limitation would preclude a direct 1 to 1 correlation between changes of activity and changes of cleaved protein levels. Given this caveat, the results from Western blotting should be limited to provide evidence in support of cleavage rather than as an exact determinant of the amount of cleaved protein.

Urinary proteases. We and other have demonstrated channel activation by exogenous and by a limited set of purified proteases. However, these results do not indicate that endogenous proteases would normally exist at sufficient levels to modify channel activity. This is a critical issue, as it would indicate activation of ENaC and CFTR in the CD by baseline levels of luminal proteases, making this regulatory process physiologically relevant under baseline conditions. Our data demonstrate significance of this process. It is important to keep in mind that these urinary proteases were collected in urine produced by euvolemic and eunatremic rats. Therefore, it is highly likely and indeed possible that rats in other Na^+ balance states would contain different levels of such proteases. Consistent with this, we have recently demonstrated that diet can induce changes to ENaC activating urinary proteases (2). Together, our current and previous data indicate that a change of proteolytic activity is a relevant regulator of cellular ion transport in the CD. It is also important to keep in mind that while the transit time of urine in the nephron is shorter than the exposure times in our experiment, the CD is continuously bathed with urine at different Na^+ content and at different Na^+ balance and, as such, acute changes of proteases may represent a main mechanism of acute control of CD transport function. This remains to be tested with urine collected at shorter time intervals and in response to experimental acute changes of Na^+ balance.

Effects of KLK1. The kinin system of proteases is a ubiquitously utilized system that processes many bioactive molecules. It has also been implicated in numerous physiological and pathological functions that range from normal cell physiology to cancer biology (10, 11, 27, 30, 35, 36). Tissue kallikrein or KLK1, as the name implies, is expressed in tissue as well as found in many biological fluids and has been known for over 100 yr to modify blood pressure, predominantly via changes to the vasculature (for a review see Ref. 10). Of interest is the expression of this enzyme in many epithelia that contains ENaC and CFTR. Of further interest is the expression of this KLK or other related KLKs in nonepithelial preparations, which may also express these channels. Whether other KLKs activate ENaC and CFTR remains to be tested; however, it is known that their substrate preference exhibits a high degree of overlap (18).

Our data indicate that KLK1 indirectly activates CFTR (see *Mechanism of activation of CFTR*) and that it favors cleavage after the first site on γENaC. We (2) have recently demonstrated that γ cleavage in vivo is an important mediator of cholesterol enhanced stimulation of ENaC in the kidney. As with the current report, differences were observed in the ability of urinary proteases to cleave the first and second sites on γENaC. Cholesterol enhanced urinary proteolytic activity toward the RKRK site (second site) with only a transient effect toward RKRR (first site). The first site is the one preferred by KLK1, indicating that the effect observed with cholesterol does not likely involve KLK1. However, our current data implicate this protease in the acute regulation of channel activity and further demonstrate the important role of γENaC in modifying channel activity in vivo.

Irrespective of the cleavage site, our data indicate a novel role of KLK1 in renal Na^+ and Cl^- transport as recently proposed by Picard et al. (38) for Na^+ transport. The stimulation of Na^+ and Cl^- transport by this protease would counteract the vasodilatory effects of this kinin. It is tempting to speculate that the balance of these two opposing effects represent a critical control of blood pressure by KLKs and that tipping the scale toward one or the other could lead to unexplained changes of pressure and hyper- or hypotension. This remains to be tested in human populations.

Mechanism of activation of CFTR. We demonstrate an indirect activation of CFTR by extracellular proteases. In all cases, the observed stimulation was biphasic and similar to that encountered with forskolin and activation of the cAMP pathway. Although we did not find direct evidence for involvement of PAR1 and PAR2, the effects of neomycin indicate a potential role of other PLC-coupled PARs. The response to neomycin may also indicate a role of a Ca^{2+} sensor (CaSR; Ref. 5). In this case, it is also known that both CD and inner medullary CD cells express an apical calcium sensing receptor (7, 40). Further, neomycin and other CaSR agonists may affect both the inositol phosphate and cAMP pathways, which are downstream effectors of CaSR stimulation. Based on the bias between these pathways, a variable response may be observed. It is interesting to note that neomycin has been reported to bias the CaSR response toward inhibition of the cAMP (39, 43). In this case, proteolysis with apical S8 proteases may inactivate CaSR leading to a diminished effect of this receptor on cAMP and activation of CFTR. This hypothesis remains to be tested.

Coordinated activation of ENaC and CFTR. The apical membranes of many epithelia including the mammalian CD express both ENaC and CFTR. The CD also contains an apical K^+ channel (ROMK). This creates a complicated and interregulated system whereby the resting apical membrane potential (E_A) can be driven toward the equilibrium potential for Na^+, Cl^- or K^+ (E_{Na}, E_{Cl}, and E_K), based on degree of activation of these three channels as predicted more than 60 yr ago by Goldman (16) and Hodgkin and Katz (20; GHK). It is well established that activation of apical ENaC drives E_A toward E_{Na} and that this depolarization (intracellular with respect to outside) drives E_A farther away from E_K leading to increased K^+ secretion. Depending on the magnitude of activation of these two channels (ENaC vs. ROMK), E_A could be driven to be either above or below E_{Cl}, driving either absorption or secretion of this counterion. This would create a system where ENaC activation to a greater extent than that of ROMK would drive E_A toward E_{Na} resulting in a membrane potential above (more depolarized) E_{Cl} and the coordinated uptake of both Na^+ and Cl^-. Conversely, ROMK activation to a greater extent than that of ENaC would drive E_A toward E_K resulting in a membrane potential below (more negative) E_{Cl} and the coordinated secretion of both K^+ and Cl^-. In this case, the magnitude and direction of current would follow the following equation derived from Ohm's law:

$$I_{CFTR} = g_{CFTR} \cdot (E_A - E_{Cl^-})$$

where E_A is the apical membrane potential predicted from the GHK equation and I_{CFTR}, g_{CFTR}, and E_{Cl-} are the current and

conductance due to CFTR and the chloride reversal potential. This equation then predicts a reversal of direction of chloride movement based on the absolute value of the algebraic sum of E_A and E_{Cl}. Similar changes could also be predicted by the GHK equation based on changes to the luminal concentration of Na^+ and K^+ resulting in a system where Cl^- would move in the same direction as the main transported ion thereby maintaining electroneutrality.

NOTE ADDED IN PROOF

Since the original submission of this work, Palmer and colleagues (Patel, AB, Chao J, Palmer LG. Tissue kallikrein activation of the epithelial Na channel *Am J Physiol Renal Physiol* 303: F540–F550, 2012) have recently demonstrated activation of ENaC in *Xenopus* oocytes by tissue kallikrein via a mechanism that involves cleavage of the γ-subunit.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.G., W.M.W., and M.S.A. conception and design of research; V.G. and M.S.A. performed experiments; V.G. and M.S.A. analyzed data; V.G., W.M.W., and M.S.A. interpreted results of experiments; V.G. and M.S.A. prepared figures; V.G., W.M.W., and M.S.A. edited and revised manuscript; V.G., W.M.W., and M.S.A. approved final version of manuscript; M.S.A. drafted manuscript.

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