Indirect Activation of the Epithelial Na⁺ Channel by Trypsin

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We tested the hypothesis that the serine protease trypsin can indirectly activate the epithelial Na⁺ channel (ENaC). Experiments were carried out in Xenopus oocytes and examined the effects on the channel formed by all three human ENaC subunits and that formed by Xenopus epsilon and human β and γ subunits (εβγENaC). Low levels of trypsin (1–10 ng/ml) were without effects on the oocyte endogenous conductances and were specifically used to test the effects on ENaC. Addition of 1 ng/ml trypsin for 60 min stimulated the amiloride-sensitive human ENaC conductance (gNa) by ~6-fold. This effect on the gNa was [Na⁺]-independent, thereby ruling out an interaction with channel feedback inhibition by Na⁺. The indirect nature of this activation was confirmed in cell-attached patch clamp experiments with trypsin added to the outside of the pipette. Trypsin was comparatively ineffective at activating εβγENaC, a channel that exhibited a high spontaneous open probability. These observations, in combination with surface binding experiments, indicated that trypsin indirectly activated membrane-resident channels. Activation by trypsin was also dependent on catalytic activity of this protease but was not accompanied by channel subunit proteolysis. Channel activation was dependent on downstream activation of G-proteins and was blocked by G-protein inhibition by injection of guanyl-5'-ylic thio phosphate and by pre-stimulation of phospholipase C. These data indicate a receptor-mediated activation of ENaC by trypsin. This trypsin-activated receptor is distinct from that of protease-activated receptor-2, because the response to trypsin was unaffected by protease-activated receptor-2 overexpression or knockdown.

It is well established that epithelial Na⁺ transport in a variety of cell types is sensitive to the actions of serine proteases. Prior to the cloning of the epithelial Na⁺ channel (ENaC), it was known that apical proteases can degrade apical membrane proteins in toad bladder and inhibit Na⁺ transport. These proteases included trypsin (1) at 1 mg/ml, as well as kallikrein (2, 3).

ENaC was cloned by Canessa et al. in 1993 and 1994 (4, 5). It is now accepted that many ENaC-expressing epithelia, including toad bladder, respond to the actions of a variety of series proteases (6–9). It is now known that lower levels of serine proteases that do not cause channel degradation actually stimulate activity. This activation is observed by a variety of physiological serine proteases (10), exogenous proteases (11), as well as by the co-expression with the serine proteases furin (6) and channel-activating proteases (CAPs) or prostasin (9). Activation of ENaC by exogenous protease or by co-expression with proteases also persists in heterologous expression systems such as Xenopus oocytes; although differences are observed between systems in that many epithelial cells require prolonged prior incubation with a protease inhibitor before observing the effects of exogenous proteases.

The mechanisms of serine protease activation remain only partially elucidated with evidence for and against limited channel cleavage or processing. In general, this has proven difficult to determine, because many of the experimental systems used to examine this mechanism only respond at protease levels close to those initially shown to degrade the channel. Adding to this complexity is the emergence of a family of receptors that are G-protein-coupled, membrane-bound, protease-sensitive receptors that may mediate some of the effects of exogenous proteases (12). Moreover, these receptors also exhibit differences in their sensitivity to exogenous proteases, which are experimental system dependent.

Supporting indirect channel activation is the finding that coexpression of CAP1 or prostasin with ENaC leads to its activation (9) but only when this protein remained glycosylphophatidylinositol-anchored and membrane-bound (13, 14). Similar effects were also observed with trypsin, but only in intact cells (15). In these experiments no evidence of ENaC protein cleavage could be found. More recently these authors also found that catalytically inactive CAP1 was still capable of activating ENaC, however, catalytic activity was necessary for the other two CAP isoforms (CAP2 and CAP3) indicating the presence of more than one mechanism of channel activation (16, 17).

Supporting channel cleavage is the finding that the cellular protease furin activates ENaC accompanied by a shift in the molecular weight of the α and γ subunits (6). Both effects could be abolished by mutation of furin processing consensus sites on these ENaC subunits, indicating that channel cleavage through the biosynthetic pathway leads to its activation. However, these mutations did not affect the response of ENaC to trypsin, and this leaves the possibility that extracellular exogenous proteases may affect ENaC via different mechanisms than the cellular proteases.
endogenous ones. The lung serine protease, elastase, and trypsin were also found to activate membrane resident ENaC in excised patches from airway epithelia indicating the possibility of channel proteolysis (7, 18).

Interestingly, exogenous elastase also stimulated ENaC expressed in oocytes, however, this response occurred in the absence of αENaC cleavage and was accompanied by γENaC cleavage that was different than that observed by normal channel biosynthetic processing (17). Moreover, protease inhibition of elastase did not affect the stimulation of ENaC observed by co-expression with CAP1, further indicating the potential of additional non-cleavage mechanisms for channel activation by exogenous proteases.

Trypsin is known to activate ENaC in oocytes at the same concentration as that used in other systems, but without the requirement for prior treatment with a protease inhibitor. However, these levels of trypsin also affect oocyte endogenous channels and are in reality close to those used to degrade channel protein. Therefore, we opted to carry out our experiments at trypsin levels that we found not to affect the endogenous channels. We report that 1 ng/ml trypsin also activated ENaC, albeit at a slower time course than that observed for the microgram/ml levels. This activation occurred in membrane-resident channels and was independent of channel self- or feedback inhibition by Na⁺. Multiple lines of evidence rule out channel cleavage and indicate a second messenger G-protein-coupled process of activation that is also coupled to membrane phospholipids. This is the first report of channel activation by exogenous protease that rules out channel subunit processing.

**EXPERIMENTAL PROCEDURES**

*Xenopus laevis Oocytes*—Oocytes were prepared as previously described (19). Briefly, surgically removed oocytes were defolliculated in Ca²⁺/-free buffer containing 1 mg/ml collagenase (type 1A, Sigma). After an overnight recovery, defolliculated oocytes were injected with cRNA for the three human ENaC subunits (referred to as αβγENaC or simply ENaC) or for exENaC and βγhENaC (referred to as εβyENaC). These, and all other clones, were inserted into the PGEM-HE oocyte expression vector previously described by Liman et al. (20). This vector contained 49 and 192 nucleotides from the 5′- and 3′-untranslated regions of the *Xenopus β*-globin gene on their respective side of the multicloning site. cRNA was synthesized from linearized plasmids using the T7 RNA polymerase Large Scale Production System (Promega, Madison, WI).

The X. laevis epsilon ENaC subunit, εENaC (accession number AJ440222), was previously described (21). A full-length clone was obtained from Open Biosystems (Huntsville, AL) and subcloned in PGEM-HE. Recordings were carried out 1–3 days after injection. Oocytes used for binding experiments substituted cRNA from an HA-tagged αENaC construct (extracellular loop tag at amino acids 161–169). All ENaC subunit cRNAs were injected at 1–2 ng each.

Oocyte incubation and recording solutions were previously described (19). Briefly, the normal recording solution (ND94) was composed of: 94 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4–7.5. This is referred to as high Na⁺ solution. For the low sodium solution, 89 mM NaCl from the previous solution was replaced by 89 mM N-methyl-d-glucamine-Cl. Experiments in these oocytes were carried out in the same solution as the incubating one. Given the constitutive Na⁺ channel activity in oocytes injected with ENaC, incubation in the low Na⁺ solution effectively clamps the intracellular Na⁺ concentration to about one-half that of extracellular [Na⁺] (22), resulting in an [Na⁺] of ~2.5 mEq in low Na⁺ conditions. Amiloride was obtained from Merck Pharmaceuticals (Rahway, NJ) and was used at 10 μM to inhibit ENaC currents. Purified bovine pancreas type 1 trypsin was obtained from Sigma (~10,000 units/mg). The response of ENaC to this trypsin was verified using sequencing grade and recombinant proteomics grade trypsin (Roche Applied Science).

**GDP Injections**—At the beginning of these experiments oocytes were impaled with two recording electrodes and a third injecting pipette as previously described (22). The injecting pipette was filled with a 10 mM solution of GDPβS. Baseline activity was established for ~20–30 min, followed by intracellular delivery of GDPβS. At the time of injection, ~25 nl of solution containing GDPβS was delivered into the oocyte. Using an average oocyte diameter of 1.1 mm, results in a volume of ~700 nl, or a dilution of ~1:29 and an estimated final concentration of ~340 μM GDPβS. Similar concentrations of this and other GTP analogs have been used by others to block oocyte-endogenous and exogenously expressed G-proteins (23–25). Injection of 25 nl of unrelated solution does not affect the baseline or amiloride-sensitive conductance (26). The injecting electrode was retained until the end of the experiment. As shown under “Results,” amiloride was added at the beginning and end of the experiment. No significant differences in the amiloride-insensitive currents were observed indicating no appreciable leak currents or conductances introduced by this maneuver.

**Surface Expression of ENaC**—Cell surface expression of HA-tagged ENaC was as previously described (27, 28). Briefly, oocytes were divided into groups of 25 oocytes, and anti-HA binding was assessed in each group separately. Each group of 25 oocytes was considered as a single experiment and was obtained from oocytes from separate frogs. Control or ENaC-expressing oocytes were divided into two major groups: untreated and trypsin-treated. Tryptsin treatment was carried out for 60 min at room temperature. At the end of this period, oocytes were washed three times with ice-cold ND-94 then transferred to tubes containing anti-HA antibody coupled to HRP (Roche Applied Science) at a final concentration of 0.357 μg/ml in ND-94, supplemented with 1% bovine serum albumin. Antibody binding was carried out for 2 h on ice. Oocytes were then washed extensively (six times) with ice-cold ND-94. Bound antibodies were indirectly detected by measuring HRP activity using Turbo-TMB HRP substrate (Pierce) according to the manufacturer’s instructions. Assaying the HRP content of the last wash solution allowed us to verify the effectiveness of washes. Experiments were conducted using ENaC-injected and non-injected oocytes to assess nonspecific binding. The ability of the assay to detect changes of surface binding was tested in oocytes injected with varying levels of ENaC cRNA (27).

**Impedance Analysis**—Dual electrode voltage clamp was carried out using TEV-200 oocyte clamp (Dagan Corp., Minneapolis, MN).
olos, MN). Impedance was continuously recorded every 10 s (19). The measurement consisted of five sequential discrete sine wave signals ranging from 55 to 390 Hz. The actual current and voltage signals were digitally acquired and used to calculate membrane impedance and consequently values of membrane capacitance ($C_m$) and conductance ($g_m$).

Patch Clamp Analysis—Conditions were as previously described (26). Briefly, oocytes were shrinked in hypertonic solution, followed by manual removal of the vitelline membrane. Experiments were carried out in the cell-attached patch clamp configuration. Only seals with resistances of $>40$ gigaohms were used. Data acquisition and analyses utilized the suite of DigiData 1322A, Axopatch 20B, and pClamp9 (Axon Instruments, Carson City, CA). Trypsin was added to the external bath after the formation of a seal and establishing baseline channel activity. This utilized a large tip electrode connected to an additional manipulator and delivered $\sim 75\, \mu l$ of ND-94 solution containing 10 ng/ml trypsin onto the surface of the oocyte. Solution delivery rate was controlled by a pressure gradient generated from a Dale 20 Pneumatic transducer (Dale Instruments). Bath solution was ND94, while the electrode solution was a modified ND94 that eliminated K$^+$). The current signal was filtered at 200 Hz and digitally acquired at 1 KHz. ENaC currents were identified from their signature long open and closed times, single channel conductance, current-voltage relation, and voltage signals were digitally acquired and used to calculate capacitance ($C_m$) and conductance ($g_m$).

$\text{PAR-2-independent Activation of ENaC by Trypsin}$

A control morpholino obtained from Gene Tools was verified also without effect. Protein knockdown with the anti-PAR-2 oligonucleotide was also confirmed (Fig. 11 and Table 2) and was used with the anti-PAR-2 oligonucleotide in the ENaC cRNA injection. 50 nl of the morpholino was injected at two different concentrations (final concentrations of $\sim 10\, \mu M$ and $100\, \mu M$) with no difference in their effect. A control morpholino obtained from Gene Tools was also without effect. Protein knockdown with the anti-PAR-2 morpholino was verified $\text{in vivo}$ by examining its effects on PAR-2-HA expression by Western blotting. The antisense morpholino was also verified to inhibit $\text{in vitro}$ translated PAR-2 (data not shown).

$\text{Oocyte Homogenization and Western Blotting}$—Injected oocytes were processed as previously described (28). Briefly, groups of 50–100 oocytes were homogenized at room temperature in 10 $\mu l/oocyte$, in buffer containing 80 mM sucrose, 5 mM MgCl$_2$, 5 mM Na$_2$PO$_4$, 1 mM Tris, pH 7.4, and a protease inhibitor mixture (Sigma-Aldrich). Oocytes were broken by trituration with 25-gauge and 27-gauge syringes. Yolk and nuclei were pelleted by centrifugation at low speed ($100 \times g$) for 5 min. Supernatants were spun at high speed ($14,000 \times g$) for 20 min at 4°C. This soluble fraction did not contain ENaC or PAR-2 and was not further used. The pellet was dissolved in homogenization buffer containing 1% Igepal CA-630 and 0.5% sodium deoxycholate (Sigma-Aldrich) at an equivalent of 2 $\mu l/oocyte$, and spun for 5 min at high speed to obtain the membrane fraction. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

Blots were blocked for 1 h at room temperature in Tris-buffered saline containing 5% nonfat dry milk and 0.1 or 0.05% Tween 20 (TBS-T) for PAR-2 and ENaC, respectively. Blots were then incubated for 1 h in blocking solution containing 50 milliunits/ml of an anti-HA HRP-conjugated antibody (Roche Applied Science), or 0.5 $\mu g/ml$ of each ENaC subunit-specific antibody (Affinity BioReagents, Golden, CO). The ENaC subunit-specific antibodies were generated against the following antigenic peptides with their relative position in the ENaC subunit shown in parentheses: L(20)MKGNKREEQGLGPE-PAAPQQPT(42) for the anti- $\alpha$, N(619)YDSLRLQPLDVIESDSEGDAI(640) for the anti- $\beta$, and L(630)RLERAFSNQLT-DTGMDLDEL(649) for the anti- $\gamma$ antibodies. After five washes, the ENaC blots were incubated for 1 h with an HRP-conjugated secondary antibody at 20 ng/ml (Pierce). Blots were then washed five times, and bound antibodies were visualized by enhanced chemiluminescence Super signal ECL (Pierce).

Unless noted otherwise, data are reported as mean $\pm$ S.E. Statistical significance was determined using Student’s $t$ test at the 99% or 95% confidence levels.

RESULTS

$\text{[Na$^+$]-Independent Activation by Trypsin}$—Trypsin is known to activate the oocyte endogenous Ca$^{2+}$-dependent Cl$^-$ channel at microgram/ml levels (29). This effect, while transient in nature, may set in motion additional downstream effects that also affect ENaC. For this reason, and the fact that these levels of trypsin are close to those previously used to digest sodium transport proteins at the apical membrane of toad bladder (1), we examined the effects of lower levels of trypsin. As shown in Fig. 1A, addition of 1 ng/ml trypsin led to a slow ($\sim 60$ min) but robust increase of ENaC activity. At this concentration, the amiloride-sensitive conductance (a measure of membrane ENaC activity) was increased by 631 $\pm 47%$ ($n = 23$) (Fig. 1B), whereas the comparable untreated time controls increased by 29.6% during the same time period. As shown in Fig. 1A, the amiloride-insensitive component was unchanged before and after trypsin, indicating the absence of effects of this concentration on oocyte endogenous channels, in contrast to that observed at higher levels of trypsin (29–32).

$\text{Statistical significance was determined using Student’s}$ $t$ $\text{test}$ $\text{at}$ $\text{the}$ $99%$ $\text{or}$ $95%$ $\text{confidence levels.}$
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FIGURE 1. Na⁺ concentration-independent activation of ENaC by trypsin. A, addition of trypsin caused a time-dependent activation of ENaC. In most experiments this increase reached a relative plateau within 60 min, however, in others as shown in this example, a small increase was still observed at this time frame. B, summary of the effects of trypsin on the amiloride-sensitive conductance. At 60 min a 6.3-fold increase of \( g_{Na} \) was observed. This effect was blocked by the addition of 20-fold excess soybean trypsin inhibitor either prior to or at the same time as the addition of trypsin (trypsin + STI). C and D, the stimulation with trypsin was independent of the intra- and extracellular [Na⁺]. Amiloride was added before and after trypsin (abbreviated as "A"). The amiloride-insensitive baseline conductance was unchanged, consistent with the absence of effects on oocyte endogenous channels. \( n = 23 \) oocytes from 14 frogs in normal Na⁺, and 9 oocytes from 6 frogs in low Na⁺, \( p < 0.01 \) for both groups.

The observed stimulation with trypsin was similar in magnitude to (and in most cases larger than) that observed by others using three to five orders of magnitude higher trypsin levels (6, 8, 9, 11, 33). As expected this ~6-fold stimulation was slower than that observed by others at the higher trypsin levels or by us at 10 ng/ml trypsin (not shown). As demonstrated below, this concentration is ideal, because it allows us to rule out channel cleavage by exogenous trypsin, and unless otherwise noted all of our experiments were limited to this concentration.

The effects of trypsin on ENaC required proteolytic activity of this serine protease as channel stimulation was blocked by 20-fold excess soybean trypsin inhibitor (see Fig. 1B for summary). These effects also occurred without changes of membrane capacitance \( (C_m) \), which averaged 0.244 ± 0.005 and 0.247 ± 0.005 microfarad \( (n = 39) \) before and after trypsin, respectively. This indicates that trypsin-mediated activation of ENaC does not likely involve effects on membrane trafficking, but rather activation of membrane resident channels, as suggested by others (7, 15). This is further demonstrated below.

To test if the effect of trypsin on ENaC is mediated via a relief of Na⁺ inhibition, as suggested by others (34), we examined this response in oocytes incubated (at the time of injection) in a low Na⁺ solution. This maneuver results in approximately a 20-fold decrease of both intra- and extracellular [Na⁺] (see “Experimental Procedures”). Under these conditions, it is expected that Na⁺ self-inhibition as well as feedback inhibition are markedly reduced (22, 35). Such a procedure is known to increase channel activity, and indeed this effect is evident from the ~2-fold stimulation of the baseline \( g_{Na} \) in the low Na⁺ solution (Fig. 1B and D).

An example of the effect of trypsin in low Na⁺ is shown in Fig. 1C, and the data are summarized in Fig. 1D. A similar increase of \( g_{Na} \) was observed irrespective of the large change in [Na⁺]. This indicates that the response to trypsin is independent of channel regulation by Na⁺. This observation is in contrast to that of others (34, 36) indicating that microgram/ml trypsin levels stimulate ENaC by eliminating the inhibitory effects of elevated [Na⁺]. However, these results are consistent with the observed reduced trypsin response in eENaC-expressing oocytes (see below), because this isoform is believed to exhibit an enhanced self-inhibition response and would otherwise be expected to also exhibit an enhanced trypsin response if trypsin stimulation of the channel was mediated by effects on self inhibition.

No Requirement for Channel Cleavage—The data with soybean trypsin inhibitor indicate the requirement for proteolytic activity. However, it is undetermined if this was limited proteolysis of the ENaC subunits or another membrane protein or receptor by trypsin. This is especially important to determine, because it is at the center of controversy regarding channel stimulation by exogenous protease, and owing to the presence of serine protease, and specifically trypsin, receptors that are oocyte endogenous (31). The requirement for channel proteolysis was tested functionally and biochemically.

To test the functional requirement for channel cleavage, we determined if direct access of trypsin to ENaC is required for channel activation. Experiments were carried out where ENaC protein was protected from trypsin by the formation of a gigahm seal in the cell-attached patch clamp configuration. In these experiments, a 10-fold higher concentration of trypsin was delivered directly onto the oocyte surface in the vicinity of the patch electrode. The volume delivered was ~1/10th that of the chamber volume, resulting in the same final trypsin concentration as the whole cell experiments.

As shown in Fig. 2, application of extracellular trypsin to the outside of the patch pipette caused marked activation of ENaC trapped within the pipette. Channel stimulation was delayed from the time of trypsin addition to the bath and was not observed in its absence, i.e. time controls. These results indicate that catalytic activity of trypsin on ENaC is not required for functional channel activation. Trypsin was also without effects on oocyte endogenous channels consistent with the absence of...
effects on the background amiloride-insensitive whole cell conductances (see Fig. 1).

The example shown in Fig. 2 was chosen for low channel activity. A stimulation of ENaC was observed irrespective of baseline activity prior to the addition of trypsin. However, in membranes with multiple channels and an elevated spontaneous ENaC activity, further stimulation by trypsin made it difficult to accurately identify individual channel levels. Therefore, data were only summarized from membrane patches with low baseline activity.

The effects observed in Fig. 2 underlie the changes of whole cell currents observed in Fig. 1. These effects are in theory attributed to stimulation of the open probability of individual channels ($P_o$), the density of membrane resident channels ($N$), or change of the single channel conductance ($g$). Given the marked change of activity and the presence of multiple additional channels levels after trypsin, it was not possible to properly identify all channel levels and calculate $P_o$, a well known issue for ENaC given the long open and closed times of the channel. Therefore, data were summarized as channel activity or $NP_o$. In the subset of experiments with low baseline $NP_o$, trypsin caused ~20-fold increase of this parameter (Fig. 3A). As evident from the raw data shown in the example in Fig. 2, and the summary in Fig. 3B, this increase of activity was due to an increase in the number of observed channel levels. This indicates that trypsin is either activating membrane-resident silent channels, or causing the insertion of new ones into the membrane. These two possibilities are tested below.

The baseline and trypsin activated current-voltage relationships are shown in Fig. 3 (C and D). A classic property of ENaC, a conductance in the 4-pS range with Na$^+$ as the charge carrier, was observed. These data rule out effects of trypsin on the single channel conductance, leaving the possibility of an effect on channel density or open probability.

To biochemically test if trypsin leads to channel processing, we examined if this protease altered the molecular weight of the three ENaC subunits. Oocytes were exposed to trypsin followed by processing for isolation of the membrane fraction (see “Experimental Procedures”). As shown in Fig. 4 trypsin was without effect on the channel proteins, thereby providing biochemical evidence ruling out channel cleavage as an obligatory process to its stimulation by exogenous trypsin. A caveat of these experiments is

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**FIGURE 2.** Indirect activation of ENaC by trypsin via effects on membrane-resident low activity channels. Cell-attached patch clamp recordings of ENaC in oocytes. Two examples are shown. A, low activity patch clamp recording with one observed ENaC level in the control ND94 period (see expanded scale on the right). Trypsin was added in ND94 solution containing a 10X higher concentration of trypsin (final concentration 1 ng/ml). After addition of trypsin, more than six channel levels are observed (see expanded scale). ENaC levels are marked by the horizontal lines. Note that events were very brief in the control period indicating a low $NP_o$. Longer events are observed after trypsin. Given the large number of ENaCs activated in this patch, these events underestimate the true magnitude of the open and closed times of the channel. In this example, endogenous channel activity was observed during the control period and was unaffected by the addition of trypsin. B, second example of ENaC activation by trypsin. Conditions were the same as in A. This patch did not contain any endogenous channels and exhibited a higher spontaneous $NP_o$ and a lower level of activation by trypsin than that shown in A. A delay in channel activation after adding trypsin is observed in both examples. Holding voltage was −100 mV and an upward deflection represents channel openings. See Fig. 3 for data summary and text for additional details.
that a small amount of plasma membrane-bound ENaC may have been processed by trypsin. In this case it may not be easily detected given the larger amount of ENaC proteins found in intracellular membranes. However, the absence of any shift to the migration of the ENaC proteins, the absence of appearance of any newly detected channel fragments, and the activation in cell attached patch clamp shown in Fig. 2, argue against this possibility.

**Activation of Membrane Resident Channels**—The patch clamp data summarized above indicate activation of silent channels and/or insertion of new ones. Although the absence of an effect on membrane capacitance is consistent with the former possibility, these results alone do not completely rule out additional trafficking effects. To test this hypothesis, surface binding experiments were carried out in the presence and absence of trypsin. This assay utilized an HA-tagged ENaC construct and subsequent binding with an HRP-conjugated anti-HA antibody in intact oocytes (see “Experimental Procedures”). As shown in Fig. 5, antibody binding in control oocytes was negligible, while easily detectable in ENaC-expressing oocytes. This signal was linearly related to ENaC expression as determined by whole cell currents in response to injection of various ENaC cRNA levels (27). Trypsin did not alter either the specific or nonspecific signals. Thus, these results indicate that trypsin increases the activity of membrane resident channels, consistent with the expectation from the absence of changes of $C_m$. This conclusion is consistent with that of others (7, 15) and is further confirmed below.

Stimulation of channel activity with trypsin was observed in both active and low activity/near silent (low $P_o$ or near zero $P_o$) patches. Given that the low $P_o$ patches exhibited a 20-fold increase of activity, while the whole cell conductance increased by 6-fold, it is intuitive that channels with higher activity exhibited a smaller stimulation by trypsin. This leads to the conclusion that the trypsin response is largely mediated via activation of silent channels. This hypothesis is tested below in experiments with ENaC.

**Reduced Activation in ENaC with High Spontaneous $P_o$**—To test the contribution of baseline channel activation to the magnitude of the trypsin response, experiments examined the regulation of $\epsilon\beta\gamma$ENaC. The $\epsilon$ subunit of ENaC was cloned from Xenopus by Babini et al. (21). At the whole cell level, this subunit was found to substitute for $\alpha$ in the formation of a Na$^+$-selective amiloride-sensitive channel. A Blast search revealed that this subunit shares a high degree of homology and appreciable identity with $\alpha$ subunits from both Xenopus and human (64% homology with either

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**FIGURE 3. Trypsin stimulates ENaC activity.** Data summarized as $N_P$, and follow the protocol established in Fig. 2 utilizing a final trypsin concentration of 1 ng/ml. The individual changes are shown in log scale in A while the summary data are shown in linear scale in B. The current-voltage relationship before and after trypsin is shown in C, and the calculated conductance is summarized in D. These data indicate a single channel conductance of $-4.1 \mu$S in Na$^+$ solution. This was unchanged by trypsin. Given that trypsin has no access to ENaC inside the patch pipette, these data indicate indirect activation of the channel by an intracellular process, as concluded from the whole cell data. $n = 12$ oocytes from 5 frogs, $p < 0.01$. See text for additional details.

**FIGURE 4. Absence of ENaC subunit protein processing with exogenous trypsin.** Oocytes were incubated in trypsin (2 ng/ml) for 60 min followed by processing as described under “Experimental Procedures.” Each lane was loaded with the equivalent membrane yield from two oocytes, and the isolated proteins were separated by SDS-PAGE and probed with antibodies specific for each of the ENaC subunits. Both ENaC and control oocytes were probed, and the ENaC-specific bands are enclosed in the white boxes. Note the absence of changes in the intensities of the specific bands and the lack of any additional potential processing fragments.
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ortholog and a 44 and 42% identity with *Xenopus* and human α, respectively).

Our initial experiments revealed that this subunit reconstitutes a channel with a high spontaneous P_o, accompanied by markedly elevated whole cell currents. Babini *et al.* (21) also indicated that this subunit exhibited an enhanced self-inhibition response by Na^+. Thus, experiments with this construct would test two hypotheses: 1) that channels with a high spontaneous P_o would exhibit a blunted trypsin response and 2) that Na^+ inhibition of ENaC does not play a role in the effects of trypsin.

The single channel properties of εβγENaC were undetermined by Babini (21). We utilized cell-attached patch clamp to determine these properties. The data are shown in Fig. 6 and demonstrate that this construct forms a channel with long openings and closings similar to those of αβγENaC. εβγENaC exhibited a high open probability (Fig. 7), consistent with the increased whole cell currents of oocytes expressing this channel. The current-voltage relationship of εβγENaC is shown in Fig. 6C. Linear regression of the I/V relationship between −120 and −60 mV predicts a single channel conductance of 4.2 pS, indistinguishable from that calculated for αβγ ENaC.

The effect of trypsin on εβγENaC is shown in Fig. 7A. Baseline g_Na was higher in these oocytes and averaged 81.0 ± 9.5 μS (n = 14). This elevated baseline conductance is consistent with the observed high activity of this construct. Trypsin increased the g_Na to 112.9 ± 17.8 μS. On a paired basis the g_Na increased by ∼35%, which was much smaller than the ∼630% increase observed with αβγENaC (18-fold difference in the magnitude of whole cell current activation). As summarized in Fig. 7B, this smaller increase is likely due to the elevated spontaneous P_o of εβγENaC, which averaged 0.858 ± 0.046 (n = 16). Moreover, the range of stimulation of the εβγENaC whole cell conductance by trypsin (a 7% decrease to a 2.3-fold increase) corresponded well with the range of spontaneous P_o (0.48 – 0.99), further demonstrating the relationship between the elevated P_o and reduced trypsin response. These data confirm the conclusion that the trypsin response is largely due to activation of membrane resident low activity channels.

The second issue tested by expressing εβγENaC is the role of Na^+ self-inhibition. This channel was reported to exhibit a higher sensitivity to Na^+ self-inhibition (21). Thus, if trypsin activated ENaC via a relief of self-inhibition, then it would be expected that the response of this channel to trypsin would be exaggerated. This is clearly not the case. Given the blunted response of εβγENaC, and the lack of an effect of a 20-fold change of [Na^+] on the response of αβγENaC to trypsin (see Fig. 1), we conclude that activation of ENaC by the exogenous serine protease trypsin does not involve a role for Na^+ self-inhibition.

A G-Protein-coupled Mechanism—Our data provide compelling evidence for an indirect mechanism of activation of ENaC by trypsin; through a mechanism that nonetheless requires catalytic activity of this protease. A potential membrane receptor candidate that may mediate this response is PAR-2. This receptor is present in many cell types that express ENaC and is
activated by numerous kidney endogenous proteases (10) and by trypsin (37). This receptor is also endogenously expressed in oocytes and is further activated by low levels of trypsin (30, 31). It is well known that this class of receptors is G-protein coupled (32, 37–39) and that the oocyte-endogenous PAR-2-mediated effects of trypsin are also coupled to a pertussis toxin-sensitive G-protein (32). To examine receptor coupling we tested: 1) the involvement of G-proteins in this response and 2) the role of endogenous PAR-2.

To test G-protein coupling of the ENaC response to trypsin, experiments were carried out in oocytes injected with GDPβS, a non-hydrolyzable di-phosphate analog of GTP. By virtue of being non-hydrolyzable, this analog will block any G-protein activation that is dependent on binding and hydrolysis of both di- and tri-phosphates. Injections were carried out after establishing the baseline amiloride-sensitive current (Fig. 8, A and B) and indicate that G-protein block virtually eliminated the stimulatory effects of trypsin. Injection of GDPβS alone was without effect on $g_{Na}$, indicating a specific effect on the trypsin induced stimulation.

To determine if trypsin stimulation of ENaC is directly coupled to G-proteins, we tested if GDPβS injection can block ENaC that is already trypsin-stimulated. As shown in Fig. 8 (C and D), addition of GDPβS did not affect the $g_{Na}$ after its stimulation by trypsin. This indicates that G-protein activation is not directly coupled to ENaC activation, and that this response likely requires the downstream activation of a signaling intermediary that is G-protein-coupled.

Activation of PARs, including those in oocytes, is known to be coupled to phospholipid signaling through the G-protein pathway (29–32). To test this hypothesis, we examined the role of phospholipase Cβ in this response. PLCβ is a logical candidate for the following reasons: 1) it is coupled to G-proteins, 2) it is known to affect ENaC activity in oocytes (40), and 3) its upstream and downstream products are biologically active lipids that regulate ENaC and may as recently reported, directly bind to the channel (41–45).

To test the role of PLC we utilized the activator m-3M3FBS. In contrast to other PLC agonists and antagonists, this agent was recently shown to selectively activate this enzyme (46). Addition of m-3M3FBS was without effects on baseline ENaC activity (Fig. 9). However, pretreatment with this activator blunted the subsequent effect of trypsin indicating that the actions of this protease involve this lipid-dependent enzyme. Upon washout of this activator, and in the continued presence of trypsin, channel activation was observed. Given that the trypsin response is largely contributed to by activation of low activity channels, these data indicate that PLCβ activation, and hydrolysis of inositol 1,4,5-bisphosphate, may preferentially affect this channel population (see “Discussion”). Irrespective of the exact mechanism, our data indicate that the effects of trypsin are mediated via a receptor coupled to a G-protein that is in turn coupled to PLCβ. These data also provide additional evidence supporting indirect channel activation by trypsin.

PAR-2-independent Activation of ENaC by Trypsin

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PAR-2-independent Mechanism—The above data engender the question of the role of PAR-2, a trypsin receptor that is also endogenously expressed in oocytes, in this response. This was tested by examining the effects of PAR-2 overexpression and knockdown.

Using a homology-based approach, we cloned \textit{Xenopus} PAR-2 (see “Experimental Procedures”). An alignment of the \textit{Xenopus} and rat PAR-2 sequences is shown in Fig. 10A. These two proteins exhibit an overall homology of 71%. Overexpression of ENaC with this receptor was without effect on the trypsin response (Fig. 10B). These results indicate that PAR-2 is not rate-limiting to the trypsin response, or that it is not involved as the trypsin receptor mediating the effects on ENaC.

Overexpression of this protein was verified using an HA-tagged PAR-2 construct as a commercial
antibody recognizing Xenopus PAR-2 is not available (see below).

To further rule out a role of PAR-2 in the effect of trypsin on ENaC, we examined the effects of endogenous PAR-2 knockdown. These experiments utilized injection of an antisense morpholino-modified oligonucleotide to inhibit PAR-2 protein levels. These modified oligonucleotides bind around the start ATG and sterically inhibit RNA translation and protein synthesis. Moreover, they are highly stable and are physically injected into each oocyte assuring very efficient protein knockdown.

The nucleotide sequence of Xenopus PAR-2 and the corresponding morpholino are shown in Fig. 11A. The efficacy of this approach in inhibiting PAR-2 synthesis is shown in Fig. 11B. This was confirmed by examining inhibition of the PAR-2-HA construct, as a commercial antibody is not available. As expected, this approach results in a near complete knockdown of PAR-2 protein levels, an effect not observed with the control oligonucleotide. Despite the marked decrease of PAR-2 protein with the antisense morpholino, the baseline ENaC activity and stimulation by trypsin were unaffected (Fig. 11, D and E). These data indicate that nanogram levels of trypsin activate ENaC via a G-protein-coupled, PAR-2-independent mechanism that is clearly independent of channel cleavage.

**DISCUSSION**

It is well accepted that a variety of exogenous serine proteases can, in a range of concentrations, activate the epithelial Na⁺ channel. Where tested, activation is found to be mediated by an increase of the activity of membrane resident channels in the absence of channel trafficking to the membrane. Despite numerous studies, there is debate as to the mechanisms of channel activation and whether it even occurs by a single mechanism. More specifically, an unresolved issue is whether channel protein cleavage is a prerequisite for activation. A second related issue of interest is the role of Na⁺ feedback inhibition in this process. Utilizing a trypsin concentration that does not affect the endogenous conductances, we demonstrate robust activation of ENaC. We rule out channel cleavage in this effect and demonstrate a role of G-protein coupled to PLCβ in this process. Utilizing multiple lines of evidence we also demonstrate that activation occurs by increasing the activity of membrane resident ENaCs in a process that is unrelated to Na⁺ inhibition.

**Activation of Low Activity Channels**—Early work using fluctuation analysis has indicated the presence of a low number of active channel protein at the membrane with the potential for a large reserve of silent, or very low activity, channels (47, 48). Similarly, patch clamp analysis of kidney tubules has also indi-
PAR-2-independent Activation of ENaC by Trypsin

cated the presence of channel populations with low and high $P_o$ (49). The role of low activity channels was unknown; however, it was speculated that they could serve as a reserve source of channels that upon activation would allow cells a large magnitude of activation of Na$^+$ transport.

Indeed, this interpretation was recently confirmed where it was concluded that trypsin activated low activity or near silent channels (7). Similarly, we also find membrane patches with low and high ENaC activity and further find that those with low activity exhibit on average a 20-fold increase with trypsin. In the face of the 6-fold increase of whole cell currents, these data indicate that activation of low activity channels contributes the most to the observed trypsin response. This is consistent with the interpretation of others (15) and indicates that channels with the lowest $P_o$ would exhibit the largest potential for stimulation by trypsin and the largest contribution to the increase of whole cell currents. This conclusion was indeed experimentally verified in our system utilizing $\varepsilon$-beta ENaC. These data, and our results that trypsin increases the $P_o$ of membrane-resident channels, indicate that channels with a high baseline $P_o$ do not respond well to activation by trypsin. Thus, the absence of a response to trypsin in some systems may simply be due to an elevated spontaneous $P_o$ of the channel. This effect may also explain why ENaC in some cell types only responds to trypsin after inhibition of transport and, potentially, a decrease of baseline spontaneous $P_o$ by protease inhibitors (9).

The larger effects of trypsin on low activity channels indicate that these channels may exhibit different sensitivity to second messenger regulation. This indeed may even be the reason why these channels may exhibit different sensitivity to second

**Role of PARS**—It is well known that a variety of extracellular proteases act via stimulation of a class of membrane receptors known as PARS or protease-activated receptors. These receptors are classified according to their ligand specificity, with trypsin-activating PAR-2. These receptors exhibit high sensitivity to their protease substrates (32, 37, 50), which allows their cleavage and activation in the absence of digestion of other integral membrane proteins. It is well known that these receptors are G-protein-coupled (30–32, 37–39). The observation that the effects of trypsin are G-protein- and PLC$\beta$-dependent provided the impetus for pursuing the role of PAR-2 in this effect.

Although our results indicate that the effects of trypsin on ENaC are receptor-mediated, we demonstrate that endogenous PAR-2 is not involved in this process. This is based on the absence of effects of PAR-2 overexpression or knockdown. It is difficult to determine the type of the receptor involved in stimulating ENaC based on the sensitivity to its ligand (e.g. trypsin, elastase, and others) alone, because this is highly variable from tissue to tissue, and among the different receptor isoforms. Furthermore, the majority of studies examining the link to PARS rely on measuring changes of [Ca$^{2+}$] as a functional assay, and thus other effects linked upstream to these receptors may have been missed altogether.

**Extracellular Processing by Trypsin**—Garty and Edelman (1) used trypsin at 1 mg/ml to irreversibly digest Na$^+$ channel protein in the apical membrane of toad bladder. Thus, it is clear that trypsin at high concentrations will digest ENaC. The question asked in the current study was: can ENaC be activated by trypsin without channel proteolysis, and if so, what is the mechanism of this activation?

To address this question we focused on the effects of low concentrations of trypsin. When comparing our results to those obtained in other systems, the exact concentration of trypsin is not critical, because it is not surprising that different systems exhibit different sensitivities to this protease. However, what is important is that 1) we used a concentration that is orders of magnitude lower than that which is known to irreversibly digest channel protein, and 2) this concentration has no effect on background conductances. Both issues are equally important, because high concentration of a protease can digest the channel irrespective of its relationship to activation, and the absence of effects on the background conductances rule out additional nonspecific effects that may also alter the response of ENaC. Using these criteria, we demonstrate indirect activation of ENaC by exogenous serine protease that is clearly not mediated by channel cleavage. It is important to point out that the oocyte system is ideal to test the role of channel cleavage, owing to their high sensitivity to trypsin. Such experiments would not have been possible in other cell types that require trypsin levels close to those causing channel digestion as they cannot rule out unrelated proteolysis.

Our findings do not and cannot rule out ENaC cleavage and activation by other mechanisms. For example, Kleyman and colleagues (6, 51) have elegantly demonstrated that furin, an endogenous serine protease, cleaves ENaC as part of normal channel maturation through the biosynthetic pathway. They also demonstrate that such limited cleavage or proteolysis is necessary for channel maturation and activation. Therefore, it is well established that channel cleavage, at least through furin, leads to its activation. The relationship between processing by furin and exogenous proteases such as trypsin is less clear as mutation of the consensus furin cleavage sites on ENaC and loss of furin activation led to inhibition of channel activity but did not prevent the channel’s activation by trypsin. Indeed, these authors found that trypsin activated the reduced activity ENaC furin mutant to similar levels as the wild-type channel. They interpreted this effect as an indication that furin and trypsin are activating ENaC via a common mechanism. A potential alternative explanation is that the effects of trypsin and furin on ENaC are divergent but are in part related as they both act via changes of the channel’s open probability. This would be consistent with the recent report that digestion of $\gamma$ENaC by exogenous elastase occurs at sites that are different than
those normally cleaved through the biosynthetic pathway (17). Moreover, addition of trypsin to in vitro translated ENaC subunits in the absence of an intact membrane, results in channel processing in the C termini of the β and γ subunits (52), further indicating differences with furin biosynthetic pathway processing, which occurs in the extracellular loop of the α and γ subunits (6, 51). In this case, is the cleavage observed by some exogenous serine proteases necessary for channel activation?

There are two potential simple answers to the above question. First, there are two separate mechanisms of channel activation; an indirect one, and a direct one that results in channel cleavage. Cleavage may then activate the channel by removing an inhibitory domain. Supporting this is the recent observation of an inhibitory domain in the β subunit, which is cleaved by furin, although not shown for trypsin (34, 53). In this case, these mechanisms would not be additive as both would work via an increase of $P_o$, giving the appearance of the same mechanism of activation.

Second, cleavage is not necessary for channel activation, and is only observed in some systems, by virtue of the low sensitivity to trypsin that requires the use of high protease levels to observe an ENaC response, or by the conventional use of the same concentration in systems that do not require such high protease levels (e.g. oocytes). These levels of exogenous serine proteases may then lead to unrelated channel proteolysis, even potentially underestimating the second messenger-mediated stimulation of ENaC. This hypothesis remains to be tested in additional systems, like the oocyte, that exhibit high sensitivity to exogenous proteases.

CONCLUSIONS

We demonstrate for the first time an indirect, receptor-mediated, second messenger activation of ENaC by an exogenous serine protease. This effect was mediated by stimulation of the open probability of membrane-resident channels. Our data rule out channel cleavage as an obligatory mechanism of activation by trypsin. The role of this mechanism in activating ENaC and Na$^+$ transport by exogenous proteases in other systems and native epithelia remains to be determined.

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