The Serine Protease Trypsin Cleaves C Termini of β- and γ-Subunits of Epithelial Na⁺ Channels*

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Received for publication, August 29, 2001, and in revised form, November 28, 2001
Published, JBC Papers in Press, December 5, 2001, DOI 10.1074/jbc.M108354200

Both extracellular and intracellular proteases can activate epithelial Na⁺ channels (ENaC). The mechanism by which serine proteases activate ENaC is unknown. We investigated the effect of the serine protease trypsin on in vitro translated and immunopurified α-, β-, and γ-rENaC subunits. Immunopurified subunit proteins were exposed to increasing concentrations of trypsin ranging from 0.002 to 2 μg/ml in Tris-buffered saline buffer for 2 h. The proteolytic mixture was subjected to SDS-PAGE and analyzed by autoradiography. Our results demonstrate that the β- and γ-subunits of ENaC were most susceptible to trypsin proteolysis, and exposure to as little as 0.002 μg/ml trypsin resulted in a reduction in the size of the β- and γ-transcripts by 7–8 kDa. By using N- and C-terminally truncated β- and γ-subunits, we determined that trypsin cleaved the C termini of both subunits, resulting in a channel structure resembling that seen in Liddle's disease. Exposure to 2 μg/ml trypsin completely digested all three subunits. Our results suggest different susceptibility of proteolytic sites of ENaC subunits to trypsin. Thus, we propose that limited intracellular proteolysis may be one of the potential physiological mechanisms of sodium channel regulation.

Epithelial sodium channels (ENaC) play a key role in the regulation of sodium balance, extracellular fluid volume, blood pressure, and fluid reabsorption. The activity of ENaC is tightly regulated in order to ensure ion and volume homeostasis of the extra- and intracellular milieu. This regulation is under the control of several hormones and intracellular factors by mechanisms that are not yet completely understood (1). The idea that proteases can modulate the activity of epithelial amiloride-sensitive Na⁺ channels is not new. Both extracellular and intracellular effects of proteases on epithelial amiloride-sensitive Na⁺ channels have been reported. Garty and Edelman (2) observed that extracellular trypsin, at a concentration of 1 mg/ml, induced an irreversible inhibition of the sodium transport in toad urinary bladder and that this effect could be prevented by amiloride. Lewis and Alles (3) studied the effects of proteases such as kallikrein, urokinase, and plasmin and observed that these proteases converted a normally highly selective amiloride-sensitive Na⁺ channel into a nonselective cation channel. Chraibi et al. (4) found that trypsin, at a concentration of 2 μg/ml, increased the amiloride-sensitive current up to 20-fold when added to the bathing solution of ENaC-expressing Xenopus oocytes. Jovov et al. (5) demonstrated that A6 cells (derived from Xenopus laevis kidney) express and secrete a kallikrein-like serine protease from their apical side. This serine protease has been cloned and named channel-activating protease (CAP) (6). Masilamani et al. (7) suggested that activation of Na⁺ currents by aldosterone is mediated by CAP1 that in turn cleaves γ-ENaC in the early portion of extracellular loop (7). Until now, this hypothesis has not been tested. An intracellular effect of trypsin on ENaC immunopurified from human lymphocytes and incorporated in planar lipid bilayers was reported by Ismailov et al. (8) who studied Liddle's disease. Liddle's disease is a form of human hypertension caused by mutations in the β- and γ-subunit of ENaC. Many of the described mutations result in truncation of the C termini of either the β- or γ-subunit of ENaC. It is likely that the increased channel activity seen in patients with Liddle's disease is due to both increased ENaC cell surface expression and increased channel open probability (9). Ismailov et al. (8) demonstrated that the addition of trypsin (0.5 mg/ml) to the putative intracellular side of lymphocyte Na⁺ channels incorporated into planar lipid bilayers increased the single channel open probability (Pₒ) in normal individuals as compared with that seen in lymphocyte Na⁺ channels obtained from patients with Liddle's disease. Intracellular addition of trypsin to lymphocyte Na⁺ channels obtained from patients with Liddle's disease did not further increase single channel Pₒ.

Recent analysis of protein motifs in some members of the DEG/ENaC family revealed the presence of proteaseodomains and protease inhibitor motifs. The presence of a Kunitz-type protease inhibitor motif that inhibits serine proteases was described by Tavernarakis et al. (10) in the N terminus of α-ENaC subunit. In addition, two nematode proteins, MEC-2 and UNC-1, contain a domain implicated in proteolysis of membrane-associated proteins (11). These finding led to the suggestion that members of the DEG/ENaC family may be regulated by, or may themselves participate in, proteolysis as an important part of the regulatory mode of these channels (11).

Despite numerous biophysical studies that demonstrated the functional effects of serine proteases on ENaC, there are no biochemical data demonstrating proteolysis of any ENaC subunit by trypsin. In this study, we showed that in vitro translated β- and γ-subunits of ENaC can be cleaved by trypsin in the nanogram/ml range and that the most susceptible trypsin cleavage sites are in the C termini of these β- and γ-subunits. We also demonstrate that 2 μg/ml trypsin (the lowest concentration used in functional studies so far) is enough to digest completely each of the three ENaC subunits.
EXPERIMENTAL PROCEDURES

DNA Constructs—The cDNAs encoding full-length epitope-tagged α-, β-, and γ-rENaC subunits and N- and C-terminally truncated β- and γ-rENaC are shown in Fig. 3 and are described in detail elsewhere (12). A polycistronic vector that contained all three ENaC subunits in one vector was used for transient transfection of 3T3 fibroblasts and was described in detail previously (13).

In Vitro Transcription, Translation, Immunoprecipitation, and Trypsinization—cDNA constructs were transcribed and translated in vitro using the TNT transcription/translation system (Promega) and subjected to immunoprecipitation in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 7.5), as described previously (12). Immunopurified proteins were then exposed to different concentrations of trypsin, ranging from 0.002 to 2 μg/ml in Tris-buffered saline (TBS) for 2 h. The composition of TBS was 150 mM NaCl, 10 mM Tris, pH 7.5. The proteolytic mixture was subjected to SDS-PAGE and analyzed by autoradiography.

Antibodies—Anti-hemagglutinin and anti-M2 monoclonal antibodies were obtained from Roche Molecular Biochemicals. The polyclonal, anti-γ-rENaC antibodies were raised against a peptide corresponding to γ-ENaC amino acids 629–650. The antibodies were used for precipitation of in vitro translated rENaC subunits as described previously (12).

Planar Lipid Bilayers—The rENaC subunits were prepared using intracellular trypsin and immunopurification as described by Jovov et al. (12). Translated proteins were reconstituted in proteoliposomes as described previously (14). Vesicles were fused to a lipid bilayer membrane composed of diphantolynosphatidylcholine-diphantolynosphatidylcholine (mole ratio 1:1) in a 2.1:1 (molar) ratio. Incorporation was done at 40 mV, and recording was done in symmetrical 100 mM NaCl, 10 mM Tris buffer, pH 7.4. Single channel currents were measured using a conventional current-to-voltage converter with a 10-gigaohm feedback resistor (Eltec, Daytona Beach, FL) as described previously (13). The identity and orientation of ENaCs in the membrane were tested at the end of each experiment by adding 0.5 μM amiloride to the bath solution. Single channel analyses were performed using pCLAMP 5.6 software (Axon Instruments Inc., Burlingame, CA) on current records low pass-filtered at 300 Hz through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) prior to acquisition using a Digidata 1200 interface (Axon Instruments Inc., Union City, CA). The actual number of functional ENaC channels in each given experiment was determined by transiently activating them (including those initially “silent”) by establishing a hydrostatic pressure gradient across the membrane (14, 16). Bilayers containing multiple channels were not analyzed.

Single Channel Recordings—The oocytes used for the patch clamp experiments were first placed in a hypertonic medium at room temperature for 5 min. The vitelline membrane was then manually removed from lymphocytes obtained from confirmed Liddle syndrome patients (8). To test whether trypsin produced comparable effects on ENaC, we transiently transfected 3T3-fibroblasts with α-, β-, γ-rENaC and recorded ENaC in excised patches using the inside-out mode of the patch clamp technique. As can be seen in Fig. 1, 5-pS, Na+-selective channels were recorded. After a 5-min exposure to 0.25 mg/ml trypsin, single channel P0 increased, and the channel remained essentially in its open state.

RESULTS

In the experiments described here, we used polyclonal antibodies against the N-terminal domain of γ-ENaC. The polyclonal and monoclonal antibodies against α- and β-ENaC were generated against synthetic peptides which correspond to amino acids 150–200 of the amino acid sequence of the human α- and β-ENaC subunits. The polyclonal antibody against γ-ENaC was raised against a synthetic peptide corresponding to human γ-ENaC amino acids 629–650.

Trypsin Cleaves γ-ENaC Subunits—Using the TNT transcription/translation system (Promega), we expressed rENaC subunits in vitro. Trypsin cleavage was achieved by adding trypsin to the reaction mixture at a concentration of 0.002–2 μg/ml, and the proteolytic mixture was subjected to SDS-PAGE and analyzed by autoradiography. The polyclonal, anti-γ-rENaC antibodies were raised against a peptide corresponding to γ-ENaC amino acids 629–650. The antibodies were used for precipitation of in vitro translated rENaC subunits as described previously (12).

Fig. 1. Representative single channel current records of wild type α-, β-, and γ-rENaC before and after bath addition of trypsin to inside-out patches of 3T3-fibroblasts expressing ENaC. Membrane potential was −100 mV. The pipette was filled with a lithium glutamate-rich solution, and the bath contained a potassium glutamate-rich solution. Solid lines indicate the zero current level before (control) and after application of 0.25 mg/ml trypsin (+ trypsin) to the same inside-out patch. In the presence of trypsin, single channel P0 increased, and the channel remained essentially in its open state.
γ-rENaC were incorporated. The single channels that were seen were indistinguishable from the wild type, i.e. no change in single channel conductance or open probability was measured, suggesting that the elimination of the early portion of the γ-subunit was not detrimental to the integrity of the functional channels recorded (data not shown). Although the mechanisms by which serine proteases activate ENaC are not clear, functional studies demonstrated that both extracellular and intracellular trypsin can activate ENaC.

Because all three rENaC subunits contain numerous trypsin proteolytic sites, our goal in these biochemical studies was to discover the most susceptible trypsin proteolytic cleavage sites within the rENaC subunits. Immunopurified, epitope tag-labeled rENaC subunits (synthesized by in vitro transcription and translation) were used as a source of subunit proteins for trypsin digestion. A schematic representation of each ENaC subunit with corresponding truncation points and positions of epitope tags in each subunit is shown in Fig. 4. The locations of the N- and C-terminal truncations of β- and γ-rENaC subunits are labeled by double horizontal lines, and the number of the last amino acid before the truncation is indicated. The position of the epitope tag in the extracellular loop of each subunit is shown as a small black box with the number of the starting and ending amino acid. We used non-glycosylated forms of in vitro translated proteins (the transcription mixture did not contain microsomes) for simplicity. Transcription of each construct in this condition resulted in a protein band of the appropriate size (Figs. 5–9, lane 1). Trypsinization was performed in TBS at 37 °C for 2 h. By using different concentrations of trypsin, we determined that 2 μg/ml trypsin was sufficient to digest completely all three subunits of ENaC (Figs. 5, A and B, 6, A and B, and 8A). To unmask the most susceptible proteolytic site in each subunit, we decreased the concentration of trypsin 1000-fold and exposed each protein subunit to four different concentrations of trypsin, namely 0.002, 0.02, 0.2, and 2 μg/ml. The proteolytic mixture was analyzed using both 8 and 15% SDS-PAGE. Proteolysis of the α-subunit by trypsin in the range of 0.002–0.2 μg/ml did not result in any visible proteolytic fragments (Fig. 5, A and B). However, 2 μg/ml trypsin completely disintegrated the α-rENaC subunit (Fig. 5, A and B). We also examined the time course of proteolysis by trypsin at 5, 10, 20, and 40 min using 0.02 μg/ml trypsin (data not shown). We did not observe any visible proteolytic fragments at these time points. These results suggest that either all α-rENaC trypsinization sites have a similar susceptibility to trypsin or our experimental conditions are not sensitive enough to detect subtle differences in trypsin effects. Tavarnarakis et al. (10) described the presence of a Kunitz-type protease inhibitor motif in the N terminus of α-ENaC subunit. It is possible that this protease inhibitor motif protects the α-rENaC subunit from proteolysis at low trypsin concentrations, but 2 μg/ml trypsin is sufficient to overcome this inhibitory effect.

![Fig. 2. Effect of trans (extracellular) trypsin on ENaC reconstituted into planar lipid bilayers. Bilayers were bathed with symmetrical 100 mM NaCl, 10 mM MOPS. Trypsin was added to trans compartment of the bilayer chamber in the concentration indicated. Holding potential was +100 mV and referred to the virtually grounded trans chamber. For illustration purposes records shown were digitally filtered at 100 Hz using pCLAMP software (Axon Instruments). Corresponding all-point amplitude histograms were constructed by pCLAMP from a record of at least 5 min in length.](http://www.jbc.org/)

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the lower band was 7–8 kDa lower than the non-cut band protein, suggesting a reduction in size due to trypsinization. Similar results were obtained by trypsinization of β-rENaC with 0.02 µg/ml trypsin at 5-, 10-, 20-, and 40-min time points (data not shown). All samples measured at each of these time points contained double bands. There was a decrease in the
using 8% SDS-PAGE. The trypsinization of H9252 2 h and analyzed using SDS-PAGE. A, analysis of proteolytic fragments using 8% SDS-PAGE. B, analysis of proteolytic fragments using 15% SDS-PAGE. Proteolysis of the α-subunit by trypsin in the range of 0.002 to 0.2 µg/ml did not result in any visible proteolytic fragments. However, 2 µg/ml trypsin completely digested the α-ENaC subunit.

To address further the question from which side (C or N terminus) trypsin cleaves β-ENaC, we used C- and N-terminally truncated constructs of β-ENaC (see Fig. 4). The N-terminally truncated construct had a deletion of 49 amino acids at the N terminus, whereas the C-terminally truncated construct had a deletion of the last 74 amino acids from the C terminus. Our hypothesis was that if trypsin cuts the C terminus of β-ENaC in close proximity to the point of truncation, the C-terminally truncated construct would be unaffected. In this case, the N-terminally truncated construct would be cut, resulting in a similar pattern as non-truncated β-ENaC (just lower molecular mass) and vice versa. The results of these experiments are shown in Fig. 7. Trypsinization of N-terminally truncated β-ENaC using 0.002–0.2 µg/ml resulted in the appearance of a double band, a higher band with the same molecular mass as the non-trypsined protein, and a lower band that is 7–8 kDa lower than the non-trypsined protein. B, trypsionization of C-terminally truncated β-ENaC. Trypsinization of C-terminally truncated β-ENaC using trypsin in the range from 0.002 to 0.2 µg/ml did not reveal the presence of any proteolytic fragments, just a single band without any shift in molecular mass. C, amino acid sequence of β-ENaC in close proximity (≤7 amino acids) to C-terminal truncation (574). Amino acids in italics are the predicted trypsin proteolytic sites.

![Trypsinization of immunopurified β-ENaC](image)

- **A**: Trypsinization of immunopurified β-ENaC. β-ENaC was transcribed and translated in vitro using TNT transcription/translation system (Promega) and subjected to immunoprecipitation in RIPA buffer. Immunopurified protein was then exposed to trypsin (Tryp) in concentrations ranging from 0.002 to 2 µg/ml in TBS buffer for 2 h and analyzed using 8% SDS-PAGE. A, analysis of proteolytic segments using 8% SDS-PAGE. B, analysis of proteolytic segments using 15% SDS-PAGE. The small (7–8 kDa) band is visible in lanes where 0.02 or 0.2 µg/ml was used for trypsinization. Detection of this 7–8 kDa band on the 15% gel suggests that the reduction in size of β-ENaC is a result of proteolytic cleavage at one side of the protein chain, as opposed to multiple cleavage sites.

- **B**: Trypsinization of immunopurified C- or N-terminally truncated β-ENaC. C- or N-terminally truncated β-ENaC was transcribed and translated in vitro (IN V) using TNT transcription/translation system (Promega) and subjected to immunoprecipitation in RIPA buffer. Immunopurified protein was then exposed to trypsin (Tryp) at concentrations ranging from 0.002 to 2 µg/ml in TBS buffer for 2 h and analyzed using SDS-PAGE. A, analysis of proteolytic fragments using 8% SDS-PAGE. B, analysis of proteolytic fragments using 15% SDS-PAGE. Proteolysis of the α-subunit by trypsin in the range of 0.002 to 0.2 µg/ml did not result in any visible proteolytic fragments. However, 2 µg/ml trypsin completely digested the α-ENaC subunit.

- **C**: Trypsinization of immunopurified C- or N-terminally truncated β-ENaC. C- or N-terminally truncated β-ENaC was transcribed and translated in vitro (IN V) using TNT transcription/translation system (Promega) and subjected to immunoprecipitation in RIPA buffer. Immunopurified protein was then exposed to trypsin (Tryp) in concentrations ranging from 0.002 to 2 µg/ml in TBS buffer for 2 h and analyzed using 8% SDS-PAGE. A, trypsinization of N-terminally truncated β-ENaC. Trypsinization of N-terminally truncated β-ENaC using 0.002–0.2 µg/ml resulted in the appearance of a double band, a higher band with the same molecular mass as the non-trypsined protein, and a lower band that is 7–8 kDa lower than the non-trypsined protein. B, trypsinization of C-terminally truncated β-ENaC. Trypsinization of C-terminally truncated β-ENaC using trypsin in the range from 0.002 to 0.2 µg/ml did not reveal the presence of any proteolytic fragments, just a single band without any shift in molecular mass. C, amino acid sequence of β-ENaC in close proximity (≤7 amino acids) to C-terminal truncation (574). Amino acids in italics are the predicted trypsin proteolytic sites.
and the lower band ran at 63 kDa (a 7–8 kDa difference in molecular mass) suggesting a reduction in size due to trypsinization. The presence of this 7–8-kDa band on a 15% gel (Fig. 8B) subsequent to trypsinization suggested that the reduction in size of γ-rENaC was a result of proteolytic cleavage at one proteolytic site, as opposed to multiple cleavage sites. The appearance of a doublet in 8% gels was also seen following trypsinization with 0.02–0.2 μg/ml trypsin (Tryp) γ-rENaC protein ran as a double band. The higher band ran at ~71 kDa (same as non-trypsinized protein) and the lower band ran at 63 kDa (7–8 kDa difference in molecular mass), suggesting a reduction in size due to trypsinization. B, analysis of proteolytic segments using 15% SDS-PAGE. The presence of a 7–8-kDa band on a 15% gel after trypsinization with 0.02–0.2 μg/ml suggests that the reduction in size of γ-rENaC is a result of proteolytic cleavage at one proteolytic site, as opposed to multiple cleavage sites.

FIG. 8. Trypsinization of immunopurified γ-rENaC. γ-rENaC was transcribed and translated in vitro (IN V) using TNT transcription/translation system (Promega) and subjected to immunoprecipitation (IP) in RIPA buffer. Immunopurified protein was then exposed to trypsin in concentrations ranging from 0.002 to 2 μg/ml in TBS buffer for 2 h and analyzed using SDS-PAGE. A, analysis of proteolytic segments using 8% SDS-PAGE. After trypsinization using 0.002–0.2 μg/ml trypsin (Tryp) γ-rENaC protein ran as a double band. The higher band ran at ~71 kDa (same as non-trypsinized protein) and the lower band ran at 63 kDa (7–8 kDa difference in molecular mass), suggesting a reduction in size due to trypsinization. B, analysis of proteolytic segments using 15% SDS-PAGE. The presence of a 7–8-kDa band on a 15% gel after trypsinization with 0.02–0.2 μg/ml suggests that the reduction in size of γ-rENaC is a result of proteolytic cleavage at one proteolytic site, as opposed to multiple cleavage sites.

Trypsin Cleaves β- and γ-rENaC

Our functional studies confirmed findings from other laboratories (4, 6, 8) that ENaC can be activated by trypsin either from intra- or extracellular sides. The doses of trypsin used in functional studies vary widely, but even the lowest doses used in functional studies (2 μg/ml trypsin) are high enough to digest completely all three in vitro translated ENaC subunits in our biochemical studies. There are at least two possible explanations for this finding. The first is that the activation of the channel seen in the functional studies is due to complete proteolysis of extra- or intracellular parts of ENaC subunits. This hypothesis was postulated for a mechanosensitive ion channel (MscL) that has a similar membrane topology as do the ENaC subunits. Ajouz et al. (19) studied the proteolytic effect of trypsin added from either the extra- or the intracellular side of a patch containing a large MscL. This channel was sensitive to mechanical stimulation in the absence of cytoskeletal attachment (19). Limited proteolysis of the C and N termini of MscL by trypsin (250 μg/ml) increased the mechanosensitivity of the channel without altering its single channel conductance. Cleavage of the external loop of the channel by trypsin (50 μg/ml) also dramatically increased the mechanosensitivity of the channel. These investigators proposed a model for MscL in which the cytoplasmic C and N termini resisted the movement of transmembrane helices. Similarly, the external connecting loops counteract the movement of transmembrane helices relative to each other. Proteolysis of all these parts of the molecule facilitates the movement of the helices, thus enhancing the sensitivity of the channel to membrane tension. It is possible...
that a similar mechanism is involved in the activation of ENaC by trypsin.

A second possible explanation is that the channel proteins in the functional and biochemical studies differ in their sensitivity to trypsin because of differences in channel environment. In functional studies, ENaC consists of heteromultimeric complexes of subunits and channel-associated proteins. These associated proteins may also be substrates for proteolysis by trypsin, and therefore, this could increase the concentration of trypsin required to see proteolytic effects in native membranes.

In addition, we and others (4, 19) have observed a time-dependent proteolysis in both functional and biochemical studies. Different exposure periods could explain, at least in part, the observed differences in the amount of trypsin required for the effects in these different studies.

Because amiloride did not prevent the stimulatory effect of extracellular trypsin, Chraibi et al. (4) suggested that the action of external proteases was on proteins associated with the channel rather than on the channel per se. The fact that external trypsin could activate ENaC in bilayers is not necessarily at variance with this interpretation, especially because much higher trypsin concentrations were required. Alternatively, the higher trypsin concentration may indicate that trypsin needs to cross the bilayer to act on the intracellular side of the channel to produce its effect.

Maslalmani et al. (7) suggest that one potential consequence of aldosterone is to activate CAP1 in turn activates ENaC by cleaving the γ-subunit somewhere in the early portion of the extracellular loop. However, the results presented in this paper are not consistent with this hypothesis for three reasons. First, our biochemical studies using in vitro translated protein do not reveal any trypsin cleavage fragments consistent with the fragmentation occurring within the extracellular loop of the γ-ENaC subunit. Second, amiloride-sensitive sodium currents in oocytes expressing N-terminal truncated (Δ2–138) γ-ENaC, along with wild type α and β, were significantly lower than those observed in oocytes expressing wild type ENaC. Third, when N-terminal (Δ2–138) γ-ENaC along with wild type α and β was incorporated into planar bilayers, no change in single channel conductance nor open probability was measured. Our results should not be extrapolated to other possible cleavage sites used by proteases other than trypsin. Alternatively, CAP1 may act on another protein associated with ENaC, as suggested by Chraibi et al. (4).

In conclusion, our biochemical results demonstrated that the two most susceptible proteolytic sites in in vitro translated rENaC subunits are in the C termini of the β- and γ-subunits. We determined that the proteolytic sites of these subunits are in very close proximity to the point of the C-terminal truncations described in Liddle’s disease. If limited intracellular proteolysis occurs in cells, it will result in increases in ENaC activity similar to what is seen in Liddle’s disease. Therefore, we postulated that limited membrane-associated proteolysis could be one of the physiological mechanisms of sodium channel regulation. Such a mechanism would require fine regulation between proteolytic activity and inhibitors. A Kunitz-type protease inhibitor motif preceding the conserved N-terminal region is present in the α-ENaC subunit (10). In addition, two nematode proteins that have been proposed to interact with and modulate degenerin channels, MEC-2 and UNC-1, contain a domain implicated in regulating proteolysis of membrane-associated proteins (11). These domains were identified using protein sequence motif analysis, and they will serve as a lead to future experiments that will test membrane-associated proteolytic complexes and its role in the regulation of the DEG/ENaC family. Our data represent the first step in biochemical proteolytic studies of the DEG/ENaC family and demonstrate the presence of two highly sensitive trypsin proteolytic sites in the C termini of the β- and γ-subunits of rENaC.

Acknowledgments—We thank Dr. A. Maximov (Department of Physiology, University of Texas Southwestern Medical Center) for participation at the early stage of this project. We thank Dr. C. Canessa (Department of Molecular and Cellular Physiology, Yale University) and Dr. B. Rossier (Institute of Pharmacology and Toxicology, Lausanne, Switzerland) for kind gifts of rENaC cDNA, and Drs. Richard Boucher and John Olsen (Department of Medicine, University of North Carolina) for the gift of the polycistronic ENaC vector. We thank Isabel Quinones and Catheleen Guy for superb work in typing the manuscript.

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J. Biol. Chem. 2002, 277:4134-4140.
doi: 10.1074/jbc.M108354200 originally published online December 5, 2001

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