Serine protease activation of near-silent epithelial Na⁺ channels

Ray A. Caldwell, Richard C. Boucher, and M. Jackson Stutts

The Cystic Fibrosis/Pulmonary Research and Treatment Center,
University of North Carolina, Chapel Hill, North Carolina 27599-7248

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Caldwell, Ray A., Richard C. Boucher, and M. Jackson Stutts. Serine protease activation of near-silent epithelial Na⁺ channels. Am J Physiol Cell Physiol 286: C190–C194, 2004. First published September 10, 2003; 10.1152/ajpcell.00342.2003.—The regulation of epithelial Na⁺ channel (ENaC) function is critical for normal salt and water balance. This regulation is achieved through cell surface insertion/retrieval of channels, by changes in channel open probability (Pₒ), or through a combination of these processes. Epithelium-derived serine proteases, including channel activating protease (CAP) and prostanin, regulate epithelial Na⁺ transport, but the molecular mechanism is unknown. We tested the hypothesis that extracellular serine proteases activate a near-silent ENaC population resident in the plasma membrane. Single-channel events were recorded in outside-out patches from fibroblasts (NIH/3T3) stably expressing rat α-, β-, and γ-subunits (rENaC), before and during exposure to trypsin, a serine protease homologous to CAP and prostanin. Under baseline conditions, near-silent patches were defined as having rENaC activity (Nₚₒ < 0.03, where N is the number of channels). Within 1–5 min of 3 µg/ml bath trypsin superfusion, Nₚₒ increased ~66-fold (n = 7). In patches observed to contain a single functional channel, trypsin increased Pₒ from 0.02 ± 0.01 to 0.57 ± 0.03 (n = 3, mean ± SE), resulting from the combination of an increased channel open time and decreased channel closed time. Catalytic activity was required for activation of near-silent ENaC. Channel conductance and the Na⁺/Li⁺ current ratio with trypsin were similar to control values. Modulation of ENaC Pₒ by endogenous epithelial serine proteases is a potentially important regulator of epithelial Na⁺ transport, distinct from the regulation achieved by hormone-induced plasma membrane insertion of channels.

Cystic Fibrosis/Pulmonary Research and Treatment Center, CB#7248, Univ. of North Carolina, Chapel Hill, NC 27599-7248 (E-mail: ray_caldwell@med.unc.edu).

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whether PA receptors were responsible for the trypsin-induced increase of ENaC $P_o$. G protein activation was inhibited by complete replacement of GDP in the pipette solution with equimolar GDP. Patches were allowed to dialyze at least 5 min before recordings were initiated. Despite GDP replacement with GDP, trypsin increased $P_o$, as shown in Fig. 3. Similarly, trypsin activation of amiloride-sensitive currents in oocytes also did not involve G protein signaling (3). The inability to wash out the trypsin effect on ENaC activity (for >20 min) is also consistent with a non-receptor-mediated action of the enzyme on $P_o$. These findings suggest that trypsin catalytic activity likely involves ENaC itself or a closely associated ENaC-regulatory protein present in 3T3 cells (see below).

The trypsin-induced increase of ENaC $P_o$ could result from a change in $N$, $P_o$, or a combination of these parameters. In records where only a single channel was observed (i.e., Fig. 2), trypsin induced a 32.8-fold increase in $P_o$ (from 0.02 ± 0.01 to 0.57 ± 0.02, $P = 0.002$, $n = 3$). The increased $P_o$ resulted from an increased open time and a decreased closed time for the channel. The infrequent channel transitions in control, and the long openings (i.e., >20 s) during trypsin exposure, precluded a quantitative analysis and comparison of channel dwell times. However, we found no evidence to support a trypsin-induced fusion of ENaC-containing vesicles with the outside-out patch of membrane (i.e., increased $N$). The patch-clamp technique is a highly sensitive detector of vesicular fusion with the ability to detect individual vesicle fusion events (10, 11).

The large-conductance fusion pore (i.e., 70 pS) created during the redistribution of membrane charge that occurs with addition of patch-surface membrane (8) could be readily detected with our recording conditions if it had occurred, but it was never observed (ENA conductance is 0.03–0.1 of the fusion pore conductance; see below). Moreover, for exocytosis to occur, vesicles along with other proteins necessary for fusion (i.e., synaptobrevin) (8) must remain intact with the excised remnant of plasma membrane. Also, patch excision was never observed (ENaC conductance is 0.03–0.1 of the fusion pore conductance; see below). The large-conductance fusion pore (i.e., 70 pS) created during the redistribution of membrane charge that occurs with addition of patch-surface membrane (8) could be readily detected with our recording conditions if it had occurred, but it was never observed (ENA conductance is 0.03–0.1 of the fusion pore conductance; see below). Moreover, for exocytosis to occur, vesicles along with other proteins necessary for fusion (i.e., synaptobrevin) (8) must remain intact with the excised remnant of plasma membrane. Also, patch excision was never observed (ENA conductance is 0.03–0.1 of the fusion pore conductance; see below).
enzyme effects on rENaC $P_o$ were inconclusive (1, 3). This lack of effect was ascribed to the characteristic highly variable ENaC $P_o$ and the selection of patches containing channels with moderate control channel activity (i.e., $P_o \sim 0.5$) (15). Indeed, we also observed wide variability of control $P_o$, from sweep to sweep in the same patch (Fig. 1C, sweeps 1–7). However, trypsin consistently increased $P_o$. We believe our ability to observe the enzyme-induced increase of channel activity stemmed from measurements of $P_o$, made before and during trypsin exposure from the same patch, coupled with amiloride superfusion to unambiguously identify the fully closed/blocked channel current level. In fact, in some experiments, we observed large baseline channel activity without ever observing the fully closed channel current level in the absence of amiloride. Confirmation of the fully closed/blocked current level is essential for accurate analysis of $P_o$, which is not always straightforward with the cell-attached patches used in previous studies that could underestimate serine protease-induced effects on $P_o$. Alternatively, the protease effect on single active channels (i.e., $P_o \sim 0.5$) is expected to be minimal, if any.

Is proteolytic cleavage of ENaC required for expression of active channels? Recently, Hughey et al. (9) showed that during channel maturation, the extracellular domains in some of mouse $\alpha$- and $\gamma$-subunits were cleaved by an endogenous, aprotinin-insensitive protease(s). Moreover, in A6 epithelia (18) and human bronchial epithelia (2), a sizable amiloride-sensitive current persists after a lengthy preincubation ($>1$ h to overnight) with aprotinin or the recombinant Kunitz-type serine protease inhibitor BAY 39-9437. Frindt et al. (7) also reported cleavage of the $\gamma$-subunit in rat cortical collecting tubule (CCT) after animals were subjected to $\geq15$ h of Na$^+$ restriction (13). Interestingly, $\gamma$-subunit cleavage correlated with expression of amiloride-sensitive whole cell currents in CCT (7), but the effect of aprotinin on whole cell currents or on the $\gamma$-subunit band pattern was not reported. In view of these findings and the results described here, it is tempting to speculate that during maturation, some fraction of $\alpha$- and/or $\gamma$-subunits are cleaved by aprotinin-insensitive protease(s) and, along with the $\beta$-subunit, emerge onto the cell surface membrane as active channels, whereas subunits not cleaved during maturation make up the near-silent pool of channels that are activated by aprotinin-sensitive extracellular proteases (i.e.,

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{G protein coupled protease-activated (PA) receptors are not involved in the trypsin-induced activation of ENaC. A: representative recording showing control (sweep 7) and 3-min trypsin exposure (sweep 16) illustrating the enzyme-induced increase of $P_o$, despite complete replacement of intracellular GTP with GDP$\beta$S. Reversible amiloride inhibition of channel activity also is shown (sweeps 17 and 18). B: summary data from 4 experiments in which trypsin increased $P_o$, with GDP$\beta$S in the pipette [GDP$\beta$S log($P_o$)] = $-1.12 \pm 0.42$ vs. trypsin + GDP$\beta$S log($P_o$) = $0.085 \pm 0.326$; $P = 0.035$). Solid lines connect data obtained from the same patch. Channel activity was recorded in 150 mM bath Li$^+$ at $-60$ mV.}
\end{figure}
CAP-1, CAP-2, trypsin). Future studies are needed to identify protease-sensitive residues in the extracellular domains of ENaC and determine how mutating these sites affects channel function. Results from such studies should be informative for understanding the physiological regulation of epithelial Na⁺ transport.

EXPERIMENTAL METHODS

Cell culture. NIH/3T3 cells were infected with retrovirus encoding cDNAs for rENaC α-, β-, and γ-subunits (18). Clones stably expressing ENaC subunits were grown at 37°C, under an atmosphere of humidified 5% CO₂ in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum, 10 μM amiloride, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 300 μg/ml G418, and 1 μg/ml puromycin. Cells for patch-clamp experiments were subcultured on 35-mm culture dishes and visually selected using a Nikon inverted microscope equipped with Hoffman modulation contrast optics.

Electrophysiology. Outside-out patches were studied. Patch-clamp signals (EPC-7; List, Darmstadt, Germany) were filtered at 0.1 kHz (−3 dB, Bessel), digitized at 1 kHz (16-bit, ITC, Instutech, Long Island, NY), and acquired with a Pentium computer running HEKA-PULSE acquisition software (Bruxton, Seattle, WA). Patch pipettes (borosilicate; Warner Instruments, Hamden, CT) were fabricated from thin-walled glass using a two-stage pull routine (Sutter Instruments, Novato, CA). Pipette resistance was 13.8 ± 0.3 MΩ (mean ± SE, n = 51). A Ag–AgCl electrode connected to the bath via a 3% agar bridge containing 1 M KCl served as the ground electrode. The diffusion potential, measured between pipette and bath solution, was 2.0 ± 0.1 mV (n = 4), and all voltages have been corrected by this amount.

Single-channel activity was recorded at membrane potentials from −100 to 0 mV with flowing bath conditions. Exchange of bath solution occurred in <100 ms with the use of a Fast-step solution exchanger (Warner Instruments). Solution flow rate was 0.2 ml/min. Bath amiloride inhibition of single channels was used to confirm ENaC identity and patch configuration. Channels observed as amiloride insensitive were not included for analysis.

Bath and pipette solutions. The standard bath solution contained (in mM) 150 Li(or Na)-aspartate, 2 MgCl₂, 1 CaCl₂, and 5 HEPES, titrated to pH 7.30 with LiOH (or NaOH). The standard pipette solution contained (in mM) 120 Tris-aspartate, 20 NaCl, 3 MgATP, 0.2 Na₂GTP, 0.1 CaCl₂, 1 EGTA, and 5 HEPES, titrated to pH 7.10 with NaOH. In selected experiments, GTP was replaced with equimolar GDPβS. Amiloride (10 μM), trypsin (type I, 10,800 U/mg, <4 U/mg chymotrypsin; Sigma), and SBTI (type I-S; Sigma) were dissolved in the bath solution. The amount of SBTI was 19-fold larger than the amount necessary to inhibit 756 units of trypsin catalytic activity (N₅0-benzoyl-L-arginine ethyl ester, substrate).

Data analysis. For records identified as containing a single channel, Pₒ was measured as the total open time of the channel, normalized to the total time of the recording at a particular test voltage. Channel transitions and open times were measured from idealized records based on 50% threshold criterion. For patches containing multiple channels, evident as two or more simultaneously opened transitions, average channel activity (NPₒ) was analyzed and obtained by integrating the area under the Gaussian curves fitted to the all-points current-amplitude histogram and normalized to the peak area of the baseline current level (i.e., closed channel current level) (4). Data analysis was performed with single-channel analysis software (TAC and TACFit; Bruxton).

Statistics. All results are reported as means ± SE, with n = no. of patches, unless otherwise stated. Because baseline ENaC activity is highly variable (15) from patch to patch, each patch served as its own control. Comparisons of NPₒ were performed using a paired t-test. The effect of trypsin on patches containing GDPβS in the pipette solution (Fig. 3) was evaluated on log-transformed NPₒ data. A P value < 0.05 was considered statistically significant.

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REFERENCES


