Endogenous Protease Activation of ENaC: Effect of Serine Protease Inhibition on ENaC Single Channel Properties

Adedotun Adebamiro,1 Yi Cheng,3 John P. Johnson,2 and Robert J. Bridges3

1Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA 15261
2Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261
3Department of Physiology and Biophysics, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064

Endogenous serine proteases have been reported to control the reabsorption of Na+ by kidney- and lung-derived epithelial cells via stimulation of electrogenic Na+ transport mediated by the epithelial Na+ channel (ENaC). In this study we investigated the effects of aprotinin on ENaC single channel properties using transepithelial fluctuation analysis in the amphibian kidney epithelium, A6. Aprotinin caused a time- and concentration-dependent inhibition (84 ± 10.5%) in the amiloride-sensitive sodium transport (I\textsubscript{Na}) with a time constant of 18 min and half maximal inhibition constant of 1 μM. Analysis of amiloride analogue blocker–induced fluctuations in I\textsubscript{Na} showed linear rate–concentration plots with identical blocker on and off rates in control and aprotinin-inhibited conditions. Verification of open-block kinetics allowed for the use of a pulse protocol method (Helman, S.I., X. Liu, K. Baldwin, B.L. Blazer-Yost, and W.J. Els. 1998. Am. J. Physiol. 274:C947–C957) to study the same cells under different conditions as well as the reversibility of the aprotinin effect on single channel properties. Aprotinin caused reversible changes in all three single channel properties but only the change in the number of open channels was consistent with the inhibition of I\textsubscript{Na}. A 50% decrease in I\textsubscript{Na} was accompanied by 50% increases in the single channel current and open probability but an 80% decrease in the number of open channels. Washout of aprotinin led to a time-dependent restoration of I\textsubscript{Na} as well as the single channel properties to the control, pre-aprotinin, values. We conclude that protease regulation of I\textsubscript{Na} is mediated by changes in the number of open channels in the apical membrane. The increase in the single channel current caused by protease inhibition can be explained by a hyperpolarization of the apical membrane potential as active Na+ channels are retrieved. The paradoxical increase in channel open probability caused by protease inhibition will require further investigation but does suggest a potential compensatory regulatory mechanism to maintain I\textsubscript{Na} at some minimal threshold value.

INTRODUCTION

Absorption of fluids and electrolytes is a function of many epithelia characterized, in many cases, by electrogenic Na+ transport where the rate limiting step is apical membrane entry mediated by the epithelial Na+ channel (ENaC) (Garty and Palmer, 1997). In addition to well-known endocrine regulation of ENaC through intracellular steroid receptors (Garty, 2000) and second messengers such as cAMP (Benos et al., 1996) and Ca\textsuperscript{2+} (Kunzelmann et al., 2001), a new means of regulation by extracellular proteases was recently described (Vallet et al., 1997) and referred to as channel activating protease (CAP) regulation of ENaC. CAP regulation of ENaC may play a role in a variety of physiological functions from blood pressure regulation (Snyder, 2002) to mucociliary clearance in the airways (Boucher, 2004) and hearing (Rossier, 2004). Three putative CAPs, CAP1 (prostasin), CAP2 (TMPRSS4), and CAP3 (matriptase) that stimulate ENaC-mediated amiloride-sensitive Na+ transport (I\textsubscript{Na}) in Xenopus oocytes have been cloned (Vuagniaux et al., 2002). These CAPs were predicted to be membrane-anchored proteins with extracellular serine protease domains. The importance of serine protease activity in ENaC regulation has been demonstrated in renal epithelial cell lines (Vallet et al., 1997; Nakhoul et al., 1998; Vuagniaux et al., 2000) and primary airway cells (Bridges et al., 2001; Donaldson et al., 2002) by serine protease inhibition.

Vallet et al. (2002) reported that both membrane anchoring and proteolytic activity were required for CAP1 activation of ENaC. However, the exogenous addition of chymotrypsin and trypsin have also been shown to stimulate I\textsubscript{Na} in Xenopus oocytes (Chraibi et al., 1998), and trypsin stimulates I\textsubscript{Na} in fibroblasts expressing ENaC (Caldwell et al., 2004). Recently, it was shown that the appearance of apparently smaller molecular weight forms of ENaC from MDCK cells heterologously expressing ENaC could be blocked by mutations that remove putative cleavage sites in ENaC α and γ subunits for the protein convertase furin (Hughey et al., 2004). These mutations were associated...
with a significant decrease of $I_{\text{sc}}$ in *Xenopus* oocytes expressing ENaC. Although exogenous proteases have no effect on the spontaneous $I_{\text{sc}}$ in several native Na+ transporting epithelia, trypsin enhanced recovery of $I_{\text{sc}}$ following inhibition by the serine protease inhibitors aprotinin and bikunin (Vallet et al., 1997; Bridges et al., 2001; Donaldson et al., 2002). Therefore, inhibition of the CAP pathway by specific protease inhibitors is sufficient to inhibit Na+ transport in numerous epithelia.

ENaC regulation by the CAP pathway could be mediated by changes in the single channel current ($I_o$), the open probability ($P_o$), or the number of active channels ($N_T$). Studies using ENaC heterologously expressed in oocytes (Chraibi et al., 1998; Adachi et al., 2001) and fibroblasts (Caldwell et al., 2004) have yielded contradictory results in regards to how extracellular proteases regulate ENaC. In an effort to further clarify how ENaC is regulated by the CAP pathway we have used the amphibian renal epithelial cell line A6 and transepithelial current fluctuation analysis. Our results demonstrate that the protease inhibitor aprotinin reversibly inhibits $I_{\text{sc}}$. The decrease in $I_{\text{sc}}$ was accompanied by a decrease in the number of open channels ($N_o$), an increase in $I_{\text{max}}$ and a paradoxical increase in $P_o$. Because only the decrease in $N_o$ can explain the decrease in $I_{\text{sc}}$ we conclude that the CAP pathway regulates sodium transport by modulating the number of active ENaCs in the apical membrane.

**MATERIALS AND METHODS**

**Cell Culture**

A6 cells were maintained in amphibian medium (Biowhitaker) and 10% FBS (GIBCO BRL) in an incubator with humidified air and 4% CO\textsubscript{2} at 28°C as previously described (Rokaw et al., 1996). The cells were expanded on plastic tissue culture dishes and then seeded on Costar Transwell permeable supports (polycarbonate membrane of 0.4 μm pore size and 1 cm\textsuperscript{2} area). Experiments were performed 14–21 d after seeding on the permeable supports and 24–48 h after media replacement.

**Short-circuit Current ($I_{\text{sc}}$) Measurement**

Costar Transwell cell culture inserts were mounted in Costar Ussing chambers and continuously voltage clamped to 0 mV with an automatic voltage clamp (Department of Bioengineering, University of Iowa). Four millivolt bipolar pulses were applied every minute, generating current deflections used to calculate the trans-epithelial resistance ($R_T$) with Ohms law. $I_{\text{sc}}$ traces were digitized at 10 Hz and recorded using a DASA 6600 acquisition board and Acquire 6600 recording software (Gould Instrument System). The bath solution was identical in the apical and basolateral chambers and contained (in mM) 100 NaCl, 2.4 KHCO\textsubscript{3}, 1 CaCl\textsubscript{2}, 5 glucose. The pH of the solution was 8.0 when gassed with ambient air. All experiments were performed at room temperature.

**Blocker-induced Fluctuation Analysis**

A6 cells on Costar Transwell inserts were placed in Costar Ussing chambers and continuously short circuited with a low noise voltage clamp previously described (Van Driessche and Lindemann, 1978). The $I_{\text{sc}}$ was high-pass filtered to remove the DC component and then subjected to anti-aliasing filtering and amplification. The resulting signal was digitized and Fourier transformed to generate power density spectra (PDS) normalized to the tissue surface area (Van Driessche and Lindemann, 1979). PDS were collected at 0.5 Hz fundamental frequency as averages of 30 sweeps over a frequency range of 0.5–350 Hz. The data was then fitted to a Lorentzian noise function $S_0/(1 + (f/f_0)^2)$ plus a “one over f” component defined as $S_1/f^\alpha$ (Paunesku et al., 2000). “One over f” noise, also called flicker noise, is the excess noise that results from fluctuations in conductance of porous membranes held away from electrical equilibrium that decreases as $1/f$ (DeFelice, 1981). $S_1$ is the low frequency power of the “one over f” noise with its decay characterized by a ranging from 0.5 to 1.5. We determined the plateau power ($S_o$) and corner frequency ($f_0$) from fits of 44 data points in the 0.5–200 Hz range. Blocker-dependent parameters were determined as previously described (Helman and Baxendale, 1990; Els and Helman, 1997; Helman et al., 1998). We determined aprotinin-dependent changes in single channel parameters using two approaches; a cumulative 6-chloro-3,5-diaminopyrazine carboxamide (CDPC) concentration step and a pulse protocol (Helman et al., 1998). CDPC was chosen because its low affinity and high off rate (see below) permit measurements of Lorentzians at relatively uninhibited states and affords a number of experimental advantages. These advantages were well described by Helman and Baxendale (1990). First, low affinity CDPC blockade minimizes the poorly understood “auto-regulatory” responses to significant current inhibition that occurs with higher affinity blockers. Second, CDPC permits simultaneous measurement of changes in steady-state current and blocker kinetics from spectral analysis necessary for the determination of the open probability from the three-state model. Third, a low affinity blocker by virtue of a high off rate reduces the relative error in the estimation of the off rate from the linear regression of the corner frequency versus blocker concentration.

The cumulative concentration step approach was performed by mounting A6 cells in a fixed bath volume (5 mL) where 4 μM aprotinin or PBS was added to the apical side. Apical and basolateral solutions were continuously mixed and oxygenated by a gas lift using air. After incubating in 4 μM aprotinin or PBS for 30 min, increasing doses of CDPC (in DMSO) were added to the apical bath to yield a cumulative increase in CDPC concentration. The concentrations used were 10, 20, 30, 40, and 50 μM CDPC. PDS were obtained 2 min after addition of each dose of CDPC and following establishment of a new steady-state $I_{\text{sc}}$. The ENaC-mediated Na+ current ($I_{\text{sc}}$) was taken as the $I_{\text{sc}}$ before minus the $I_{\text{sc}}$ remaining after addition of 10 μM amiloride and usually accounted for >90% of the $I_{\text{sc}}$. Blocker rate coefficients were calculated from the linear regression of $2\pi f_o$ vs. blocker concentration using a pseudo-first order kinetic description where $2\pi f_o = k_{\text{off}} + k_{\text{on}} \cdot [B]$ ($k_{\text{on}}$ and $k_{\text{off}}$ are the apparent on- and off-coefficients of the blocker (B) interacting with the open channel) and the dissociation constant was calculated as $K_B = k_{\text{off}}/k_{\text{on}}$. For the pulse protocol, the Ussing chamber was modified to reduce the apical volume to 1.5 mL. The cells were continuously perfused at 5 mL/min with ringer containing 10 μM CDPC. At indicated time points, PDS were obtained, the solution was switched to one with 30 μM CDPC, PDS were again obtained, and then the solution was switched back to one with 10 μM CDPC. Three experimental periods were evaluated: (1) control, 30 min of perfusion without aprotinin; (2) aprotinin, 45 min of perfusion with a solution containing 10 μM aprotinin; and (3) washout, 30 min of perfusion without aprotinin. PDS were obtained at 10 and 30 μM CDPC at 10, 20, and 30 min of the control and washout periods and at 25, 35, and 45 min of the aprotinin period. The blocker rate coefficients were calculated from the two blocker concentrations and corner frequencies as:
where $f_1(30)$ and $f_1(10)$ are the corner frequencies at 10 and 30 μM CDPC and $k_D = 2f_1(10) - k_o \cdot 10 \cdot \mu M$. The single channel current amplitude ($i_{Na}$) was calculated as:

\[
i_{Na} = \frac{S_o \cdot (2\pi f_1(10))^2}{4 \cdot i_{Na}(10) \cdot k_o \cdot 10 \cdot \mu M},
\]

(1)

where $i_{Na}$ is the amiloride-sensitive current thus allowing us to determine the number of open channels at 10 μM CDPC as $N_o = i_{Na}(10) / i_{Na}$. It has been extensively verified that CDPC and its analogs interact with ENaC-mediated amiloride-sensitive Na$^+$ transport in a manner described satisfactorily by a three-state model (Lindemann and Van Driessche, 1978; Li and Lindemann, 1983; Abramcheck et al., 1985; Els and Helman, 1989, 1997; Helman and Baxendale, 1990; Els et al., 1991; Baxendale-Cox et al., 1997; Helman et al., 1998; Becchetti et al., 2002). The use of a closed-open blocked three-state model instead of two-state or more complicated four-state models is not the focus of this work and arguments for the use of a three-state model of CDPC blockade of $i_{Na}$ in A6 cells can be found in Helman and Baxendale (1990) and Helman et al. (1998). Therefore, the open probability ($P_o$) was calculated assuming a three-state model where the blocker interacts with the open state using:

\[
P_o = \frac{1 - \frac{i_{Na}(30)}{i_{Na}(10)} \cdot K_d}{30 \cdot \mu M \frac{i_{Na}(30)}{i_{Na}(10)} - 10 \cdot \mu M},
\]

(2)

where $i_{Na}(10)$ and $i_{Na}(30)$ are the amiloride-sensitive current at 10 and 30 μM CDPC (Helman et al., 1998). The number of active channels ($N_o$) was calculated from

\[
N_o = N_s \cdot \left( \frac{1}{P_o} + \frac{10}{K_d} \right).
\]

(3)

**Cell Surface Biotinylation and Western Blot Analysis**

Confluent A6 cell monolayers grown on permeable filters (Costar; 75 mm diameter) were treated with PBS, 10 μM aprotinin for 1 h, or 100 mM aldosterone for 6 h and then kept on ice. Cell surface biotinylation and Western blot analysis of ENaC subunits were performed according to the modified method as previously described (Gottardi et al., 1995; Hanwell et al., 2002). In brief, cells were washed three times in ice-cold PBS-CM (PBS with 1 mM MgCl$_2$ and 0.1 mM CaCl$_2$ and incubated with 1.5 mg/ml EZ-link Sulfo-NHS-S-S-biotin (Pierce Chemical Co.) and detected with X-Omat Blue XB-1 imaging film (Kodak). Signal intensities were quantified with scanning densitometry (Bio-Rad Laboratories).

**Results**

**Effect of Aprotinin on $i_{Na}$**

Apical administration of 10 μM aprotinin resulted in a decrease of $i_{Na}$. Short circuit current traces of the effects of PBS and aprotinin on A6 cell sodium transport are shown in Fig. 1 (A and B). After a 25–30-min equilibration period following initiation of transepithelial voltage clamping, PBS or aprotinin was added to the apical side for 50 min followed by 10 μM amiloride to obtain a measure of net electrogenic sodium transport mediated by the amiloride-sensitive sodium channel ENaC ($i_{Na}$). We verified that under these experimental conditions >90% of the $i_{Sc}$ was amiloride sensitive (unpublished data). Thus any change in the $i_{Sc}$ can be attributed to a change in $i_{Na}$. The $i_{Na}$ taken after the equilibration period is referred to as the control $i_{Na}$. As can be seen, aprotinin caused a time-dependent decrease in the $i_{Sc}$ that was not observed in the PBS (vehicle control) treated cells. The decrease in $i_{Na}$ caused by aprotinin was apparent following a variable short lag phase of ~30 s. In this subset of experiments, the PBS-treated monolayers had a control $i_{Na}$ of 8.4 ± 1.36 μA/cm$^2$ (n = 12) that was not significantly changed (8.9 ± 1.08 μA/cm$^2$, P > 0.05) 50 min after addition of PBS but was reduced to 0.8 ± 0.13 μA/cm$^2$ upon addition of 10 μM amiloride. Parallel experiments with 10 μM aprotinin added to the apical side had control $i_{Sc}$ of 7.5 ± 1.25 μA/cm$^2$ (n = 12) that was reduced to 2.2 ± 0.55 μA/cm$^2$ (P < 0.01) 50 min after addition of aprotinin and further reduced to 0.5 ± 0.12 μA/cm$^2$ with the addition of 10 μM amiloride. Thus $i_{Na}$ was reduced from 8.1 ± 1.1 μA/cm$^2$ in the PBS-treated cells to 1.7 ± 0.53 μA/cm$^2$ in the aprotinin-treated cells. These results are summarized in Fig. 1 C.
The inhibition of \( I_{Na} \) by aprotinin was time dependent. The decrease in \( I_{Na} \) as a percentage of control \( I_{Na} \) following addition of 10 \( \mu \)M aprotinin was fitted to an exponential decay (% control = \( a \exp(-t/\tau) + b \)) as illustrated in Fig. 2 A. Using the period starting 5 min and ending 45 min after addition of aprotinin, \( \tau \) was 18 ± 1.2 min. Aprotinin (10 \( \mu \)M) inhibited 84 ± 10.5% of the control \( I_{Na} \) in this subset of filters. The effect of aprotinin on \( I_{Na} \) was also concentration dependent (Fig. 2 B). The half-maximal inhibition constant (\( K_{1/2} \)), determined by fitting the \( I_{Na} \) as a percentage of control \( I_{Na} \) versus aprotinin concentration to a simple inhibition curve (% control = \( aK_{1/2}/(K_{1/2} + [\text{aprotinin}]) + b \); see Fig. 2 legend), was 1.0 ± 0.13 \( \mu \)M, with a maximal inhibition of 80 ± 12%. The kinetic fits suggest a high-affinity inhibition of the majority of the \( I_{Na} \) by aprotinin. RT was maintained after addition of 10 \( \mu \)M aprotinin. The decrease in \( I_{Na} \) caused by aprotinin was accompanied by a tendency toward increasing RT particularly in filters that had relatively high control \( I_{Na} \). The RT before addition of PBS and aprotinin was 5.15 ± 0.46 and 4.90 ± 0.57 kΩ cm\(^2\) respectively. Following the addition of PBS and aprotinin for 50 min the RT was 4.6 ± 0.46 and 6.1 ± 0.53 kΩ cm\(^2\), respectively, thus the integrity of the epithelial monolayer was not compromised by aprotinin incubation.

Effect of Aprotinin on Blocking of ENaC by the Amiloride Analogue CDPC

The blockade of \( I_{Na} \) by CDPC for PBS and aprotinin-treated A6 was first measured by a cumulative concentration response determination using a step protocol. PBS or 4 \( \mu \)M aprotinin was introduced to the apical side of the A6 filters for 30 min before commencing the CDPC concentration step protocol, which was performed over 30 min. The concentration of 4 \( \mu \)M aprotinin was used because this concentration was capable of significantly inhibiting \( I_{Na} \) but sufficient current remains at the higher CDPC blocker concentrations to ensure adequate measurement of the Lorentzian component in the PDS. As shown in Fig. 3 A, PBS-treated cells had significantly higher \( I_{Na} \) (6.1 ± 0.40 \( \mu \)A/cm\(^2\)) compared with aprotinin-treated cells (2.8 ± 0.59 \( \mu \)A/cm\(^2\), \( P < 0.01 \)) at 0 \( \mu \)M CDPC and also at all other concentrations studied (10, 20, 30, 40, and 50 \( \mu \)M). CDPC caused a concentration-dependent decrease in \( I_{Na} \) in both PBS- and aprotinin-treated A6 cells, thus the sensitivity to blockade of \( I_{Na} \) by CDPC was maintained in the presence of aprotinin. The PDS in both PBS- and aprotinin-treated A6 had Lorentzian components that varied with CDPC concentration (Fig. 4, A and B). As expected from a smaller \( I_{Na} \), the Lorentzian \( S_o \) values were also significantly decreased in aprotinin-treated cells (\( P < 0.01 \)) at the blocker concentrations studied. The \( S_o \) values remained biphasic with respect to blocker concentration in both the PBS and aprotinin studies. The data were fitted to the equation describing the \( S_o \) of a two-state kinetic scheme and a biphasic curve was obtained. The \( S_o \) maxima were at 18.7 ± 0.28 \( \mu \)M and 15.5 ± 0.71 \( \mu \)M CDPC for PBS- and aprotinin-treated filters.
A6, respectively (Fig. 3 B). Since the maximum value of $S_0$ occurs at the blocker concentration of 0.5$k_{\text{off}}/k_{\text{on}}$, the lack of a change in the blocker concentration at maximum $S_0$ in the absence or presence of aprotinin showed the ratio $k_{\text{off}}/k_{\text{on}}$ and hence the dissociation constant of the blocker was unaltered. The second indication came from measurement of the $2\pi f_c$ values at the CDPC concentrations used. For both PBS- and aprotinin-treated A6 cells, the $2\pi f_c$ increased linearly with respect to blocker concentration. A linear regression of the data demonstrated that the apparent rate
plus low frequency “1/f” noise. The solid lines represents the fit of the data to the sum of Lorentzian

Figure 4. Typical current noise power spectral density in the absence (solid squares; 3.87 µA/cm² Iₙa) and presence (open squares; 1.67 µA/cm² Iₙa) of aprotinin are shown at 10 µM CDPC (A) and 30 µM CDPC (B). Corner frequencies were 47.6 and 50.2 Hz (A) (at 10 µM CDPC) in and 72.4 and 72.7 Hz (B) (at 30 µM CDPC) in the absence and presence of aprotinin, respectively. The solid lines represents the fit of the data to the sum of Lorentzian plus low frequency “1/f” noise.

coefficients were not different (Fig. 3 C). The k.off and k.on were 242 ± 5.40 s⁻¹ and 7.8 ± 0.19 µM⁻¹s⁻¹ for PBS experiments (n = 8). These constants were not significantly different in aprotinin experiments (n = 6) at 240 ± 3.78 s⁻¹ and 8.6 ± 0.15 µM⁻¹s⁻¹, respectively (P > 0.05). The blocker dissociation constant Kₐ was 31.2 and 28.1 µM CDPC for PBS- and aprotinin-treated A6 cells, respectively. The biphasic dependence of Sₒ on blocker concentration and the linear dependence of 2πfc on blocker concentration indicated a simple pseudo first order blocker–ENaC binding in PBS- and aprotinin-treated A6. Consequently, only two concentration points were required to determine the blocking kinetics as well as the single channel parameters as previously described (Helman et al., 1998). To avoid autoregulatory responses of Iₙa to prolonged blocker-induced decreases in Iₙa caused by cumulative increases in blocker concentration (Abramcheck et al., 1985; Tang et al., 1985; Els et al., 1991), a pulse-protocol approach was employed to measure the single channel properties.

Single Channel Properties: CDPC Pulse Protocol
To avoid blocker-induced autoregulation of ENaC and to study the single channel properties of the same cells in control and aprotinin-treated periods as well as measure reversibility of the aprotinin effect, a pulse protocol approach was employed pulsing between 10 and 30 µM CDPC (Helman et al., 1998). Typical PDS in control and aprotinin-treated periods at 10 and 30 µM CDPC are shown in Fig. 4. The two CDPC concentrations were chosen because they were close to the Sₒ maxima as shown in the step protocol experiments (Fig. 3 B), thus providing the highest signal for the Lorentzian component in the PDS while producing measurable changes in Iₙa and significant changes in 2πfc. Secondarily, 10 and 30 µM CDPC could be used in both the control and aprotinin periods because the blocker kinetics were identical in the absence and presence of aprotinin. The Iₙa, iNa, Pₒ, and Nₒ were measured before (control), while perfusing with aprotinin (aprotinin), and following removal of aprotinin from the apical side (washout) in eight filters as illustrated in Fig. 5. The Iₙa was 3.4 ± 0.17 µA/cm² under control conditions and was reduced to 1.4 ± 0.17 µA/cm² 35 min after addition of 10 µM aprotinin (P < 0.01). Following washout of aprotinin, Iₙa recovered to control levels (3.2 ± 0.11 µA/cm²). The recovery appears to be complete by 10 min after washout as subsequent time points did not show further increases in Iₙa. The iNa was 0.46 ± 0.037 pA in the control period, increased to 0.70 ± 0.069 pA (P < 0.05) 35 min after addition of aprotinin, and returned to 0.45 ± 0.018 pA following removal of aprotinin. The Pₒ was 0.23 ± 0.031 before aprotinin addition, approached a maximum of 0.41 ± 0.075 (P < 0.05) 15 min after aprotinin addition, and then returned to 0.23 ± 0.019 after removal of aprotinin. The changes in Nₒ paralleled changes in Iₙa with Nₒ of 8.03 ± 0.812 before, 2.26 ± 0.523 (P < 0.01) after aprotinin addition, and 7.21 ± 0.445 million channels/cm² following washout of aprotinin. Since changes in Iₙa cannot be explained by changes in iNa or Pₒ, these changes must result from changes in NₖT as shown in Fig. 6. NₖT was 42 ± 8.24 million channels/cm² before aprotinin addition. NₖT was reduced to 8.93 ± 3.351 million channels/cm² after addition of aprotinin and returned to 34.4 ± 5.418 million channels/cm² following aprotinin washout.

Relationship between Single Channel Properties and Iₙa
To examine how the single channel properties change with Iₙa, we quantified their relationship to Iₙa by lin-
ear regression analysis using the experimental dataset shown in Fig. 5. Because of the apparent differences in the relationship of the single channel properties to $I_{\text{Na}}$ in the control, aprotinin, and washout conditions we determined the regressions using data points in each condition. The linear parameters are summarized in Table I. The values of $i_{\text{Na}}$ versus $I_{\text{Na}}$ in the control and aprotinin-treated conditions are plotted in Fig. 7 A. Over the range of control $I_{\text{Na}}$ ($2.78$–$4.17 \mu \text{A/cm}^2$), $i_{\text{Na}}$ decreased with increases of $I_{\text{Na}}$ ($r = 0.62$). The $i_{\text{Na}}$ also decreased with increases of $I_{\text{Na}}$ ($r = 0.80$) over the aprotinin $I_{\text{Na}}$ range ($0.87$–$2.08 \mu \text{A/cm}^2$). However, the slope was approximately threefold steeper and the intercept $\sim 50\%$ higher during the aprotinin treatment period compared with the control period. No correlation was observed between $i_{\text{Na}}$ and $I_{\text{Na}}$ during the wash condition ($r = 0.02$). Plotted also are the $P_0$ values versus $I_{\text{Na}}$ in the control and aprotinin-treated conditions (Fig. 7 B). The $P_0$ also decreased with increases of $I_{\text{Na}}$ ($r = -0.46$) in the control condition as well as in the presence of aprotinin ($r = -0.75$). In the presence of aprotinin, the slope was approximately fourfold steeper and the intercept increased by $\sim 100\%$. No correlation was observed between $P_0$ and $I_{\text{Na}}$ in the wash condition ($r = -0.17$). We also plotted the $N_0$ versus $I_{\text{Na}}$ in the control and aprotinin-treated conditions (Fig. 7 C). We found that $N_0$ increased with increases of $I_{\text{Na}}$ in the control condition ($r = 0.86$). This positive correlation remained in the aprotinin ($r = 0.92$) and wash conditions ($r = 0.61$) so that transport is determined primarily by changes of $N_0$ in each condition studied.

Cell Surface Expression
The above results indicate that aprotinin alters the number of active channels in the apical membrane. To determine if this effect is also reflected in changes in ENaC apical membrane protein levels we performed cell surface protein biotinylation studies. We biotinylated apical membrane proteins of A6 cells grown on permeable supports (75 mm diameter). Biotinylated proteins were recovered with streptavidin–agarose beads...
and we determined subunit density by Western blotting using specific antibodies to each of the ENaC subunits α, β, γ as described by Alvarez de la Rosa et al. (2002, 2004), Gottardi et al. (1995), and Hanwell et al. (2002). Western blots were examined from A6 cells treated with PBS or 10 μM aprotinin for 1 h, or cells treated with 100 nM aldosterone for 6 h. There were two immunoreactive bands for the α subunit at 85 and 65 kD, two for the β subunit at 115 and 100 kD, while one predominant band at 90 kD was observed for the γ subunit. As shown in Fig. 8, quantification of the relative changes in apical membrane subunits indicated that aldosterone increased subunit density two to fourfold as previously reported by Alvarez de la Rosa et al. (2002). In contrast, aprotinin had no detectable effect on the cell surface density of any of the three ENaC subunits. The results summarized in Fig. 8 represent three independent experiments. Alvarez de la Rosa et al. (2002) reported that aldosterone increased cell surface expression of ENaC subunits proportional to an observed increase in INa. These results confirm that it is possible by biochemical methods to detect changes in NT and thereby INa when A6 cells are stimulated with aldosterone. In contrast, the large decreases in NT in the presence of aprotinin did not correlate with a biochemically detectable decrease in cell surface subunit density.

**DISCUSSION**

Aprotinin is a 6.5-kD protein that is a potent and reversible Kunitz type inhibitor of several serine proteases including trypsin, plasmin, and kallikreins (Vogel and Werle, 1970). The inhibition of Na+ transport by apical administration of aprotinin has been reported in toad urinary bladder (Orce et al., 1980), the A6 cell line (Vallet et al., 1997), the mouse cortical collecting duct cell mpkCCD14 cell line (Liu et al., 2002), human bronchial epithelial cells (Bridges et al., 2001; Donaldson et al., 2002), and rat and mouse lung alveolar epithelial cells (Planes et al., 2005). In all cases, aprotinin inhibition of INa was much slower than the effect caused by the ENaC blocker amiloride. Direct inhibitors of ENaC such as extracellular cations (Chraibi and Horisberger, 2002; Caci et al., 2003) and covalent modification of thiol groups (Snyder, 2000; Snyder et al., 2000) typically demonstrate a rapid time course. Also, evidence of blockade in the form of aprotinin-induced Lorentzians in the PDS or apparent changes in the CDPC-induced Lorentzian that may result from aprotinin competitive inhibition of ENaC (Li et al., 1982) were absent. Inhibition of INa by aprotinin was evident within 10 min of addition and approached a nonzero nadir with time. The inhibition was characterized by a time constant of ~18 min. The plateau level of inhibition was concentration dependent with high affinity and a maximum inhibition of 70–80%. The micromolar affinity reported here is consistent with measurements performed on the urinary toad bladder (Orce et al., 1980). The concentration dependence and time course suggests that aprotinin inhibits a specific protease-dependent pathway that regulates a large fraction of the epithelial Na+ transport. The identity of this regulatory protease(s) is a matter of some conjecture.

Kallikrein-like protease activity has been identified at the apical surface and in the apical medium of A6 cells forming a tight monolayer as well as from toad urinary bladder (Jovov et al., 1990). The kallikrein-like protease activity was inhibited by aprotinin at a concentration that inhibited Na+ transport in A6 cells and toad bladder (Margoliou and Chao, 1980). Further, exogenous addition of trypsin and chymotrypsin have been shown to activate INa in epithelia pretreated with protease inhibitors and activate Na+-mediated currents in *Xenopus* oocytes expressing ENaC (Vallet et al., 1997; Bridges et al., 2001; Donaldson et al., 2002; Liu et al., 2002). Vallet et al. (1997), using expression cloning methods, identified a channel activating protease (CAP) from A6 cells that increased ENaC-mediated INa.
in *Xenopus* oocytes. The mammalian homologue of xCAP1 is prostasin/hCAP1 and coexpression of prostasin with ENaC also increases Na\(^+\)-mediated currents (Vuagniaux et al., 2000; Adachi et al., 2001). Similar to the secretion of kallikrein-like proteases by amphibian renal cells, prostasin has been found to be secreted in an aldosterone-regulated manner in rat urine (Narikiyo et al., 2002). Bikunin, a Kunitz type serine protease inhibitor with two Kunitz domains, has a submicromolar potency for inhibiting Na\(^+\)/H\(^+\)-mediated transport in human bronchial epithelia (Bridges et al., 2001) and this correlates with the nanomolar inhibition constant reported for aprotinin inhibition of prostasin enzymatic activity in vitro (Shipway et al., 2004). These findings support the idea that prostasin and CAP1 are endogenous proteases that can regulate epithelial Na\(^+\)/H\(^+\) transport. Consistent with this view is that RNA silencing of CAP1 was sufficient to inhibit INa in the CF15 airway epithelial cell line to the extent observed with aprotinin on A6 and HBE cells (Tong et al., 2004). Expression cloning has identified two other such CAPs in mammalian renal cells that are capable of stimulating current in *Xenopus* oocytes coexpressing ENaC in an aprotinin-sensitive manner (Vuagniaux et al., 2002). Another possible protease that may regulate Na\(^+\)/H\(^+\) transport is the ubiquitous convertase furin, which modulated ENaC-mediated currents in the Chinese hamster ovary expression system (Hughey et al., 2004). However furin is not inhibited by aprotinin; consequently, the aprotinin inhibition of INa cannot be attributed to a furin pathway.
of channel activation in A6 cells and human bronchial epithelia. The relative importance of the individual proteases and the mechanism of their action in regulating \(Na^+\) transport are yet to be determined. The studies reported here were designed to investigate the mechanism of action of apro tinin inhibition of epithelial \(Na^+\) transport and thereby how apro tinin-sensitive proteases regulate \(Na^+\) transport.

**Single Channel Properties**

\(Na^+\) transport is a function of the single channel properties and can be expressed as \(I_{Na} = N_T * P_o * i_{Na}\). Using noise analysis we studied the apical \(Na^+\) channels in the presence and absence of apro tinin and found that the decrease in \(I_{Na}\) caused by apro tinin can be explained by a decrease in the number of open channels \(N_o\) and these effects of apro tinin were fully reversible upon apro tinin washout. In contrast, apro tinin caused both \(i_{Na}\) and \(P_o\) to increase, and thus these changes cannot account for the decrease in \(I_{Na}\). The \(i_{Na}\) averaged 0.45 pA under control conditions in the present study, which is similar to previously reported values in short-circuited A6 cells by noise analysis (Helman et al., 1998; Alvarez de la Rosa et al., 2004). Given the \(i_{Na}\) of 0.45 pA under control conditions and a single channel conductance of 4-6 pS for the highly selective \(Na^+\) channel in A6 cells (Palmer, 1992; Puoti et al., 1995), a driving force of \(<90\) mV can be estimated for apical \(Na^+\) entry. A 90-mV effective electromotive force for \(Na^+\) movement across the apical membrane has been reported in A6 cell monolayers with resistances and \(I_{Na}\) in the range that we have measured (Granitzer et al., 1991). The average \(i_{Na}\) presented here is therefore consistent with the characteristic driving forces across the apical membrane of A6 cell monolayers and the conductance of sodium channels. Application of apro tinin to the apical bath caused a significant increase in \(i_{Na}\). Therefore, the inhibition of \(I_{Na}\) by apro tinin cannot be explained by changes in \(i_{Na}\). By definition, \(N_o = N_T * P_o * i_{Na}\), where \(N_T\) is the number of electrically detectable or active channels gating between the open and closed states. A decrease in \(N_o\) may arise from a decrease in \(N_T\) or a decrease in \(P_o\) of the active channels. The \(P_o\) of active channels at the apical membrane was measured and in the control condition averaged 0.2. This value of \(P_o\) is well within the range that has been measured by noise analysis and patch clamping for ENaC (Helman and Kizer, 1990). With application of apro tinin, the \(P_o\) doubled. The increase in \(P_o\) would be expected to increase \(N_o\) and \(I_{Na}\); however, apro tinin causes a reduction in \(N_o\) and \(I_{Na}\). Therefore, apro tinin inhibition of \(I_{Na}\) is explained by a decrease in \(N_T\) (Fig. 5).

To further elucidate the relationship between \(I_{Na}\) and the single channel properties we examined the individual data points from our pulse-protocol experiments. Considering the control conditions, we found a weak negative correlation between \(i_{Na}\) and \(I_{Na}\). A negative correlation is expected because increased \(Na^+\) transport rates depolarize the cells, reducing the electromotive force across the \(Na^+\) channels (Granitzer et al., 1992; Blazer-Yost and Helman, 1997). The strength of the correlation is expected to be weak because the driving force across the apical membrane is variable due in part to a high variation in the basolateral resistance from one monolayer to another (Granitzer et al., 1991). Following apical administration of apro tinin, the negative correlation between \(i\) and \(I_{Na}\) became stronger. Such a correlation cannot explain the decrease in \(I_{Na}\). It however suggests that the effect on \(i_{Na}\) is not simply due to the presence of apro tinin but follows the amount of inhibition of \(I_{Na}\) caused by the presence of apro tinin. It was found that a negative correlation occurs between the ratio \(I_{Na}\)control/\(I_{Na}\)aprotinin and \(i_{Na}\)control/\(i_{Na}\)aprotinin, thereby confirming that inhibition of \(I_{Na}\) by apro tinin produces the steep inverse relationship seen in the presence of apro tinin. In a similar manner, the \(P_o\) from cells under control conditions showed significant variation but a weak negative correlation was evident. This increasing \(P_o\) with decreasing \(I_{Na}\) may be related to a previously reported hyperpolarization-induced increase in ENaCPo (Palmer and Frindt, 1988, 1996). As observed in the case of \(i_{Na}\), with apro tinin treatment, the negative correlation between \(P_o\) and \(I_{Na}\) was intensified, supporting the notion that the \(P_o\) change accompanies the decreasing \(I_{Na}\) and is not simply due to the presence of apro tinin. For \(N_o\), we found a strong positive correlation with \(i_{Na}\) both in the control condition and the in the presence of apro tinin. Since the apical conductance has been shown to be strongly positively correlated with \(I_{Na}\) in A6 cells (Granitzer et al., 1991; Helman and Liu, 1997) and the apical conductance is in large part given by the product of \(N_o\) and the single channel conductance, it is expected that under control conditions \(N_o\) would have a positive correlation with \(i_{Na}\). This correlation persists following treatment with apro tinin. The decrease in \(I_{Na}\) following apro tinin treatment is only explained by a decrease in \(N_o\) that results from a decrease in \(P_o\).

**Mechanism of Inhibition of \(N_o\) by Aprotinin**

Out of several possible explanations for the decrease in \(N_o\) caused by apro tinin, an intriguing explanation is that at the apical membrane there is a turnover of active channels into inactive channels in the presence of apro tinin. The inactive channels can be considered to be “capped” while the active channels are “uncapped.” Because multiple proteases have been shown to increase ENaC activity in a variety of systems in a manner dependent on their proteolytic activity, it is possible that ENaC is the substrate for proteolytic uncapping.
Alternatively, ENaC unapping may occur through proteolysis of a closely associated ENaC regulatory protein, proteolytic generation of a stimulatory ligand, protein–protein interactions of ENaC extracellular domain and the protease, or through generation of intracellular second messengers. Activation of the trypsin receptor protease-activated receptor 2 (PAR2) does not stimulate ENaC (Danahay et al., 2001) and ENaC activation is independent of PAR2 (Chraibi et al., 1998). Furthermore, ENaC activation by serine proteases is independent of G protein–coupled receptors in oocytes (Chraibi et al., 1998) and fibroblasts (Caldwell et al., 2004), yet to date all known PARs are G protein–coupled receptors. There is no finding to support an intracellular second messenger requirement for the protease-mediated unapping of ENaC. Protein–protein interactions in the extracytoplasmic domain between the protease dipeptidyl aminopeptidase-like protein, DPPX, and neuronal A-type K⁺ channels has been implicated in the redistribution of the neuronal channels from the ER to the plasma membrane as well as in the regulation of channel gating (Nadal et al., 2003). The function of DPPX is not associated with proteolytic activity; however it remains to be determined that the protease-like domain is required for DPPX channel-regulating activity. It is difficult to imagine that apically administered aprotinin is rapidly trafficked to the ER where it disrupts a possible DPPX-like interaction of ENaC with a serine protease or that once disrupted, reconstitution and trafficking occur on the time scale observed with exogenous protease reactivation of aprotinin inhibited Iₙa in epithelia cells. Trafficking from a compartment closer to the apical membrane or direct activation by the protein–protein interaction may cause the unapping event. If aprotinin inhibits a protease-dependent trafficking of ENaC to the apical membrane from intracellular pools, then the physical channel density is expected to decrease. The quantitation of apical membrane ENaC subunits by cell surface biotinylation, however, shows that the steady-state densities of α, β, and γ subunits were unchanged in the presence of aprotinin. Thus it appears unlikely that aprotinin mediates ENaC trafficking to alter steady-state sodium current in A6 cells. Recent investigations suggest that the biochemically detected apical membrane protein subunit density may be one to two orders of magnitude greater than electrically detectable channels (Alvarez de la Rosa et al., 2004). Although the aldosterone-mediated changes in subunit density and Iₙc are detectable, a similar correlation could not be detected for the aprotinin-mediated changes in Iₙc. These results may mean aprotinin does not alter cell surface expression of ENaC subunit or that any changes are beyond the resolution of present biochemical methods. The high level of subunit protein at the apical surface would also impair the detection of proteolytically cleaved ENaC subunits.

There is, however, some evidence to support the idea that unapping may occur through proteolysis of ENaC. A low apparent molecular weight form of the γ subunit immunoreactive band on Western blots was induced by aldosterone (Masilamani et al., 1999). The new size was consistent with excision of an amino-terminal region immediately after the first transmembrane domain. Aldosterone up-regulation of CAP1 (Narikiyo et al., 2002) and subsequent ENaC cleavage may explain the observations by Masilamani et al. (1999). Molecular weight reductions attributable to proteolytic cleavages in α and γ subunits in MDCK cells expressing ENaC were recently reported and found critical for channel activity (Hughey et al., 2003, 2004). In A6 cells, a fast and slow migrating α ENaC immunoreactive band has been reported with the fast form seen primarily at the apical membrane (Alvarez de la Rosa et al., 2002). However, this fast migrating band form appears to result from the formation of stable disulfide bonds following maturation. Although extensive processing of ENaC resulting in the change of the apparent molecular weight of its subunits have been observed, the data remains inconclusive as to whether ENaC is proteolytically cleaved in A6 cells and whether the cleavage results in the phenomenon we term unapping. Independent of the mechanism, protease-mediated unapping was apparently irreversible in oocyte studies, suggesting that channels cannot be recapped (Chraibi et al., 1998).

A simple hypothesis that explains aprotinin inhibition of Iₙa is that endocytosis of unapped channels (i.e., active channels) is responsible for the loss of current in the presence of aprotinin, as aprotinin prevents the unapping of capped channels (i.e., inactive channels) newly inserted into the apical membrane. The half-life (11–17 min) measured for the α, β, and γ ENaC subunits in the A6 apical membrane (Alvarez de la Rosa et al., 2002) is in good agreement with the time constant (18 min) for the decay in Iₙc following administration of aprotinin. Taking changes in Iₙc as a measure of the changes in Nₜ, the decrease is consistent with the retrieval of active channels from the apical membrane. This implies that aprotinin prevents a constitutive replacement of active channels while retrieval of already activated channels continues leading to an apparent turnover of unapped channels into capped channels. If this notion is correct, aprotinin should not cause a change in cell surface protein subunit density, an expectation consistent with the biotinylation studies shown in Fig. 8. However, much higher half-life values have been reported for ENaC subunits (>24 h for α and γ, 6 h for β) in the apical membrane of A6 cells (Weisz et al., 2000; Kleyman et al., 2001). The discrep-
ancies in the measurements of ENaC subunit half-lives at the apical membrane are yet to be resolved. Since subunit half-life measurements do not necessarily correlate with the actual half-life of an active channel, we emphasize that the remarkable agreement of the estimate of the time constant for the inhibition of $I_{\text{Na}}$ by aprotinin with the biochemical half-life observed by Alvarez de la Rosa et al. (2002) provides a working hypothesis whereby we can test the requirement of trafficking in the regulation of ENaC by endogenous proteases.

A protease-mediated uncapping that increases the $N_T$ is at odds with recent findings that trypsin stimulates increases in "NP o", which is equivalent to $N_o$ (Caldwell et al., 2004). However, the increase in $N_P$ was only demonstrated for channels with very low $N_P$, resulting in 10–100-fold increases of $N_P$. Inhibition by aprotinin did not reveal the presence of these 100-fold lower $P_o$ "near-silent" channels. If these very low $P_o$ channels were present in the absence or in the presence of aprotinin, they probably contribute a very small fraction to the total $I_{\text{Na}}$. If the near-silent channels are present, they do not explain the aprotinin-insensitive current and would not be detected in the presence of high $P_o$ channels remaining in the membrane. From the perspective of transepithelial blocker–induced fluctuation analysis, the near-silent channels if present are virtually inactive. Consequently, the reduction of $N_T$ measured here is not inconsistent with a turnover of channels with high $P_o$ to near-silent channels in the presence of aprotinin. However, our results suggest that the higher $P_o$ channels observed in the presence of aprotinin results either from functional heterogeneity of the Na$^+$ channels in the apical membrane or from compensatory increases in the $P_o$ of active channels remaining in the membrane.

In conclusion, our data suggest there is an aprotinin-sensitive protease in the apical membrane that serves to uncap newly inserted inactive ENaC channels. It remains of interest to show whether the size of the un-capped (active) channel pool is regulated by direct proteolytic activation of apical membrane channels or via the proteolysis of coprotein(s) that in turn regulate channel activity. The possible involvement of intermediate second messengers in the protease-mediated regulation of ENaC channels will also require further investigation.

We are grateful to W. Van Driessche (K.U. Leuven, Leuven, Belgium) for providing the A6 cells. This work was supported by National Institutes of Health grants R01-IR01DK61639 (to R. Bridges) and NRSFSCIDK015311 (to A. Adebamiro) and by the UNCF-Merck graduate research fellowship award (to A. Adebamiro).

Olaf S. Andersen served as editor.
Els, W.J., and S.I. Helman. 1989. Regulation of epithelial sodium channel densities by vasopressin signalling. *Cell. Signal.* 1:533–539.

Els, W.J., and S.I. Helman. 1997. Dual role of prostaglandins (PGE2) in regulation of channel density and open probability of epithelial Na+ channels in frog skin (R. pipiens). *J. Membr. Biol.* 155:75–87.

Els, W.J., S.I. Helman, and T. Mencio. 1991. Activation of epithelial Na channels by hormonal and autoregulatory mechanisms of action. *J. Gen. Physiol.* 98:1197–1220.

Garty, H. 2000. Regulation of the epithelial Na+ channel by aldosterone: open questions and emerging answers. *Kidney Int.* 57:1270–1276.

Garty, H., and L.G. Palmer. 1997. Epithelial sodium channels: function, structure, and regulation. *Physiol. Rev.* 77:359–396.

Gottardi, C.J., L.A. Dunbar, and M.J. Caplan. 1995. Biotinylation and assessment of membrane polarity: caveats and methodological concerns. *Am. J. Physiol.* 268:F285–F295.

Granitz, M., T. Leal, W. Nagel, and J. Crabbe. 1991. Apical and basolateral conductance in cultured A6 cells. *Pflugers Arch.* 417:463–468.

Granitz, M., W. Nagel, and J. Crabbe. 1992. Basolateral membrane conductance in A6 cells: effect of high sodium transport rate. *Pflugers Arch.* 420:559–565.

Hanwell, D., T. Ishikawa, R. Saleki, and D. Rotin. 2002. Trafficking and cell surface stability of the epithelial Na+ channel expressed in epithelial Madin-Darby canine kidney cells. *J. Biol. Chem.* 277:9772–9779.

Helman, S.I., and L.M. Baxendale. 1990. Blocker-related changes of channel density. Analysis of a three-state model for apical Na channels of frog skin. *J. Gen. Physiol.* 95:647–678.

Helman, S.I., and N.L. Kizer. 1990. Apical Sodium Ion Channels of Tight Epithelia as Viewed from the Perspective of Noise Analysis. *Vol. 37. Academic Press, San Diego.* 117–155.

Helman, S.I., and X. Liu. 1997. Substrate-dependent expression of Na+ transport and shunt conductance in A6 epithelia. *Am. J. Physiol.* 273:C434–C441.

Helman, S.I., X. Liu, K. Baldwin, B.L. Blazer-Yost, and W.J. Els. 1998. Time-dependent stimulation by aldosterone of blocker-sensitive ENaCs in A6 epithila. *Am. J. Physiol.* 274:C947–C957.

Hughey, R.P., J.B. Bruns, C.L. Kinlough, K.L. Harkerod, Q. Tong, M.D. Carattino, J.P. Johnson, J.D. Stockand, and T.R. Kleyman. 2004. Epithelial sodium channels are activated by furin-dependent proteolysis. *J. Biol. Chem.* 279:18111–18114.

Hughey, R.P., G.M. Mueller, J.B. Bruns, C.L. Kinlough, P.A. Poland, K.L. Harkerod, M.D. Carattino, and T.R. Kleyman. 2003. Maturation of the epithelial Na+ channel involves proteolytic processing of the α- and γ-subunits. *J. Biol. Chem.* 278:37073–37082.

Jovov, B., N.K. Wills, P.J. Donaldson, and S.A. Lewis. 1990. Vectorial secretion of a kalikrein-like enzyme by cultured renal cells. *I. General properties. Am. J. Physiol.* 259:C869–C882.

Kleyman, T.R., J.B. Zuckerman, P. Middleton, K.A. McNulty, B. Hu, X. Su, B. An, D.C. Eaton, and P.R. Smith. 2001. Cell surface expression and turnover of the α-subunit of the epithelial sodium channel. *Am. J. Physiol. Renal Physiol.* 281:F213–F221.

Kunzelmann, K., R. Schreiber, and A. Boucherot. 2001. Mechanisms of the inhibition of epithelial Na+ channels by CFTR and purinergic stimulation. *Kidney Int.* 60:455–461.

Li, J.H., and B. Lindemann. 1983. Competitive blocking of epithelial sodium channels by organic cations: the relationship between macroscopic and microscopic inhibition constants. *J. Membr. Biol.* 76:235–251.

Li, J.H., L.G. Palmer, I.S. Edelman, and B. Lindemann. 1982. The role of sodium-channel density in the natriferic response of the toad urinary bladder to an antidiuretic hormone. *J. Membr. Biol.* 64:77–89.

Lindemann, B., and W. Van Driessche. 1978. The mechanism of Na uptake through Na-selective channels in the epithelium of frog skin. *Membrane Transport Processes.* 1:155–179.

Liu, L., K.S. Hering-Smith, F.R. Schiro, and L.L. Hamm. 2002. Serine protease activity in m-1 cortical collecting duct cells. *Hypertension.* 39:860–864.

Margoliou, H.S., and J. Chao. 1980. Amiloride inhibits mammalian renal kallikrein and a kallikrein-like enzyme from toad bladder and skin. *J. Clin. Invest.* 65:1343–1350.

Masalmani, S., G.H. Kim, C. Mitchell, J.B. Wade, and M.A. Knepper. 1999. Aldosterone-mediated regulation of ENaC α, β, and γ subunit proteins in rat kidney. *J. Clin. Invest.* 104:R19–R25.

Nadal, M.S., A. Ozaita, Y. Amarillo, E. Vega-Saenz de Miera, Y. Ma, W. Mo, E.M. Goldberg, Y. Misumi, Y. Ikehara, T.A. Neubert, and B. Rudy. 2003. The CD26-related dipeptidyl aminopeptidase-like protein DPPX is a critical component of neuronal A-type K+ channels. *Neuron.* 37:449–461.

Nakhoul, N.L., K.S. Hering-Smith, C.T. Gambala, and L.L. Hamm. 1998. Regulation of sodium transport in M-1 cells. *Am. J. Physiol.* 275:F998–F1007.

Narikiyo, T., K. Kitamura, M. Adachi, T. Miyoshi, K. Washita, N. Shiraiishi, H. Nonoguchi, L.M. Chen, K.X. Chai, J. Chao, and K. Tomita. 2002. Regulation of prostanoid by aldosterone in the kidney. *J. Clin. Invest.* 109:401–408.

Orce, G.G., G.A. Castillo, and H.S. Margoliou. 1980. Inhibition of short-circuit current in toad urinary bladder by inhibitors of glandular kallikrein. *Am. J. Physiol.* 239:F459–F465.

Palmer, L.G. 1992. Epithelial Na channels: function and diversity. *Annu. Rev. Physiol.* 54:51–66.

Palmer, L.G., and G. Frindt. 1988. Conductance and gating of epithelial Na channels from rat cortical collecting tubule. Effects of luminal Na and Li. *J. Gen. Physiol.* 92:121–138.

Palmer, L.G., and G. Frindt. 1996. Gating of Na channels in the rat cortical collecting tubule: effects of voltage and membrane stretch. *J. Gen. Physiol.* 107:35–45.

Pannecou, T.G., B.L. Blazer-Yost, C.J. Vlahos, and S.I. Helman. 2000. LX-294002-inhibitable PI 3-kinase and regulation of baseline rates of Na+ transport in A6 epithelia. *Am. J. Physiol. Cell Physiol.* 279:C236–C247.

Planes, C., C. Levyraz, T. Uchiha, M.A. Angelova, G. Vuagniaux, E. Hummel, M.A. Matthay, C. Clerici, and B.C. Rossier. 2005. In vitro and in vivo regulation of transepithelial lung alveolar sodium transport by serine proteases. *Am J Physiol Lung Cell Mol Physiol.* 288:L1099–L1109.

Puoti, A., A. May, C.M. Canessa, J.D. Horisberger, L. Schild, and B.C. Rossier. 1995. The highly selective low-conductance epithelial Na channel of Xenopus laevis A6 kidney cells. *Am. J. Physiol.* 269:C188–C197.

Rokaw, M.D., D.J. Benos, P.M. Palevsky, S.A. Cunningham, M.E. West, and J.P. Johnson. 1996. Regulation of a sodium channel-associated G-protein by aldosterone. *J. Biol. Chem.* 271:4941–4946.

Rossier, B.C. 2004. The epithelial sodium channel: activation by membrane-bound serine proteases. *Proc. Am. Thorac. Soc.* 1:4–9.

Shipway, A., H. Danahay, J.A. Williams, D.C. Tully, B.J. Backes, and J.L. Harris. 2004. Biochemical characterization of prostanoid, a channel activating protease. *Biochem. Biophys. Res. Commun.* 324:953–963.

Snyder, P.M. 2000. Liddle’s syndrome mutations disrupt CAM-mediated translocation of the epithelial Na+ channel to the cell surface. *J. Clin. Invest.* 105:45–53.

Snyder, P.M. 2002. The epithelial Na+ channel: cell surface insertion and retrieval in Na+ homeostasis and hypertension. *Endocr. Rev.* 23:258–275.

Snyder, P.M., D.B. Bucher, and D.R. Olson. 2000. Gating induces a
conformational change in the outer vestibule of ENaC. *J. Gen. Physiol.* 116:781–790.

Tang, J., F.J. Abramcheck, W. Van Driessche, and S.I. Helman. 1985. Electrophysiology and noise analysis of K⁺-depolarized epithelia of frog skin. *Am. J. Physiol.* 249:C421–C429.

Tong, Z., B. Ilekk, V.J. Bhagwandin, G.M. Verghese, and G.H. Caughey. 2004. Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 287:L1928–L1935.

Vallet, V., A. Chraibi, H.P. Gaeggeler, J.D. Horisberger, and B.C. Rossier. 1997. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature.* 389:607–610.

Vallet, V., C. Pfister, J. Loffing, and B.C. Rossier. 2002. Cell-surface expression of the channel activating protease xCAP-1 is required for activation of ENaC in the *Xenopus* oocyte. *J. Am. Soc. Nephrol.* 13:588–594.

Van Driessche, W., and B. Lindemann. 1978. Low-noise amplification of voltage and current fluctuations arising in epithelia. *Rev. Sci. Instrum.* 49:53–57.

Van Driessche, W., and B. Lindemann. 1979. Concentration dependence of currents through single sodium-selective pores in frog skin. *Nature.* 282:519–520.

Vogel, R., and E. Werle. 1970. Handbook of Experimental Pharmacology. Bradykinin, Kallidin, and Kallikrein. Vol. 25. Springer, Berlin. 213–249.

Vuagniaux, G., V. Vallet, N.F. Jaeger, E. Hummler, and B.C. Rossier. 2002. Synergistic activation of ENaC by three membrane-bound channel-activating serine proteases (mCAP1, mCAP2, and mCAP3) and serum- and glucocorticoid-regulated kinase (Sgk1) in *Xenopus* oocytes. *J. Gen. Physiol.* 120:191–201.

Vuagniaux, G., V. Vallet, N.F. Jaeger, C. Pfister, M. Bens, N. Farman, N. Courtois-Courty, A. Vandewalle, B.C. Rossier, and E. Hummler. 2000. Activation of the amiloride-sensitive epithelial sodium channel by the serine protease mCAP1 expressed in a mouse cortical collecting duct cell line. *J. Am. Soc. Nephrol.* 11:828–834.

Weisz, O.A., J.M. Wang, R.S. Edinger, and J.P. Johnson. 2000. Non-coordinate regulation of endogenous epithelial sodium channel (ENaC) subunit expression at the apical membrane of A6 cells in response to various transporting conditions. *J. Biol. Chem.* 275:39886–39893.