Sodium self-inhibition of human epithelial sodium channel: selectivity and affinity of the extracellular sodium sensing site

Vincent Bize and Jean-Daniel Horisberger

Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland

Submitted 27 February 2007; accepted in final form 27 July 2007

Bize V, Horisberger J-D. Sodium self-inhibition of human epithelial sodium channel: selectivity and affinity of the extracellular sodium sensing site. Am J Physiol Renal Physiol 293: F1137–F1146, 2007. First published August 1, 2007; doi:10.1152/ajprenal.00100.2007.—The epithelial Na⁺ channel (ENaC) is present in the apical membrane of “tight” epithelia in the distal nephron, distal colon, and airways. Its activity controls the rate of transepithelial sodium transport. Among other regulatory factors, ENaC activity is controlled by the concentration of extracellular Na⁺, a phenomenon named self-inhibition. The molecular mechanism by which extracellular Na⁺ concentration is detected is not known. To investigate the properties of the extracellular Na⁺ sensing site, we studied the effects of extracellular cations on steady-state amiloride-sensitive outward currents in Na⁺-loaded oocytes expressing human ENaC and compared them with self-inhibition of inward current after fast solution changes. About half of the inhibition of outward Na⁺ currents was due to self-inhibition itself and the rest might be attributed to conduction site saturation. Self-inhibition by extracellular Li⁺ was similar to that of Na⁺ except for slightly slower kinetics. Ionic selectivity of the inhibition for steady-state outward current was Na⁺ ≥ Li⁺ > K⁺. We estimated an apparent inhibitory constant (Kᵢ) of ~40 mM for extracellular Na⁺ and Li⁺, and found no evidence for a voltage dependence of the Kᵢ. Protease treatment induced the expected increase of the amiloride-sensitive current measured in high-Na⁺ concentrations which was due, at least in part, to abolition of self-inhibition. These results demonstrate that both self-inhibition and saturation play a significant role in the inhibition of ENaC by extracellular Na⁺ and that Na⁺ and Li⁺ interact in a similar way with the extracellular cation sensing site.

The epithelial sodium channel (ENaC) is a key component of the transepithelial sodium transport in the aldosterone-sensitive distal nephron, the structure responsible for the maintenance of sodium balance, in distal colon, and in airways. The regulation of the transepithelial reabsorption of sodium is mediated mostly through the control of the channel density at the apical membrane of the epithelial cells, which involves several hormones: the adrenal mineralocorticoid aldosterone in distal nephron and colon (15), but also vasopressin and insulin in the distal nephron (10). It also depends on ENaC open probability, which is highly variable and the control of which is still poorly understood (19, 20).

The activity of ENaC is strongly regulated by sodium itself, via two distinct phenomena, feedback inhibition and sodium self-inhibition. The first mechanism displays a slow time course (time constant 10–20 min) due to the increase of the intracellular sodium concentration (1), involves a PY motif in the COOH terminus of the β- and γ-subunit of ENaC, and leads to modulation of apical membrane ENaC density by ubiquitin-mediated control of ENaC trafficking (12, 25).

The second phenomenon, initially described ~30 yr ago in amphibian epithelia (8), was demonstrated by the recording of a large peak of sodium inward current quickly relaxing to a lower steady-state value following the rapid increase of extracellular sodium concentration from 0 to 100 mM (8, 9). Self-inhibition has also been deduced from the observation of differences in the Kᵢ for extracellular sodium of the amiloride-sensitive current between macroscopic current and single-channel current (8, 20). Self-inhibition, the decrease of the inward sodium current observed upon sodium addition or fast amiloride removal, has rapid kinetics, with a time constant of ~3 s, and is temperature dependent, the maximum effect being observed at the mammalian physiological temperature (6). Moreover, as self-inhibition has been detected in vitro, in a heterologous expression system expressing the three α-, β-, and γ-subunits of ENaC, it is most probably intrinsic to ENaC itself and not due to accessory/associated proteins.

Several pieces of evidence show that ENaC can be activated by a proteolytic treatment, such as by trypsin in Xenopus laevis oocytes (7). It has also been shown that the members of the channel activating proteases (membrane-bound serine proteases) are able to increase the amiloride-sensitive current when coexpressed with ENaC in X. laevis oocytes (26, 27). The effects of proteases have been attributed, at least in part, to the relief of self-inhibition after cleavage of the extracellular loops of the channel by trypsin (6) or furin (22).

The physiological role of self-inhibition would be to prevent excessive sodium reabsorption when the luminal sodium concentration rises to high values. Depending on the rate of water and Na⁺ reabsorption, the luminal concentration of Na⁺ may indeed be highly variable in the distal parts of the nephron. Self-inhibition may also ensure a homogenous reabsorption all along the cortical collecting duct (20).

The molecular mechanism of self-inhibition is still unknown. First, it has been confirmed that self-inhibition was directly linked to the extracellular sodium concentration and not to an increase of intracellular sodium (6). As the kinetics (of the order of 0.3 s) are too fast for an internalization of the channels, it has been supposed that the phenomenon was linked to a decrease of the open probability of ENaC. On the other hand, the kinetics are much too slow for a direct block of the pore by sodium ions. Thus the existence of an extracellular Na⁺ sensing site distinct from the conduction site has been proposed (6, 8). Several recent studies (21, 23) have explored...
the possible role of structural elements by observing the effects of site-directed mutations in the extracellular loop of ENaC subunits on self-inhibition. However, the results of these studies do not allow to determine whether the self-inhibition modifying mutations have their effects by altering the Na⁺ sensing site itself or by modification of any subsequent event leading eventually to changes in the channel open probability. Thus the precise nature of the extracellular Na⁺ sensing site and the mechanism responsible for signal transmission to the channel gate control remains unknown.

The aim of this study was thus to characterize the physiological properties of the putative extracellular sodium sensing site in terms of its ionic selectivity and affinity. In addition, we also explored the mechanisms of the effect of proteases on this process.

MATERIALS AND METHODS

Expression of human α-, β-, and γ-ENaC in X. laevis oocytes. Human ENaC (hENaC) was expressed in X. laevis oocytes. It has been shown that the biophysical properties of the channel in this system are similar to those of the channel in native epithelial tissues (5).

Stage V–VI oocytes were obtained from ovarian tissue of female frogs anesthetized by immersion in MS 222 (2 g/l; Sandoz, Basel) according to a procedure approved by the Service Vétérinaire Cantonal of the Canton de Vaud.

The three α-, β-, and γ-hENaC subunits are cloned in the pBSK vector, linearized by NOTI, and capped cRNA was in vitro synthesized by SP6 polymerase. Equal amounts (1 ng) of each subunit in a vector, linearized by NOTI, and capped cRNA was in vitro synthesized by SP6 polymerase. Equal amounts (1 ng) of each subunit in a total volume of 50 nl were injected into each oocyte as previously described (4, 5). For the test of the effect of the channel activating protease-1 on self-inhibition, oocytes were coinjected with hENaC and mouse CAP-1 (2.5 ng).

The injected oocytes were incubated for 1–2 days in a modified Barth’s saline solution [in mM: 85 NaCl, 1 KCl, 2.4 NaHCO₃, 0.8 MgSO₄, 0.5 Ca(NO₃)₂, 0.4 CaCl₂, 2 HEPES, 0.8 NaOH, 10 µg/ml sodium penicillin, 5 µg/ml streptomycin sulphate, pH 7.2]. The high-Na⁺ concentration was chosen to enable a large Na⁺ loading during the ENaC expression time.

Electrophysiological measurements. The oocytes expressing ENaC were studied using the two-electrode voltage clamp technique using a Dagan TEV voltage clamp amplifier (Dagan, Minneapolis, MN), the Digidata 1322 digitizer, and the PClamp 9 data acquisition and analysis package (Axon Instruments, Molecular Devices, Sunnyvale, CA).

Changes of the perfusion solution were performed by means of a six-way stopcock. The composition of the solutions were (in mM) 100 N-methyl-D-glucamine (NMDG)-Cl, 0.82 MgCl₂, 0.41 CaCl₂, 10 NMDG-HEPES, 5 BaCl₂, 10 TEA-Cl for the NMDG 100 mM solution; NMDG-Cl was replaced by 100 mM KCl, 100 mM NaCl, 100 mM LiCl in, respectively, the potassium, sodium, and lithium solutions. To determine amiloride-sensitive currents, we added amiloride (Sigma) in a separated fraction of each test solution at a final concentration of 10 µM (from a stock solution at 10 mM). For the estimation of the affinity of the sensing site, solutions with concentrations of sodium or lithium between 1 and 120 mM were prepared by replacing NMDG⁺ by Na⁺ or Li⁺. Oocytes were exposed to each Na⁺ (or Li⁺) concentration for 1 to 2 min before current-voltage (I-V) curves were recorded in the absence and in the presence of 10 µM amiloride. In the experiments with different extracellular Na⁺ concentrations, the intracellular Na⁺ concentration could be calculated from the reversal potential (obtained by linear interpolation between the current values nearest to 0) of the amiloride-sensitive current.

In the experiment with proteases, the oocytes were exposed to 5 µg/ml trypsin (Sigma) in the 100 mM NMDG⁺ solution for 3 min during the measurements.

The electrophysiological experiments were carried out under voltage clamp conditions at a holding potential of −20 mV. This −20 mV was chosen to have a stable current recording while limiting changes of intracellular Na⁺ due to Na⁺ inflow. The protocol used to record the I-V curves consisted of a series of 30-ms voltage steps, each starting from a command potential of −20 mV, ranging from −100 to +50 mV with increments of 30 mV. To take into account the ENaC current rundown in the experiments including series of consecutive measurements such as those with different concentrations of Na⁺ or Li⁺, I-V curves in the 100 mM NMDG⁺ solution were recorded at the beginning and at the end of the protocol for each oocyte and a linear time-dependent rundown factor was calculated (the amiloride-sensitive current at +50 mV decreased by an average of ~7.5% between the first and last measurement in NMDG⁺ for an experiment duration of 8–10 min). The value of this time-dependent decrease was used to make a time-dependent correction of current values for each oocyte at each membrane potential.

I-V model fitting and calculation of sodium outward currents. The study of self-inhibition is made difficult due to the fact that the modification of the extracellular cation concentration may not only modulate the open probability of the channel but also inevitably leads to an inward current through the open channel, when the extracellular cation is permeant and acts as charge carrier. We reasoned that this problem could be minimized by measuring currents at highly positive membrane potential (+50 mV) in Na⁺-loaded oocytes. Under these conditions, the current through the open channel is mostly an outflow of Na⁺ and is only weakly dependent on the extracellular cation concentration. However, even at +50 mV, addition of an external permeant cation results in an inward current. Thus to increase the precision of our estimation of the inhibition by extracellular cation, and also to obtain inhibition values at potentials more negative than +50 mV, we made the assumption that the current through the open channel obeyed the Goldman-Hodgkin-Katz (GHK) model and calculated the current due to the outward movement of Na⁺ ([Naoutward]), a current that should be completely independent of charge carrying by the extracellular cation. As illustrated in Fig. 1, [Naoutward] was
calculated from the total amiloride-sensitive current minus the inward cation current due to the flow of the extracellular cation into the cell.

First, the GHK equation (11) was used to obtain the intracellular Na\(^+\) concentration and the \(P_{Na}\) values in the presence of 100 mM extracellular Na\(^+\). Intracellular Na\(^+\) was considered stable within the duration of our measurements.

\[
I_s = \frac{P_{Na}^2 \cdot \frac{F^2}{RT} \cdot [S]_o - [S]_i \cdot \exp\left(-z_\text{S} \cdot \frac{F}{RT}\right)}{1 - \exp\left(-z_\text{S} \cdot \frac{F}{RT}\right)}
\]

with \(I_s\) the current mediated by the ion \(S\), \(P\) the permeability of the membrane for \(S\), \(z_\text{S}\) the charge of \(S\), \(E\) the membrane potential, \(F\) the Faraday constant, \(R\) the gas constant, \(T\) the absolute temperature, and \([S]_o\) and \([S]_i\), respectively, the intra- and extracellular \(S\) concentrations.

Then, using this intracellular Na\(^+\) concentration value, it was possible to obtain the \(P_{Na}\) parameter of the membrane with uninhibited channels using the same equation and the amiloride-sensitive current recorded in the absence of external permeant cation (with the NMDG-Cl solution).

We first had to verify that NMDG\(^+\), our reference nonpermeant cation, had no inhibitory effect by itself and was indeed nonpermeant by recording amiloride-sensitive current in a 100-mM NMDG-Cl solution and in a solution in which NMDG-Cl was replaced by an isotonic saccharose solution (180 mM). The amiloride-sensitive I-V curves were similar in these two solutions, NMDG-Cl solution 737 ± 157 nA vs. sucrose solution 645 ± 99 nA (\(n = 14\), \(P > 0.2\) by Student’s t-test for paired data, no statistically significant difference).

As shown in Fig. 1, it is possible to consider separately the current resulting from the outflow (dotted line) and from the inflow (dashed line) of cation. For instance, in the case of extracellular Li\(^+\), the extracellular cation is permeant and different from the intracellular cation. In this case, to determine \(P_{Na}\), we used the following equation in which the current is the sum of an outward Na\(^+\) current and an inward Li\(^+\) current, assuming that the ion currents through the open channel obey the GHK current equation, in particular flow independence

\[
I = \frac{P_{Na}^2 \cdot \frac{F^2}{RT} \cdot [Na]_o - [Na]_i \cdot \exp\left(-z_{\text{Na}} \cdot \frac{F}{RT}\right)}{1 - \exp\left(-z_{\text{Na}} \cdot \frac{F}{RT}\right)}
\]

\[
+ \frac{P_{Li}^2 \cdot \frac{F^2}{RT} \cdot [Li]_o - [Li]_i \cdot \exp\left(-z_{\text{Li}} \cdot \frac{F}{RT}\right)}{1 - \exp\left(-z_{\text{Li}} \cdot \frac{F}{RT}\right)}
\]

leading to the following simplified equation (considering \(z_{\text{Na}}\) and \(z_{\text{Li}}\) are equal to 1)

\[
I = \frac{P_{Na}^2 \cdot \frac{F^2}{RT} \cdot [Na]_o - [Na]_i \cdot \exp\left(-z_{\text{Na}} \cdot \frac{F}{RT}\right)}{1 - \exp\left(-z_{\text{Na}} \cdot \frac{F}{RT}\right)}
\]

In this equation, \(P_{Li}/P_{Na}\) was assumed to have a value equal to 1.6 according to published data (13).

Thus in each case we could calculate by best fit to the GHK equation the \(P_{Na}\), or the outward Na\(^+\) current in the presence of various extracellular cations. We could then estimate channel inhibition by extracellular cations either by comparing the \(P_{Na}\_\text{inward}\) or the values of the \(P_{Na}\) parameter determined in the presence and in the absence of extracellular K\(^+\), Na\(^+\), or Li\(^+\).

Cation affinity and voltage dependence. To obtain affinity values of the extracellular cations for the self-inhibition site, we recorded the amiloride-sensitive I-V curves at various extracellular concentrations (0, 1, 3, 10, 30, 100, and 120 mM). To analyze these data, we used the following assumptions. 1) The current of the open channel obeys the GHK equation. 2) Self-inhibition occurs through a change of the open probability of the channel, following occupancy of a single inhibitory site. 3) The affinity of the cation for the self-inhibition site might be voltage dependent.

The inhibition by extracellular cation was described by the following equation

\[
1 - \frac{[S]_o}{[S]_o + K_i(0) \cdot \exp\left(-\delta \cdot \frac{E}{F}\right)}
\]

in which \(K_i(0)\) is the inhibition constant at 0 mV, \(\delta\) is the fractional distance into the membrane electrical field at the binding site according to the Woodhull formalism (28) and \([S]_o\), the extracellular cation concentration, and \(F, R, \text{and } T\) have their usual meaning as above.

Thus for each set of data (7 extracellular cation concentrations times 6 membrane potentials = 42 amiloride-sensitive current values) measured with each oocyte, we obtained the best-fitting values for the three parameters \(P_{Na}, K_i(0), \text{and } \delta\) using the least-square method provided by the FindFit routine of Mathematica (Wolfram Research, Champaign, IL) to the following equations:

\[
I = \left(1 - \frac{[Na]_o}{[Na]_o + K_i(0) \cdot \exp\left(-\delta \cdot \frac{E}{F}\right)} \cdot \frac{P_{Na} \cdot E \cdot \frac{F^2}{RT}}{1 - \exp\left(-\frac{E}{F}\right)}\right)
\]

with \([Na]_o\), the intracellular Na\(^+\) concentration and \(k (=P_{Li}/P_{Na})\) equal to 1 or 1.6 in case of extracellular Na\(^+\) or Li\(^+\), respectively (see I-V model fitting and calculation of sodium outward currents).

Self-inhibition measurements by transient current recording after fast solution exchange. The amplitude and the kinetics of self-inhibition were also studied in Na\(^+\) - and Li\(^+\)-containing solutions using the time course of the amiloride-sensitive current recorded after a fast solution exchange from a solution containing no permeant cation (NMDG\(^+\) solution) to Na\(^+\) or Li\(^+\) solutions. The experimental protocol and data analysis were in all aspects similar to those reported earlier (6). Briefly, oocytes expressing human \(\alpha 1\beta\gamma\) ENaC were exposed to the 100 mM NMDG\(^+\) solution under voltage clamp at ~60 mV, and the current was recorded during a first solution change for a 100 mM Na\(^+\) (or Li\(^+\)) solution for ~40 s and then 10 \(\mu\)M amiloride was added to record the baseline value of the amiloride-resistant current. The same maneuver was repeated with the other cation Li\(^+\) (or Na\(^+\)). A similar number of recordings was carried out with Na\(^+\) and with Li\(^+\) for the first measurement. The current amplitude decrease observed after the fast solution exchange was analyzed as described (6) using a two-conformation model with an active (A) and an inactive (I) state linked by two first-order rate constants, an activation (\(k_a\)) and an inactivation (\(k_i\)) rate constant.

\[
k_i \Rightarrow A \Rightarrow I
\]

and the equation describing the time course of the current during the equilibration between the active and the inactive state after self-
inhibition takes place upon exposure to Na\(^+\) or Li\(^+\) and taking into account a slow rundown phenomenon described by the \(k_{\text{down}}\) constant

\[
I(t) = I_{\text{max}}(0) \frac{k_i e^{-\frac{t}{k_{\text{down}}}}}{k_i + k_e} (1 - k_{\text{down}} \cdot t) \tag{6}
\]

The best-fitting parameter \(I_{\text{max}}, k_i, k_e, \text{ and } k_{\text{down}}\) were then obtained for each current recording by fitting equation 6 using the least-square best-fit routine of the Kaleidagraph software (Synergy Software, Reading, PA) as described (6).

**RESULTS**

**Ionic selectivity of the sensing site.** To determine the inhibition produced by extracellular cations on ENaC activity, we recorded the \(I-V\) curves of the amiloride-sensitive current in the presence of chloride salts of a nonpermeant cation, NMDG\(^+\), and of Na\(^+\), Li\(^+\), and K\(^+\) as shown in Fig. 2A. At +50 mV, it can be observed on this plot that perfusion of hENaC-expressing oocytes with extracellular K\(^+\), Na\(^+\), and Li\(^+\) lead to a decrease of the amiloride-sensitive current compared with the nonpermeant and noninteracting cation NMDG\(^+\). The outward component of the Na\(^+\) current (\(I_{\text{Naoutward}}\)) was calculated as described in MATERIALS AND METHODS by subtracting an estimated inward current from the measured current, and Fig. 2B shows an inhibitory effect of extracellular cations on the \(I_{\text{Naoutward}}\) at +50 mV.

We noticed that perfusion of 100 mM potassium leads to a 18% inhibition of \(I_{\text{Naoutward}}\) compared with the current recorded in the NMDG-Cl solution, sodium to a 66% inhibition and lithium to a 59% inhibition, a value slightly but significantly smaller than that of sodium (see Fig. 2B). Figure 2C shows inhibition estimated from the change of the \(P_{\text{Na}}\) parameter of the GHK equation (corresponding to the membrane permeability for sodium). A 29, 71, and 70% inhibition were obtained for K\(^+\), Na\(^+\), and Li\(^+\), respectively. In this case, the inhibition was similar for Na\(^+\) and Li\(^+\) and significantly larger than the inhibition by K\(^+\).

Taken together, these parameters show that extracellular Na\(^+\) strongly decreases the channel activity, as it is also the case for Li\(^+\). K\(^+\) has a smaller yet significant effect.

**Apparent affinity and voltage dependence of the extracellular cation binding site.** To determine the apparent affinity of Na\(^+\) and Li\(^+\) for the extracellular inhibitory site, we measured the effect of increasing concentrations of extracellular Na\(^+\) or Li\(^+\) on the amiloride-sensitive current (see Fig. 3) and used model fitting as described in MATERIALS AND METHODS to obtain estimates of the apparent affinity and possible voltage dependence of this affinity. The intracellular Na\(^+\) concentrations estimated from the reversal potential of the amiloride-sensitive current indicate that intracellular Na\(^+\) increased from ~60 to 100 mM when extracellular Na\(^+\) was changed from 3 to 120 mM (Fig. 3C). These changes most probably reflected modification of Na\(^+\) concentrations in the submembrane unstirred layers (1) rather than the bulk cytosolic concentration because Na\(^+\) influx obtained by integrating the amiloride-sensitive membrane current over the 1- to 2-min exposure to various extracellular Na\(^+\) concentrations does not yield fluxes large enough to modify Na\(^+\) concentration in the whole cytosolic volume.

We did not attempt to determine the affinity for K\(^+\) because only a 18% inhibition could be observed with 100 mM K\(^+\) indicating a very low affinity effect. It would have not been possible to use high enough concentrations in our whole cell configuration to obtain reliable affinity values.

The mean best-fitting values of the parameters are shown in Table 1. For both, Na\(^+\) and Li\(^+\), the voltage dependence factor
Fig. 3. Ionic affinity of the sensing site. Amiloride-sensitive I-V curves recorded in the presence of increasing Na\(^{+}\) (A) or Li\(^{+}\) (B) concentrations. Data points are means ± SE of normalized values (the reference being the amiloride-sensitive current value at +100 mV with 100 mM extracellular Na\(^{+}\)) for Na\(^{+}\): n = 12, for Li\(^{+}\): n = 14. The solid lines are the best-fitting models using equation 5 with the parameters values given in Table 1. C: mean values (n = 12) of intracellular Na\(^{+}\) concentration (Na\(^{+}\)in) calculated from the reversal potential of the amiloride-sensitive current when the oocytes were exposed to various extracellular Na\(^{+}\) concentrations (Na\(^{+}\)ext). The values at 1 mM Na\(^{+}\) are not shown because the reversal potential was often not defined at this concentration.

| Table 1. Comparison of best-fitting parameters of inhibition by extracellular Na\(^{+}\) and Li\(^{+}\) |
|-----------------|-----------------|-------|-------|
|                 | Na\(^{+}\) (n = 12) | Li\(^{+}\) (n = 14) |
|                 | V dep | no V dep | V dep | no V dep |
| \(K_{i}(0)\), mM | 41 ± 8 | 44 ± 8 | 43 ± 4 | 53 ± 6 |
| \(P_{Na}\) | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.2 ± 0.2 | 1.2 ± 0.2 |
| \(\delta\) | −0.028 ± 0.008 | −0.052 ± 0.026 | 43 ± 7 | 47 ± 5 |

Results are means ± SE. The best-fitting inhibition parameters [half inhibition constant at 0 mV \(K_{i}(0)\), amiloride-sensitive Na\(^{+}\) permeability \(P_{Na}\), and fractional electrical distance \(\delta\), see MATERIALS AND METHODS equations 4 and 5] were obtained by model fitting using a value of 1.6 for the \(P_{Na}/P_{Li}\) ratio. All current data were normalized to the current recorded at +100 mV in the presence of 100 mM Na\(^{+}\), thus \(P_{Na}\) values have no unit. The columns headed by “V dep” indicate the result of the fitting procedure using a potential voltage dependence (equation 5). The columns headed by “no V dep” indicate the result of the fitting procedure without voltage dependence (\(\delta\) value set to 0.0). There was no significant statistical difference between the different \(P_{Na}\) values (Student’s t-test). The inhibition constant calculated from the values of the amiloride-sensitive current at +50 mV is indicated in the last line of the table.

\(\delta\) has a value very close to zero and thus the apparent affinity of the inhibition site can be considered as voltage independent. The values for the parameters obtained by fitting to a model without the voltage dependence factor are given in the right column of each cation (no V dep). The apparent affinity for the self-inhibition site is similar for extracellular Na\(^{+}\) and Li\(^{+}\) with values ~40 mM. In each case, \(P_{Na}\) (i.e., the membrane permeability for sodium, without inhibition) is constant and not significantly different from 1. Moreover, these data also show that our estimate of 1.6 for the \(P_{Li}/P_{Na}\) is correct: the estimated \(P_{Na}\) values are the same in case of extracellular Na\(^{+}\) or Li\(^{+}\). Using a lower value of 1.3 as reported by others (24) for the \(P_{Li}/P_{Na}\) ratio yielded a slightly higher mean \(K_{i}\) for Li\(^{+}\) 75 ± 10 mM with \(P_{Li}/P_{Na} = 1.3\) vs. 53 ± 6 mM with \(P_{Li}/P_{Na} = 1.6\).

The apparent affinity for extracellular inhibition by Na\(^{+}\) or Li\(^{+}\) was also calculated from the total amiloride-sensitive current at +50 mV (see Table 1) yielding similar \(K_{i}\) values.

Self-inhibition by Na\(^{+}\) and Li\(^{+}\) in transient current measurements. The results of the self-inhibition measurements by transient current recordings after fast solution exchange are illustrated by an example of current trace in Fig. 4A and summarized by the mean values of the four kinetic parameters \(I_{max}\), \(k_{on}\), \(k_{off}\), \(k_{down}\) in Fig. 4B. Also shown are the amplitudes of the inhibition due to the fast self-inhibition itself, calculated from the kinetic model as \(k_{d}/(k_{c} + k_{d})\), and the “steady-state” inhibition, i.e., the measurement measured 25 s after the solution change, defined as 1 – \(I_{d}/I_{max}\) (\(I_{LS} = \) amiloride-sensitive current at 25 s) a measurement that includes part of the slow rundown component as well. The maximal inward current of Li\(^{+}\) is 1.48-fold larger than that recorded with Na\(^{+}\), a ratio close to that recorded of 1.70-fold than can be calculated for the Li\(^{+}\)-to-Na\(^{+}\) current ratio from the single-channel conductance data published by Palmer and Frindt (18) for the rat renal ENaC, at an extracellular concentration of 100 mM Na\(^{+}\) or Li\(^{+}\) and a membrane potential of −60 mV. As shown in Fig. 4C, the activation constant \(k_{a}\) was similar for Na\(^{+}\) and Li\(^{+}\), which is expected as this constant to describe the rate of the conformation change going from the cation-inhibited channel to cation-free open channel, while the inactivation constant \(k_{i}\) seems to be slightly (but significantly) slower, as if occupancy
of the self-inhibition site by Na\(^+\) or Li\(^+\) had some influence on the kinetic behavior of the channel. Finally, the slow component of the current rundown (estimated from the \(k_{\text{down}}\) parameter) was found to decrease faster in the presence of Li\(^+\) compared with Na\(^+\) as extracellular cation. As the effect of lithium on the feedback inhibition has not been studied in detail up to now, we cannot determine whether this faster effect is related to the larger amount of lithium ions entering the cell (due to the larger conductance of ENaC for Li\(^+\) than for Na\(^+\)) or whether lithium ions have a different effect on the intracellular cation sensing mechanism that is responsible for activating feedback inhibition.

**Effect of proteases on self-inhibition.** We also studied the effects of proteases on the inhibition of the outward amiloride-sensitive current by extracellular Na\(^+\). Figure 5A shows \(I-V\) curves of the amiloride-sensitive current obtained before and after treatment of hENaC-expressing oocytes by 5 \(\mu\)g/ml trypsin with and without 100 mM extracellular Na\(^+\). As described earlier (7), we observed that trypsin treatment induced an increase of the amiloride-sensitive current (5-fold) recorded in the presence of extracellular Na\(^+\) at \(-100\) mV.

Figure 5, B and C, shows that adding 100 mM external Na\(^+\) resulted in a much stronger inhibition of the Na\(^+\) conductance...
before than after treatment by trypsin, when this change was estimated by the $I_{Na_{\text{outward}}}$ ($33 \pm 3$ vs. $73 \pm 3\%$, Fig. 5B, $P < 0.001, n = 12$) or by the decrease of the calculated permeability ($33 \pm 3$ vs. $69 \pm 3\%$, Fig. 5C, $P < 0.001, n = 12$).

However, it is to be noticed that trypsin produced a significant stimulation of $I_{Na_{\text{outward}}}$ and $P_{Na}$ even in the absence of extracellular Na$^+$. Thus the effect of trypsin cannot be entirely attributed to removal of self-inhibition, but it must have some other component.

Figure 6 shows similar results obtained by comparing oocytes coexpressing or not the protease CAP-1. As for trypsin, the coexpression of this protease and hENaC led to an activation of the current recorded, as already demonstrated (26), with both extracellular solutions (NMDG$^+$ and Na$^+$) compared with a control group of oocytes expressing hENaC alone (see Fig. 6A for the $I-V$ curves, a 1.6-fold increase for the NMDG$^+$ solution at $+50$ mV and a 4.3-fold increase with the 100 mM sodium perfusion solution at $-100$ mV), Figure 6, B and C, shows that perfusing 100 mM extracellular Na$^+$ led to a much stronger decrease of the Na$^+$ conductance in the group of oocytes expressing hENaC alone ($n = 11$) than in the group coexpressing hENaC and CAP-1 ($n = 10$). This change was estimated by the $I_{Na_{\text{outward}}}$ ($82 \pm 7$ vs. $31 \pm 2\%$, Fig. 6B, $P < 0.001$) or by the decrease of the Na$^+$ membrane permeability ($75 \pm 5$ vs. $29 \pm 2\%$, Fig. 5C, $P < 0.001$).

These observations show that serine proteases can abolish or at least reduce the sodium self-inhibition of ENaC.

**DISCUSSION**

In this study, we attempted to characterize the extracellular cation binding site that is responsible for the sodium self-inhibition observed with ENaC. As explained in more details in MATERIALS AND METHODS, we chose to study this phenomenon by measuring steady-state amiloride-sensitive outward currents in the presence of various extracellular cation concentrations in Na$^+$-loaded oocytes because under these conditions, the outward current through open channels depends mostly on the high intracellular Na$^+$ and the error due to the inflow of extracellular cation is minimized and this error can be further reduced if the inflow of cation can be calculated (under a few specific assumptions) and subtracted. In these steady-state current measurements, there are, however, other factors that have to be taken into account in the interpretation of the results.

First, the possible saturating occupancy of a conduction site, when the extracellular cation concentration is raised, will result in the leveling off of the single-channel conductance and an apparent decrease of the membrane permeability. Saturation of the current through ENaC has been indeed well-described based on single-channel current measurements for inward currents and shows a $K_m$ of $15–20$ mM for Na$^+$ and $50$ mM for Li$^+$ (18). Measurement of outward current at a roughly constant intracellular Na$^+$ should reduce the changes in the occupancy of conduction site(s) but, depending on the number of cation binding sites in the channel conduction pathway, the location of these sites, and the interactions between these sites, it is possible that rising extracellular Na$^+$ or Li$^+$ may result in a high rate of occupancy of an extracellular site resulting in a decrease of the channel conductance.

Because steady-state measurements cannot distinguish between the effects of saturation of a conduction site and self-
Fig. 6. Effect of CAP-1 coexpression on self-inhibition. A: I-V curves obtained for oocytes expressing hENaC alone (filled symbols, n = 11) or coexpressing hENaC and CAP-1 (open symbols, n = 10) with 2 different perfusion solutions: 100 mM NMDG and 100 mM Na. Solid lines are best-fitting curves using equation 1. B: relative outward currents I_{Na_{outward}}/I_{Na^+} at +50 mV, where I_{Na^+} is the amiloride-sensitive current recorded for the group of oocytes expressing hENaC alone, when the 100 mM NMDG solution is perfused. C: relative permeability P_{Na^+}/P_{Na^+}. For the 2 histograms, filled columns represent values determined for extracellular NMDG, and open columns for extracellular Na. * * \( p < 0.005 \) and * * \( p < 0.05 \) (Student’s t-test for paired data when comparing NMDG and Na or unpaired data when comparing with or without CAP-1).

inhibition related to occupancy of another type of site, we also compared the effect of 100 mM Na and Li after fast solution exchange. These measurements allow to distinguish the effects of conduction site occupancy, which must have a very fast time course, orders of magnitude faster than the time resolution of our measurements, and self-inhibition, which has a time constant of a few seconds and can be observed by this technique (6). The results of these measurements (summarized in Fig. 4) indicate that self-inhibition occurs with extracellular Li as well as with extracellular Na, although with some kinetic differences. The saturation of the conduction site cannot be inferred quantitatively from these measurements but the fact that the ratio of the Li maximal current to Na maximal inward current is close to the Li to Na current ratio observed in single-channel conductance data (18) suggests that saturation by site occupancy affects similarly Na and Li currents.

A second problem with steady-state measurements may result from the fact that amiloride-sensitive currents measured in X. laevis oocytes expressing ENaC are not very stable in time but tend to “run down.” At least part of this rundown can be attributed to the “feedback inhibition” related to the increase of intracellular Na that occurs because of large inflow of Na (1, 10, 12). We did indeed observe a rundown in our inward current measurements (see Fig. 4) for Li and for Na and this rundown appeared faster in the measurements with extracellular Li than with Na when estimated by the \( k_{down} \) parameter of our kinetic model. However, our experimental conditions in the steady-state current measurements were chosen to exclude this effect. First, a linear rundown factor was measured and taken into account for each measurement and, second, the −20-mV holding potential and the very short clamping time at high negative potential should have limited the oocyte loading with Na or Li.

The total reduction in the steady-state outward current upon raising Na or Li from 0 to 100 mM (and after exclusion of the effect due to channel rundown) amounts to ~60% (Fig. 2B) with a slightly but significantly smaller inhibition for Li. When measured as a decrease of the inward current from the fast solution change experiments, self-inhibition can be estimated to be ~30% for Na and ~20% for Li for the same change in concentration (Fig. 4E) using a measure \( [k_0/k_{down}] \) excluding the effect of conduction site saturation and channel rundown. Comparison of these values indicates that about half (or slightly less than half in the case of Li) of the current decrease may be due to self-inhibition itself and the rest to conduction site saturation.

Ionic selectivity of the sensing site. We first determined the selectivity of this extracellular sodium sensing site: Na ≥ Li > K. We indeed observed a strong inhibition by sodium and lithium amounting to ~60 to 70% of the amiloride-sensitive outward Na current at an extracellular concentration of 100 mM. Extracellular potassium at the same 100-mM concentration produced a small (~20%) but statistically significant inhibition of the outward Na current. Because of the small size of this effect, we could not study further the mechanism of this inhibition by K. We can make two hypotheses 1) potassium acts with a very low affinity on the sodium sensing site and 2) potassium produces a low-affinity direct blockade of the pore of ENaC, as proposed in a channel pore model (16). The mechanism of ENaC block by extracellular K would then be different from the “self-inhibition” phenomenon.
For Na\(^+\) and Li\(^+\), because of the difficulty of observing the inhibitory effects of cations that are also charge carriers for the same channel, we estimated the inhibition by the extracellular cation using three different variables: the total amiloride-sensitive currents, the amiloride-sensitive sodium outward currents, and also the amiloride-sensitive permeability (P) parameter given by model fitting to the GHK current equation. These three variables displayed the same trend of inhibition by extracellular cations with the Na\(^+\) ≈ Li\(^+\) > K\(^+\) selectivity. This selectivity is different from the ionic selectivity of the conducting site of ENaC: Li\(^+\) > Na\(^+\) ≫ K\(^+\) (17) that involves the selectivity filter in the pore region of the channel (14). This difference in the ionic selectivity between the conduction site and the self-inhibition site suggests that these two sites are different.

Apparent affinity and voltage dependence of the extracellular cation binding site. In a second part of our study, we used model fitting to estimate the apparent affinity of the self-inhibition site for Na\(^+\) and Li\(^+\) and a possible voltage dependence of this affinity. This analysis did not provide any support for a significant voltage dependence of the extracellular cation effect. The \(K_m\) for saturation of the conduction site has been shown almost voltage independent (18). Our measurements of the effects of extracellular Na\(^+\) for which conduction site saturation and self-inhibition contribute roughly equally do not detect any voltage dependence and indicate that self-inhibition itself is not voltage dependent either. Thus our results suggest that the site responsible for self-inhibition must be located outside the transmembrane electrical field in the extracellular part (and not in the transmembrane part) of the ENaC protein, in accordance with location of mutations influencing self-inhibition in the extracellular part of the protein (21, 23).

We obtained apparent inhibitory constants for both cations ~40 mM, consistent with the decrease of the current recorded with 100 mM extracellular Na\(^+\) and Li\(^+\) as described above. This apparent affinity has a lower value than what had previously been determined for Na\(^+\) (~100 mM) (6). There are, however, several differences between these two sets of measurements that might explain the higher value that we obtained earlier. First, the inhibition due to high extracellular Na\(^+\) or Li\(^+\) is due not only to self-inhibition but also to conduction site saturation and this fact could well explain the difference. Second, our former measurements considered only the effect of various extracellular Na\(^+\) concentrations on the rate constant of the inhibition by extracellular Na\(^+\) (6) while we are now considering the relationship between extracellular Na\(^+\) concentration and the self-inhibition amplitude. Finally, it is difficult to evaluate the contribution of differing experimental conditions such as Na\(^+\)-loaded vs. not loaded oocytes or different holding potential.

Our present results do not indicate a large difference in the apparent affinity of the inhibition by extracellular Na\(^+\) and Li\(^+\), in contrast with the more than threefold difference in the apparent affinity of these two cations for the conduction site of the single-channel current (18). As the contribution of conduction site saturation does not seem to be very different between Na\(^+\) and Li\(^+\), as discussed above, this observation also supports the hypothesis that the inhibitory sodium sensing site is distinct from the conduction site.

Effect of proteases on self-inhibition. Activation of ENaC by the effect of added extracellular proteases, or by the coexpression of ENaC with proteases such as CAP-1, has been observed in several expression systems and also in native epithelia (3, 26) but the mechanism of this activation is still not fully understood. Several pieces of evidence support the hypothesis that this activation is due to a direct effect of the extracellular protease with ENaC and that it is related to changes in the channel open probability rather than changes of channel density or single-channel current (7) but modulation of the channel density has also been proposed from studies of the effect of protease inhibitors (2). Consistent with earlier observations using a different approach (6), we observed that after exposure to the effect of extracellular proteases, either acute treatment with extracellular trypsin or coexpression of hENaC with the channel activating protease-1, inhibition by high extracellular Na\(^+\) was reduced from ~70 to ~30% (see Fig. 5). This reduction of the inhibition amplitude has been noticed by all three methods that we used to estimate self-inhibition, the total amiloride-sensitive currents, the sodium outward currents, and the sodium permeability. The fact that an inhibition by high extracellular Na\(^+\) can still be observed after exposure to protease is consistent with our interpretation that the current decrease is due to both self-inhibition, a factor that would be abolished by protease treatment, and saturation of the conduction site, a factor that might not be modified by protease treatment. Thus our present results confirm that the abolition of the self-inhibition is at least one of the mechanisms by which extracellular proteases can activate ENaC. The effects of trypsin observed in the absence of extracellular Na\(^+\) (or Li\(^+\)) indicate that the effects of proteases on ENaC are not due only to a removal of self-inhibition but may also be due to other types of effect such as a modulation of the channel density for instance, as suggested by others (2). We do not know yet whether the effects of extracellular proteases would affect the extracellular cation sensing site itself, another element in the channel protein responsible for transmitting the self-inhibition information to the channel gating mechanism, or the gating mechanism.

In summary, our results support the hypothesis that the Na\(^+\) sensing site responsible for transmitting the information about the extracellular Na\(^+\) concentration to the channel gating mechanism has a low and voltage-insensitive affinity for extracellular Na\(^+\) or Li\(^+\). This site appears to be different from the channel conduction site and to be located in the extracellular domain rather than in the transmembrane part of the channel protein.

ACKNOWLEDGMENTS

We are grateful to L. Schild and S. Kellenberger for reading the manuscript and useful comments.

GRANTS

This work was supported by Swiss National Fund Grant 2100-061553.

REFERENCES


