

# Na Self Inhibition of Human Epithelial Na Channel: Temperature Dependence and Effect of Extracellular Proteases

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**ABSTRACT** The regulation of the open probability of the epithelial Na<sup>+</sup> channel (ENaC) by the extracellular concentration of Na<sup>+</sup>, a phenomenon called “Na<sup>+</sup> self inhibition,” has been well described in several natural tight epithelia, but its molecular mechanism is not known. We have studied the kinetics of Na<sup>+</sup> self inhibition on human ENaC expressed in *Xenopus* oocytes. Rapid removal of amiloride or rapid increase in the extracellular Na<sup>+</sup> concentration from 1 to 100 mM resulted in a peak inward current followed by a decline to a lower quasi-steady-state current. The rate of current decline and the steady-state level were temperature dependent and the current transient could be well explained by a two-state (active-inactive) model with a weakly temperature-dependent ( $Q_{10}^{\text{act}} = 1.5$ ) activation rate and a strongly temperature-dependant ( $Q_{10}^{\text{inact}} = 8.0$ ) inactivation rate. The steep temperature dependence of the inactivation rate resulted in the paradoxical decrease in the steady-state amiloride-sensitive current at high temperature. Na<sup>+</sup> self inhibition depended only on the extracellular Na<sup>+</sup> concentration but not on the amplitude of the inward current, and it was observed as a decrease of the conductance at the reversal potential for Na<sup>+</sup> as well as a reduction of Na<sup>+</sup> outward current. Self inhibition could be prevented by exposure to extracellular protease, a treatment known to activate ENaC or by treatment with *p*-CMB. After protease treatment, the amiloride-sensitive current displayed the expected increase with rising temperature. These results indicate that Na<sup>+</sup> self inhibition is an intrinsic property of sodium channels resulting from the expression of the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of human ENaC in *Xenopus* oocyte. The extracellular Na<sup>+</sup>-dependent inactivation has a large energy of activation and can be abolished by treatment with extracellular proteases.

**KEY WORDS:** ENaC • activation energy • trypsin • protease • sodium transport

## INTRODUCTION

The fine regulation of sodium balance in terrestrial vertebrates is realized mostly by a tight control of Na<sup>+</sup> urinary excretion. In the distal segments of the nephron, Na<sup>+</sup> ions are reabsorbed by passing through highly selective, amiloride-sensitive epithelial Na<sup>+</sup> channels (ENaC)\* located in the apical membrane of tubule cells. Thus, the regulation of Na<sup>+</sup> excretion occurs mostly by the control of the density and the open probability of these apical membrane Na<sup>+</sup> channels. The surface density of ENaC is under a complex hormonal control involving transcriptional and posttranscriptional regulation of its synthesis and insertion, and retrieval of active channels from the apical membrane (Garty and Palmer, 1997; Horisberger, 2001; Rotin et al., 2001). The control of the open probability of ENaC is until now not well understood: the sodium channels of the apical membrane of the cortical collecting tubule have a highly variable open probability, suggesting

the hypothesis of high and low open probability modes (Palmer and Frindt, 1996; Palmer et al., 1998).

The extracellular concentration of Na<sup>+</sup> has a marked influence on the amiloride-sensitive current and this influence can be explained by at least two different mechanisms, feedback inhibition and self inhibition (Garty and Palmer, 1997).

Feedback inhibition, a phenomenon with a time course of minutes or tens of minutes, is related to the intracellular Na<sup>+</sup> concentration. A rise in intracellular Na<sup>+</sup> concentration, whether it is due to an increased Na<sup>+</sup> entry (for instance by an increased luminal Na<sup>+</sup> concentration) or by a decreased basolateral extrusion (for instance by inhibition of the Na,K-pump) results in a slow decrease of the amiloride-sensitive current. Several reports have proposed a role for intracellular calcium and PKC in this regulation (Garty and Palmer, 1997). With heterologous expression in *Xenopus* oocyte, an intracellular Na<sup>+</sup>-dependent run down has been observed and shown to be dependent on the presence of a “PY” motif in the COOH-terminal of ENaC (Kellenberger et al., 1998), and to be related to ubiquitin-mediated endocytosis (Staub et al., 2000). Thus, feed-back inhibition seems to result mostly from the control of ENaC density at the cell surface, even though the contribution of a change in open probability cannot be excluded.

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\*Abbreviations used in this paper: ASIC, acid-sensing ion channel; BIG, benzimidazolylguanidine; ENaC, epithelial Na<sup>+</sup> channel; NMDG, N-methyl-D-glucamine; *p*-CMB *p*-chloro-mercuribenzoate.

Self inhibition, a much faster phenomenon, has been observed as a peak inward current that relaxes to a lower value with a time course of a few seconds after a sudden increase of the extracellular  $\text{Na}^+$  concentration in amphibian "tight" epithelia (Lindemann and Voute, 1976; Fuchs et al., 1977; Garty and Benos, 1988). In these tissues, and also in mammalian colon (Luger and Turnheim, 1981), self inhibition can be abolished by apical treatment with a number of seemingly unrelated compounds, such as sulfhydryl reagents, organic cations, and detergents (Lindemann and Voute, 1976; Li and Lindemann, 1983; Li et al., 1986). Self inhibition has also been inferred from an observed discrepancy in the  $K_m$  value (for the  $\text{Na}^+$  concentration dependence of the amiloride-sensitive current) between a high  $K_m$  value (no or late saturation) for instantaneous estimates of the  $\text{Na}^+$  current and low  $K_m$  values (early saturation) for steady-state amiloride-sensitive current (Fuchs et al., 1977). A similar discrepancy has been observed between a high  $K_m$  for single channel current estimates by noise analysis or by patch clamp recordings and a low  $K_m$  for macroscopic steady-state current (Van Driessche and Lindemann, 1979; Lindemann, 1984; Palmer et al., 1998). This discrepancy indicates a decrease of the open probability of ENaC with rising extracellular  $\text{Na}^+$  concentrations. Because of its fast time course, and of the absence of detectable increase in intracellular  $\text{Na}^+$  concentration, self inhibition has been attributed to the extracellular  $\text{Na}^+$  ions rather than to the influence of the extracellular on the intracellular  $\text{Na}^+$  concentration (Lindemann, 1984). However, the available data do not specify the localization of the  $\text{Na}^+$  sensor site or exclude the existence of an intracellular site that would be reactive to  $\text{Na}^+$  concentration in an intracellular subcompartment in which transient  $\text{Na}^+$  concentration changes would be difficult to detect. As self inhibition has been observed only with native tissues, it is not yet clear if this phenomenon is associated with the three subunits of ENaC itself or if associated proteins are required.

In this study we have explored the self inhibition phenomenon using heterologous expression of human  $\alpha$ ,  $\beta$ , and  $\gamma$  ENaC in *Xenopus* oocytes, characterized its temperature dependence and demonstrated that it can be abolished by extracellular treatment with proteases. We also demonstrate that self inhibition is not related to the entry of  $\text{Na}^+$  into the cell but only to the extracellular  $\text{Na}^+$  concentration.

## MATERIALS AND METHODS

### *Expression of Human $\alpha$ , $\beta$ , and $\gamma$ ENaC in *Xenopus* Oocytes*

The hENaC subunits, provided by P. Barbry, were subcloned into the pBSK vector, linearized by NOT I, and capped cRNA was synthesized in vitro by SP6 polymerase. Equal amounts (0.2–0.5 ng) of each subunit in a total volume of 50 nl were injected

into stage V-VI *Xenopus laevis* oocytes as described previously (Canessa et al., 1993, 1994). Oocytes were obtained from ovarian tissue of female frogs anesthetized by immersion in MS 222 (2 g/L; Sandoz).

The injected oocytes were incubated for 1–2 d in a low  $\text{Na}^+$  solution (in mM: NaCl 10, KCl 2.0,  $\text{CaCl}_2$  0.7,  $\text{MgCl}_2$  0.8, N-methyl-D-glucamine [NMDG] Cl 80, NMDG-HEPES 10.0, pH 7.4). The low  $\text{Na}^+$  concentration was chosen in order to prevent excessive  $\text{Na}^+$  loading during the time needed for ENaC expression. In one series of measurements, in which a high intracellular  $\text{Na}^+$  was requested in order to measure outward current, the oocyte were incubated in a 90 mM  $\text{Na}^+$  incubation solution similar to that described above, except that NMDG-Cl was replaced by NaCl. In some cases, the oocytes were exposed to 2–5  $\mu\text{g}/\text{ml}$  trypsin (from Sigma-Aldrich) either for 2–3 min during the measurements or by addition of trypsin to the 10 mM  $\text{Na}^+$  incubation solution for 10–20 min before the measurements.

### *Electrophysiological Measurements*

Oocytes were studied by the standard two-electrode voltage-clamp technique by means of a Dagan TEV voltage clamp apparatus (Dagan Corp.). The perfusion chamber, with a cross section of 3 by 3 mm and a length of 35 mm was perfused by gravity at a 12–15 ml/min rate. Changes of the  $\text{Na}^+$  concentration were performed by means of a six way stopcock that was linked to the chamber by a PE tubing with a total dead space of <100  $\mu\text{l}$ . Amiloride addition was realized by injection of a 300- $\mu\text{M}$  amiloride solution directly in the tubing between the stopcock and the chamber (thereby reducing the dead space) using a constant volume syringe injection pump. The concentrated amiloride solution was injected at 1/30 of the global perfusion rate resulting in a 10  $\mu\text{M}$  amiloride final concentration. The composition of the control solution was (in mM) Na 100, Cl 22.4, gluconate 100, NMDG 5, Cl 86,  $\text{HCO}_3^-$  2.4, HEPES 10,  $\text{Mg}^{2+}$  1.0,  $\text{Ca}^{2+}$  0.4,  $\text{Ba}^{2+}$  5.0, TEA 10.0, pH 7.4 (adjusted with NMDG base or HEPES acid). Low  $\text{Na}^+$  solutions (1 or 2 mM  $\text{Na}^+$ ) were obtained by NMDG gluconate replacement of  $\text{Na}^+$  gluconate. Barium and TEA were used to decrease the background  $\text{K}^+$  conductance. The experiments were performed at a holding potential of –60 mV, except for a few cases for which a different potential was used as indicated in the text of the RESULTS section.

Most experiments were performed at room temperature (24–26°C). In one set of experiments, several bottles of the same solution were maintained at temperature ranging from 0 to 45°C. The temperature was measured in the bath using a digital thermometer (TTX 1090; Ebro) the sensing tip of which was placed in the flow of the solution 5–10 mm downhill from the oocyte. Actual bath temperature was noted at the time of each current recording and ranged from 12 to 34°C (the decrease range of temperature was due to temperature change from passage through the tubing and the stopcock). In some experiments, the bath temperature was continuously recorded using the voltage output of a modified thermistor-based temperature control unit (YSI Mod 73A) calibrated with the TTX 1090 digital thermometer. The thermistor was placed in the flow of the solution ~5 mm downhill from the oocyte.

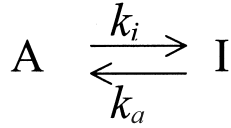
Current and voltage were recorded continuously on a paper chart recorder and the current elicited by fast amiloride removal or  $\text{Na}^+$  concentration increase was recorded during 40–60 s episodes by means of a TL1 DMA/pCLAMP data acquisition system (Axon Instruments, Inc.) for quantitative analysis.

### *Model Fitting*

The relaxation of the current after rapid amiloride removal or sudden  $\text{Na}^+$  concentration increase was analyzed by the expo-

nential fit routine of the PClamp package and according to the following model.

This model postulates the existence of two conformations of ENaC, an active (A) and an inactive (I) conformation linked by two first order rate constants, an activation ( $k_a$ ) and an inactivation ( $k_i$ ) rate constant.



(SCHEME 1)

The maximal current ( $I_{max}$ ) can be recorded when all the channels are in the active state. In addition, to take into account the run-down phenomenon, the total number of channels was assumed to decrease with time. Because of the short time frame of the current recording, this run-down was assumed to decrease as a linear function of time:

$$I_{max}(t) = I_{max}(0) \cdot (1 - k_{down} \cdot t).$$

Assuming that at  $t = 0$  all the channels are in the active state, the amplitude of the current as a function of time is then given by

$$I(t) = I_{max}(0) \frac{k_a + e^{-(k_a + k_i)t} k_i}{k_a + k_i} (1 - k_{down} \cdot t). \quad (1)$$

As the removal of amiloride, or the addition of  $\text{Na}^+$ , was not instantaneous, the origin of time ( $t = 0$ ) was set at the inflection point of the rising phase of the current amplitude after the removal of amiloride or the addition of  $\text{Na}^+$ . The best fitting parameter  $I_{max}$ ,  $k_a$ ,  $k_i$ , and  $k_{down}$  were then obtained for each current recording by fitting Eq. 1 using the least square best fit routine of the Kaleidagraph® software (Synergy Software) as described in the examples of Fig. 1. Shortly after the solution exchange, the current amplitude depends in a complex manner on the rate of amiloride removal (or the rate of  $\text{Na}^+$  concentration increase) and the inactivation and activation pro-

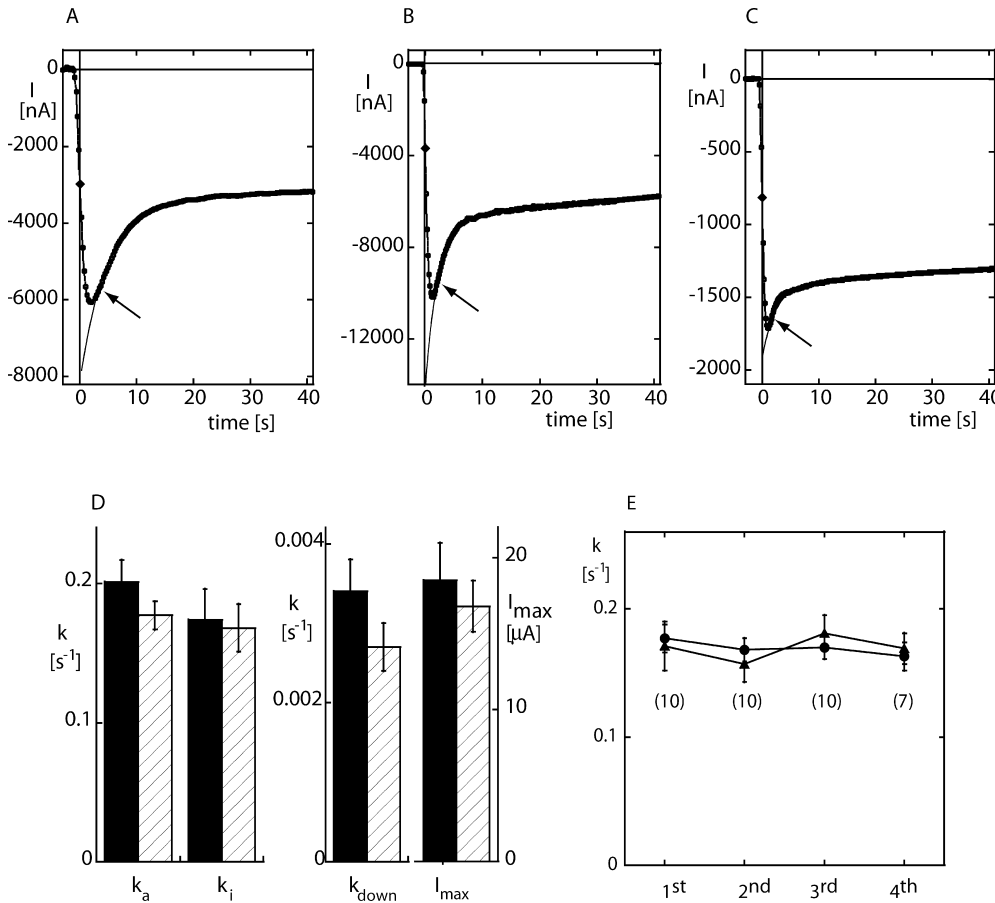


FIGURE 1. Sodium self-inhibition with ENaC expressed in *Xenopus* oocyte. Examples of current recordings at  $-60$  mV holding potential showing the peak current and its relaxation after (A) the sudden removal of amiloride in the presence of  $100$  mM  $\text{Na}^+$ , or (B and C) a  $1$ – $100$  mM increase in  $\text{Na}^+$  concentration. The original data (collected at the rate of  $5$  points/s) are indicated by the small black circles. The solid line, representing the best fitting curve (according to Eq. 1 presented in the MATERIALS AND METHODS) was obtained by fitting the current values from the inflexion point (arrow) of the current amplitude decline to the  $40$ -s point ( $\sim 160$  data points). In the example of A, the best fitting parameters were (a) the maximal current extrapolated to time  $t = 0$ ,  $I_{max}$   $8596$  nA, (b) the activation constant  $k_a$   $0.086$  s<sup>-1</sup>, (c) the inactivation constant  $k_i$   $0.127$  s<sup>-1</sup>, and (d) the run down factor  $k_{down}$   $0.0016$ . The origin of time was set to the inflexion point of the rising phase of the current ampli-

tude (indicated by a black diamond). C presents a similar recording obtained with an oocyte-expressing rat  $\alpha\beta\gamma$  ENaC following the same experimental maneuver as in B, i.e., a sudden increase of the  $\text{Na}^+$  concentration from  $1$ – $100$  mM. A current relaxation with a similar time course as with human ENaC (A and B) was also observed after the initial peak; however, the amplitude of the decrease was smaller. D shows the mean values of the kinetics parameters  $k_a$ ,  $k_i$ ,  $k_{down}$ , and  $I_{max}$  obtained by model fitting in  $11$  and  $6$  measurements using amiloride removal (black bars) and  $\text{Na}^+$  concentration increase (hatched bars), respectively. There were no statistically significant differences between the mean values obtained by amiloride removal (black bars) and those obtained in  $\text{Na}^+$  concentration increase experiments. E shows the results obtained for repeated measurements after amiloride removal under the same condition on the same oocyte. A recuperation period of at least  $60$  s in the amiloride-containing solution was allowed between each measurement. The kinetic parameters  $k_a$  (black circles) and  $k_i$  (black triangles) obtained in  $10$  first, second, and third measurements and in  $8$  fourth measurements are shown. There was no significant trend with time nor statistically significant differences between the values obtained at the first, second, third or fourth measurement for both these parameters. The number of measurements is indicated in parentheses.

cesses. For this reason, the initial part of the current trace were not used for the kinetic model fitting. We only used the data after the inflexion point of the current declining phase as described in Fig. 1.

### Chemicals

Trypsin (type XI), novobiocin, and *p*-chloro-mercuribenzoate (*p*-CMB) were purchased from Sigma-Aldrich.

## RESULTS

### Self Inhibition after Rapid Na Addition or Amiloride Removal

Fig. 1 shows examples of current traces illustrating the self inhibition phenomenon and the principle of the data analysis by model fitting. After rapid removal of amiloride (Fig. 1 A) or after a sudden increase in the Na<sup>+</sup> concentration from 1.0 to 100 mM (Fig. 1, B and C) the inward current increased to a peak and then relaxed to a lower value. It usually did not reach a steady-state value but presented a slow quasi-linear decline during the 1-min duration of the recording.

As described in the MATERIALS AND METHODS section, each current trace was analyzed by best fitting to a model assuming that immediately after the removal of amiloride or after the increase of the Na<sup>+</sup> concentration the channels are in a high open probability state, and then they equilibrate between a high (active) and a low open probability (inactive) state. The rate of the relaxation to the new equilibrium should be equal to the sum of the forward and backward reaction. Thus, as a check of the consistency of the results obtained by model fitting, we verified that the rate constant of the exponential fit of the current amplitude decrease was similar to the sum of the  $k_i$  and  $k_a$  parameters of the model. Table I shows that this was indeed the case.

Amiloride removal or 1–100 mM Na<sup>+</sup> concentration increase yielded very similar kinetics of the current relax-

ation as shown in Fig. 1 D. There was no significant difference in any of the four best fitting parameters  $k_a$ ,  $k_i$ ,  $k_{down}$ , and  $I_{max}$ . This observation indicates that immediately after amiloride removal, the channels are in the same active state as the channels exposed to a low extracellular Na<sup>+</sup> concentration. When measurements were repeated on the same oocyte, there were no consistent changes with time in the kinetic parameters of the self inhibition (Fig. 1 E); only the maximal current tended to decrease with repeated measurement, as expected from the slow “run-down” phenomenon (unpublished data). This observation allowed us to perform repeated measurements on the same oocyte under different conditions to study the effects of these conditions.

### Solution Exchange Rate

To estimate the solution exchange rate around the oocyte, we have chosen to use the time course of the ENaC-carried current under conditions in which self inhibition has been abolished, i.e., after trypsin treatment or in the presence of *p*-CMB (see below). Each current trace was analyzed and the rising phase of the current was fitted to a single exponential yielding values for the rate of replacement of NMDG<sup>+</sup> by Na<sup>+</sup>. The pooled values of the experiments after trypsin treatment (see Fig. 5 A) and with *p*-CMB (see Fig. 5 C) yielded a mean rate constant of the current rising phase of  $2.16 \pm 0.16 \text{ s}^{-1}$  ( $n = 25$ ), which, taking into account the nonlinear relationship between current and Na concentration (see Fig. 4 C, below) correspond to a solution exchange rate of  $1.71 \pm 0.13 \text{ s}^{-1}$  (or a half-life of 0.40 s).

### Temperature Dependence

The temperature dependence of the self inhibition kinetics was examined by studying the time course of the current relaxation after amiloride removal. Measurements were performed 3–5 times on the same oocyte at different temperatures. Although the first measurement was always performed at room temperature (24–26°C), the sequence of the temperature tested varied for each measurement. Example of current traces obtained successively at five different temperatures on the same oocyte are shown in Fig. 2 A. The rate of decrease after the peak current was much slower at low temperature and faster at high temperature. In addition, the steady-state current, when reported to the peak value, was also temperature dependent with a steady-state value nearly equal to the peak value at low temperature and a lower final value compared with the peak value at high temperature. It was also apparent that the “run-down,” the slow decrease that follows the fast relaxation phase after the peak current, was more pronounced at high temperature.

Best fitting kinetic parameters were obtained by model fitting for 46 measurements obtained in 11 oocytes, with measurement at 3 to 5 different tempera-

TABLE I

Comparison of the  $k_i$  and  $k_a$  Parameters Obtained by Model Fitting with the Rate Constant Obtained from the Exponential Fit of the Relaxation of the Current Amplitude ( $k_{exp}$ )

	Amiloride removal ( $n = 12$ )	1 → 100 Na <sup>+</sup> ( $n = 7$ )
	$s^{-1}$	$s^{-1}$
$k_a$	$0.19 \pm 0.02$	$0.26 \pm 0.06$
$k_i$	$0.16 \pm 0.02$	$0.16 \pm 0.02$
$k_a + k_i$	$0.34 \pm 0.03$	$0.40 \pm 0.05$
$k_{exp}$	$0.35 \pm 0.02$	$0.42 \pm 0.06$

Comparison of the  $k_i$  and  $k_a$  parameters obtained by model fitting with the rate constant obtained from the exponential fit of the relaxation of the current amplitude ( $k_{exp}$ ). Results from experiments with amiloride removal (left column) or with Na<sup>+</sup> concentration increase (right column) are shown. The results for the Na concentration changes were obtained in a subgroup of the oocytes used for the measurements with amiloride removal. Results are mean  $\pm$  SE.



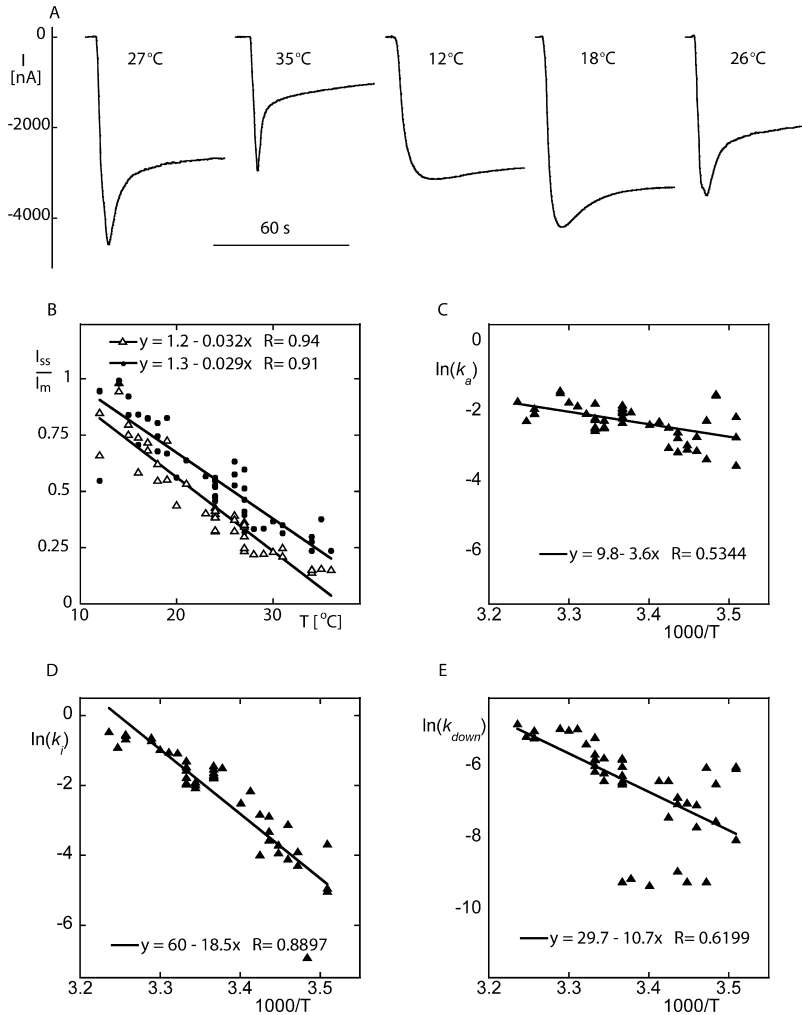


FIGURE 2. Temperature dependence of the self-inhibition. A shows five sequential current recordings of the effect of amiloride removal in a 100 mM Na<sup>+</sup> solution obtained with the same oocyte at different temperatures. Intervals of at least 1 min, during which the temperature was changed, were allowed to occur between each episode of amiloride removal (not depicted). The peak current ( $I_{peak}$ , the largest recorded inward current value) and the steady-state current value ( $I_{ss}$ , i.e., the value measured at 40 s), and the kinetic parameters ( $k_a$ ,  $k_i$ ,  $k_{down}$ , and  $I_{max}$ ) of the current relaxation were obtained from each current recording. B shows the temperature dependence of steady-state current reported either to the peak current ( $I_{ss}/I_{peak}$ , open triangles) or to the maximal current  $I_{ss}/I_{max}$ , black dots). C is the Arrhenius plots of the activation rate ( $k_a$ ), D is the plot of the inactivation rate ( $k_i$ ), and E the plot of the run down factor ( $k_{down}$ ).

tures ranging from 12 to 36°C on each oocyte. There was a significant positive correlation between each of the rate constants  $k_a$ ,  $k_i$ , and  $k_{down}$  and temperature ( $k_a$  vs.  $T$ :  $R = 0.44$ ,  $P < 0.01$ ;  $k_i$  vs.  $T$ :  $R = 0.91$ ,  $P < 0.001$ ;  $k_{down}$  vs.  $T$ :  $R = 0.62$ ,  $P < 0.001$ ). There was no correlation between the maximal current and the temperature, possibly because of the large variation in the level of expression in each oocyte and because of the effect of the run down. However, as shown in Fig. 2 B, there was a strong negative correlation between the quasi steady-state current amplitude, i.e., the value taken at 40 s and the temperature when this amplitude was expressed as a fraction of the peak current (the largest recorded current amplitude) ( $R = -0.94$ ,  $P < 0.001$ ) or as a fraction of the maximal current ( $I_{max}$ ; the parameter of the kinetic model that represent the amplitude of current extrapolated at time 0) ( $R = -0.91$ ,  $P < 0.001$ ).

The temperature dependence of the kinetic parameters were analyzed using the Arrhenius plot (Fig. 2, C–E). There was a statistically significant correlation between the logarithm of the rate constant,  $\ln(k)$ , and the reciprocal of the temperature for each of the three

rate constants  $k_a$ ,  $k_i$ , and  $k_{down}$  (values of  $R$  shown in Fig. 2, C–E;  $P < 0.001$  in each case).

The activation energy ( $E_a$ ) was determined from the slope of the regression line (sArr) of the Arrhenius plot for each rate constant according to equation

$$E_a = -sArr \cdot R,$$

with  $R = 8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ .

The inactivation rate ( $k_i$ ), i.e., the parameter describing the phenomenon responsible for the decrease of the current after exposure of the channel to a high Na<sup>+</sup> concentration, was strongly temperature dependent with an activation energy of 154 KJ.mol<sup>-1</sup>, corresponding to a  $Q_{10}$  of  $\sim 8$ . In contrast, the activation rate ( $k_a$ ) was only weakly temperature dependent with an activation energy of 30 KJ.mol<sup>-1</sup> ( $Q_{10}$  of  $\sim 1.5$ ). The steeper temperature dependence of the inactivation rate explains the negative correlation between the steady-state value and the temperature and, thus, the paradoxical temperature dependence of the human ENaC-mediated current (Askwith et al., 2001), as described later in

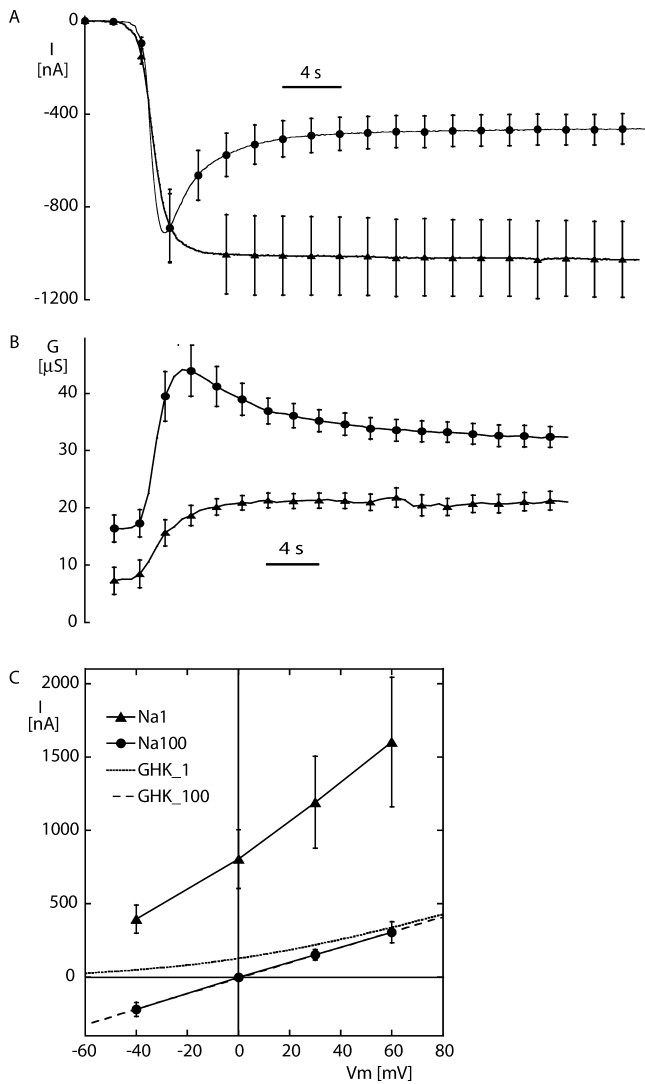


FIGURE 3. Self inhibition is not related to the inward  $\text{Na}^+$  current. (A) Mean values of the amiloride-sensitive current recorded upon removal of amiloride at a membrane potential of  $-15$  mV in a  $100$  mM  $\text{Na}^+$  solution (black triangles,  $n = 22$ ) and at  $-120$  mV in a  $2$  mM  $\text{Na}^+$  solution (black filled circles,  $n = 29$ ). The SE values are indicated only for points at 2-s intervals. (B) Mean conductance of the whole oocyte membrane during amiloride removal in a  $100$  mM (circles,  $n = 15$ ) and in a  $1$  mM (triangles,  $n = 9$ )  $\text{Na}^+$  solution. The first data points show the conductance in the presence of  $10$   $\mu\text{M}$  amiloride. The holding potential was set at the reversal potential of the amiloride-sensitive current and the conductance was measured, three times per second, from the current changes induced by a  $10$  mV (from  $5$  to  $-5$  mV from the holding potential) voltage step. (C) Amiloride-sensitive I-V curves for potentials ranging from  $-40$  to  $60$  mV were obtained by subtraction of I-V curves obtained in the absence and in the presence of  $50$   $\mu\text{M}$  amiloride, in  $1$  mM ( $n = 11$ ) and  $100$  mM ( $n = 11$ )  $\text{Na}^+$  solutions. The dashed line represents the Goldman-Hodgkin-Katz (GHK) current equation:

$$I = P \frac{VF^2 [Na]_i - [Na]_o \exp(VF/RT)}{RT (1 - \exp(VF/RT))},$$

for a  $100$  mM extracellular  $\text{Na}^+$  concentration ( $[Na]_o$ ) and with the following best fitting parameters:  $\text{Na}^+$  permeability

more detail. It is interesting to note that the “run-down” rate also appeared temperature dependent with a activation energy of  $89$   $\text{KJ.mol}^{-1}$  ( $Q_{10}$  of  $\sim 3.3$ ), which is not too surprising considering that this run down is attributed to a complex surface protein retrieval process (see INTRODUCTION).

#### Role of Extracellular $\text{Na}^+$ Concentration Versus Rate of $\text{Na}^+$ Inflow on Self Inhibition

It has been difficult to exclude the possibility that self inhibition was due to a local (in some kind of submembrane compartment) and transient increases in intracellular  $\text{Na}^+$  secondary to the increase in  $\text{Na}^+$  influx following the increase of the extracellular  $\text{Na}^+$  concentration. The following three sets of experiments were designed to address this question.

First, we have attempted to observe self inhibition following similar changes of the flow of  $\text{Na}^+$  into the cell upon amiloride removal either in the presence of a high  $\text{Na}^+$  extracellular concentration and a small holding potential, or with a low  $\text{Na}^+$  concentration and a large negative potential. Measurements at low and high  $\text{Na}$  concentration were obtained successively on the same oocytes. If self inhibition was related to the flow of  $\text{Na}^+$  into the cell across the membrane, a similar self inhibition should be observed in both cases. The results of these experiments are shown in Fig. 3 A. When amiloride was removed from a  $100$  mM  $\text{Na}^+$  solution at a small negative potential ( $-10$  to  $-20$  mV), an initial peak current of  $\sim 900$  nA was recorded, which then declined to a value of  $\sim 500$  nA, indicating the presence of self inhibition similar to what has been described above. In contrast, when amiloride was removed from a  $2$  mM  $\text{Na}^+$  solution at  $-120$  mV, a steady inward current of  $\sim 1,000$  nA was recorded without any relaxation to a smaller value. As the amplitude of this current was at least equal or even slightly larger than that of the peak current recorded with the high  $\text{Na}^+$  concentration, these experiments demonstrate that self inhibition is not determined by the flow of  $\text{Na}^+$  into the cell but rather by the extracellular  $\text{Na}^+$  concentration.

Second, we have monitored the cell membrane conductance close to the  $\text{Na}^+$  reversal potential in order to observe self inhibition in the absence of net  $\text{Na}^+$  flow through ENaC. The reversal potential of the amiloride-sensitive current was first determined by repeated exposure to  $10$   $\mu\text{M}$  amiloride and manually adjusting

( $P$ ) =  $1.47 \times 10^{-4}$   $\text{cm.s}^{-1}$  and intracellular  $\text{Na}^+$  concentration ( $[Na]_i$ ) =  $94$  mM ( $V$  is the membrane potential, and  $R$ ,  $T$ , and  $F$  have their usual meaning). The dotted line shows the current values calculated from the GHK equation with the same parameters when the extracellular  $\text{Na}^+$  concentration has been set to  $1$  mM.

the holding potential until no change in holding current could be detected upon addition or removal of amiloride. Na<sup>+</sup>-loaded oocytes (i.e., exposed to a 100 mM Na<sup>+</sup> incubation solution) were used in order to avoid that reversal membrane potential would be too large and positive. The oocyte was held at the reversal potential in the amiloride-containing solution for 1 min and then amiloride was removed suddenly while the membrane conductance was repeatedly measured (three times per second) by recording the current during two 50-ms voltage steps at +5 and -5 mV from the holding (reversal) potential. In the 100 mM Na<sup>+</sup> solution (circles), the amiloride-sensitive current reversal potential was  $-2.9 \pm 0.8$  mV ( $n = 15$ ), indicating that the intracellular Na<sup>+</sup> concentration was close to 100 mM. Fig. 3 B shows that self inhibition was clearly observed by a secondary decline of the membrane conductance after the initial increase observed upon amiloride removal. The relaxation of the conductance had approximately the same time course as the relaxation of the current observed at -60 mV under the same conditions. There was no current change upon amiloride removal because the holding potential was equal to the reversal potential for Na<sup>+</sup>. When the same maneuver was repeated with some of the same oocytes in a 1 mM Na<sup>+</sup> solution (triangles), with a holding potential equal to the reversal potential under these conditions ( $-110 \pm 1$  mV,  $n = 9$ ) no secondary decrease of the current could be observed. These results confirm that self inhibition is dependent on the extracellular Na<sup>+</sup> concentration itself, but not on the influx of Na<sup>+</sup> into the cell.

Third, we have measured the effect of extracellular Na<sup>+</sup> on the outward amiloride-sensitive conductance. To observe sizeable outward currents, we used oocyte with a very high Na<sup>+</sup> concentration, i.e., preincubated in a 100 mM Na<sup>+</sup> solution. From a holding potential of 20 mV, a series of four I-V curves with short (20 ms) voltage steps at -40, 0, 30, and 60 mV were recorded with and without amiloride in a 1 mM and, in the same oocytes, in a 100 mM extracellular Na<sup>+</sup> solution. Short voltage steps were chosen in order to prevent activation of other types of conductance by the strong depolarization, such as the depolarization-induced Na<sup>+</sup> conductance of the *Xenopus* oocyte (Baud et al., 1982). A concentration of amiloride of 50  $\mu$ M was used to ensure full channel block even at positive membrane potentials as amiloride block is known to be voltage dependent (Palmer, 1984). The resulting amiloride-sensitive I-V curves (Fig. 3 C) show that a reversal potential close to 0 was recorded in the 100 mM Na<sup>+</sup> solution, indicating that the intracellular Na<sup>+</sup> concentration was indeed close to 100 mM in these oocytes. Fitting the four current points to the Goldman-Hodgkin-Katz current equation indicated a intracellular concentration of Na<sup>+</sup> averaging 94 mM. With 1 mM extracellular Na<sup>+</sup>, there

was, as expected, a large shift of the reversal potential toward negative values because of the large outwardly directed Na<sup>+</sup> concentration gradient. The amplitude of the current and the slope of the I/V curve was, however, much larger than expected. At high positive membrane potentials, the current should be dominated by the outward movement of Na<sup>+</sup> ions, depending mostly on the intracellular Na<sup>+</sup> concentration which was similar in both cases; thus, the two I-V curves should converge at high positive membrane potential (as expected from the classical Goldman-Hodgkin-Katz constant field model shown in dotted line in Fig. 3 C). The cord conductance measured between 30 and 60 mV was significantly larger in the presence of 1 mM Na,  $13.7 \pm 4.3$   $\mu$ S, than in the presence of 100 mM extracellular,  $5.1 \pm 1.2$   $\mu$ S ( $n = 11$ ,  $P < 0.05$ , paired Student's *t* test), indicating that self inhibition affects also outward current flowing through ENaC.

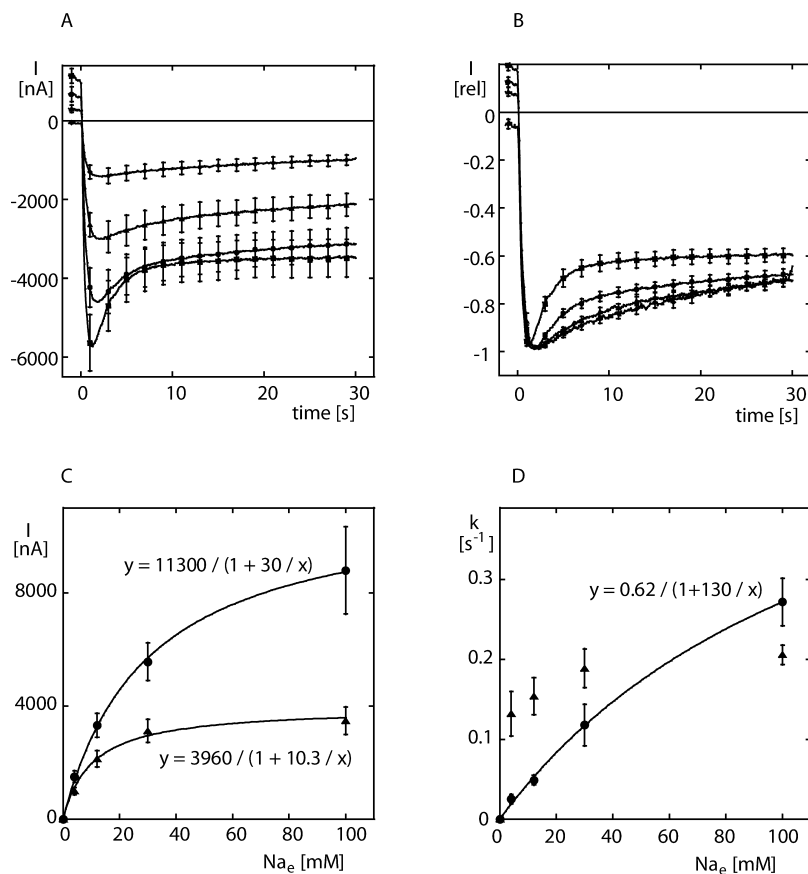
#### *Sodium Concentration Dependence of Self Inhibition*

The dependence on the extracellular Na<sup>+</sup> concentration was evaluated in a series of experiments in which four Na<sup>+</sup> concentration jumps, from 1 to 4, 1 to 12, 1 to 30, and 1 to 100 mM, were tested successively on the same oocytes. As shown in Fig. 4, A and B, increasing Na<sup>+</sup> concentration resulted in larger peak and steady-state currents. Fig. 4 B also shows that current decline after the peak amplitude, i.e., self inhibition, was clearly more pronounced at high than low Na<sup>+</sup> concentrations. The kinetic parameters were obtained by model fitting of the mean current values shown in Fig. 4 A. The Na<sup>+</sup> concentration dependence of the steady-state current (Fig. 4 C) demonstrates the high apparent affinity ( $k_M = 10.3$  mM) usually observed for the macroscopic current (Palmer et al., 1998). The same panel also shows that the  $I_{max}$  current, i.e., the extrapolated value of the current before the occurrence of self inhibition, has a lower apparent affinity with a  $k_M$  of 30 mM, closer to what has been reported for the Na<sup>+</sup> concentration dependence of the single channel current (Palmer et al., 1998; Kellenberger et al., 1999). Fig. 4 D shows that  $k_i$  was strongly Na<sup>+</sup> concentration dependent but with a rather low affinity, with an estimated  $k_{1/2}$  of more than 100 mM. Even though this value is obviously not very precise as the maximal Na<sup>+</sup> concentration used was of the same magnitude as the estimates  $k_{1/2}$ , the difference of  $k_i$  between the current traces recorded at 30 and 100 mM Na<sup>+</sup> strongly suggests that the affinity is rather low. The  $k_a$  values were only weakly Na<sup>+</sup> concentration dependent.

#### *Effect of Trypsin on Na<sup>+</sup> Self Inhibition*

Trypsin, and other serine proteases, have been shown to increase the activity of ENaC expressed in *Xenopus*

FIGURE 4. Sodium concentration dependence of self inhibition. A shows the mean amiloride-sensitive current recorded after 1 to 4 (losanges), 1 to 12 (triangles), 1 to 30 (circles), and 1 to 100 (squares) mM Na<sup>+</sup> concentration jumps. The solid line is in each case the mean of nine current values (measured at 0.3-s interval) and the errors bars indicate the SEM every 2 s. Because most of the variation in the current values originate in the variable level of expression of the channel in individual oocytes, we have also presented the same results as values normalized to the highest current value (peak current) recorded during each 30 s measurement in B. (C) Na<sup>+</sup> concentration dependence of the extrapolated maximal current ( $I_{max}$ , circles) obtained by model fitting of the individual data and of the steady-state current (triangles), i.e., the current value 30 s after the Na<sup>+</sup> concentration change. (D) Na<sup>+</sup> concentration dependence of the activation and inactivation constant ( $k_a$ , triangles and  $k_i$ , circles). In C and D, the solid line is the Michaelis-Menten relation with the best fitting parameters indicated in the equations printed in the graph. This relation is not shown for  $k_a$  because the concentration dependence was very weak for this parameter.



oocytes (Chraïbi et al., 1998). Proteases were found to be expressed in the same cells, most probably in the same apical membrane in which ENaC is found, first in *Xenopus* kidney A6 cell line (Vallet et al., 1997) and then also in mammalian collecting tubule cells (Vuagniaux et al., 2000). These findings strongly suggest a regulatory role for these proteases. In the following experiments, we have tested the hypothesis that extracellular proteases activate ENaC by interfering with the self inhibition process.

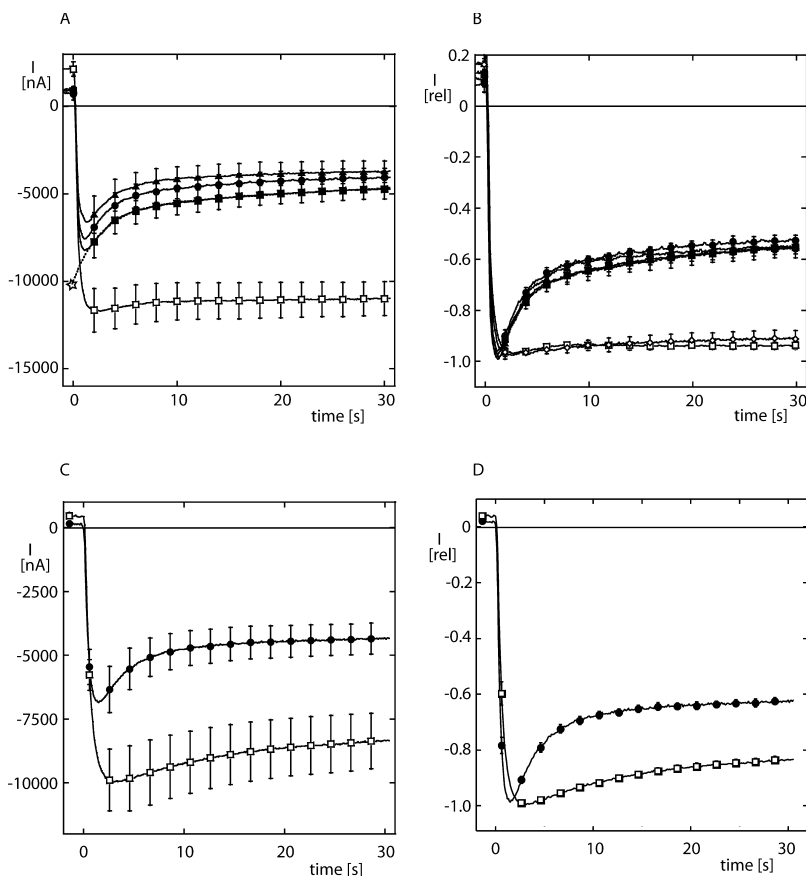
The effect of a 1–100 mM Na<sup>+</sup> concentration change was first measured under our standard conditions and then a second time in the same oocytes, in a group of oocytes incubated in the 10 mM Na<sup>+</sup> solution containing 5 μg/ml trypsin for 5 min and a control group of oocytes incubated in the same solution without trypsin. In a third group, trypsin was added in the presence of 10 μM amiloride. As shown in Fig. 5 A, the usual current relaxation was observed at the time of the first measurement but no or only a small decrease of the current amplitude was seen upon Na<sup>+</sup> addition at the time of the second measurement in trypsin-treated oocytes. The steady state amiloride-sensitive current (i.e., the current measured 30 s after the Na<sup>+</sup> concentration increase) was more than twice larger than the control (before trypsin) value in trypsin-treated oocytes, whereas it was slightly smaller in control oocytes. As shown in Fig. 5 B, the presence of

amiloride during the incubation time with trypsin did not prevent the effect of the protease and the results were similar in the trypsin and the trypsin plus amiloride groups. The extrapolated maximal current ( $I_{max}$  obtained by model fitting, indicated by a star in Fig. 5 A) had an amplitude similar, although somewhat smaller, than the steady state value after trypsin treatment.

#### Effects of *p*-CMB, Novobiocin, and Benzimidazolylguanidine

A number of various organic and inorganic compounds have been shown to abolish self inhibition in several model epithelia (Lindemann and Voute, 1976; Luger and Turnheim, 1981; Li and Lindemann, 1983; Li et al., 1986; Garty and Benos, 1988). We have tested the effect of three of these chemicals on the amiloride-sensitive current due to human ENaC expressed in *Xenopus* oocytes. Two of these compounds, novobiocin, and benzimidazolylguanidine (BIG), resulted in a concentration-dependent and rapidly reversible (amiloride-like) inhibition of weak amplitude. The inhibition reached  $27 \pm 4\%$  ( $n = 5$ ) and  $22 \pm 2\%$  ( $n = 6$ ) of the total amiloride-sensitive current at the highest concentration tested of 1 mM for novobiocin and BIG, respectively. Because of this dominant inhibitory effect, these compounds were not tested further for their effect on self inhibition. The mercurial compound *p*-chloro-mercuribenzoate (*p*-CMB) induced a significant increase of the steady-state amil-





amiloride during the exposure to trypsin did not make any detectable difference. C and D show the mean current values after a 1–100 mM Na<sup>+</sup> concentration change recorded in the absence and in the presence of 0.5 mM *p*-CMB. In all four panels, the solid line is in each case the mean current value (measured at a sampling rate of 3 s<sup>-1</sup>) and the errors bars indicating ±SEM are shown with the symbols every 2 s. The data are the results of nine pairs of measurements; control are indicated by circles and measurements with *p*-CMB by white squares.

oride-sensitive current and its effects were studied by recording the current evolution after 1–100 mM Na<sup>+</sup> concentration jump in the absence and in the presence of 0.5 mM *p*-CMB (this was the maximal concentration that we could solubilize in our experimental solution) successively in the same oocytes. As shown in Fig. 5, C and D, 0.5 mM *p*-CMB decreased the rate of current decline as well as the inhibition of the steady-state current, demonstrating a marked decrease of self inhibition. Lower concentrations had a smaller effect (unpublished data) and a full inhibition could not be reached because of solubility problems.

#### Effect of Temperature on the Steady-State Amiloride-sensitive Current

The effect of a sudden change in the temperature of the bath solution on the amiloride-sensitive current was studied. As shown in Fig. 6, a sudden increase of temperature from a cold (10–12°C) to a warm (31–34°C) solution resulted in a biphasic response: a fast increase in the current amplitude was followed by a

slower relaxation to a steady-state value lower than the value in the cold solution. Return to the cold solution resulted in the mirror image effect, a fast decrease followed by a slow relaxation to a steady-state amplitude larger than that recorded in the warm solution. In the presence of amiloride, raising the temperature only resulted in a small increase of the baseline current, which can be understood as temperature activation of the many endogenous ion-conductive pathways present in the oocyte membrane. After treatment with trypsin, the effect of a rise in temperature was completely different: only a large increase of the current amplitude to a steady-state value was observed. Thus, while in the control situation the ENaC carried current decreased with rising temperature; there was a clear increase in current amplitude with a higher temperature after protease treatment.

#### Self Inhibition in ENaC from other Species

We have also attempted to observe self inhibition in ENaC from other species expressed in *Xenopus* oocytes.

FIGURE 5. Effect of protease treatment and *p*-CMB on self inhibition. Mean amiloride-sensitive current during the 30 s after a sudden increase from 1 to 100 mM Na<sup>+</sup> (at time *t* = 0) are shown as absolute values in A and as values relative to the peak current in B. In A, the experimental group is represented by black squares and open squares, before and after trypsin treatment (5 min in a 5 μg/ml trypsin solution), respectively. The results of a subgroup in which trypsin was added alone (*n* = 11) and another subgroup in which trypsin was added in the presence of 10 μM amiloride (*n* = 4) were similar and are pooled. The control group (*n* = 8) is represented by black circles and black triangles, respectively, before and after a 5-min incubation in a solution without trypsin. The dotted line shows the theoretical curve with the best fitting parameters obtained from the mean current data of the experimental group before trypsin treatment (*k<sub>a</sub>* 0.15 s<sup>-1</sup>, *k<sub>i</sub>* 0.21 s<sup>-1</sup>, *k<sub>down</sub>* 0.0061, *I<sub>max</sub>* 9979 nA). The extrapolated current value at time 0 (*I<sub>max</sub>*) is indicated by a star. Because most of the variation in the current values originate in the variable level of expression of the channel in individual oocytes, we have also presented the same results as values normalized to the highest current value recorded during each 30-s measurement (peak current). In this panel the values of the trypsin and trypsin+amiloride groups are represented separately by squares and diamonds, respectively (black symbols before trypsin, open symbols after trypsin). Presence of

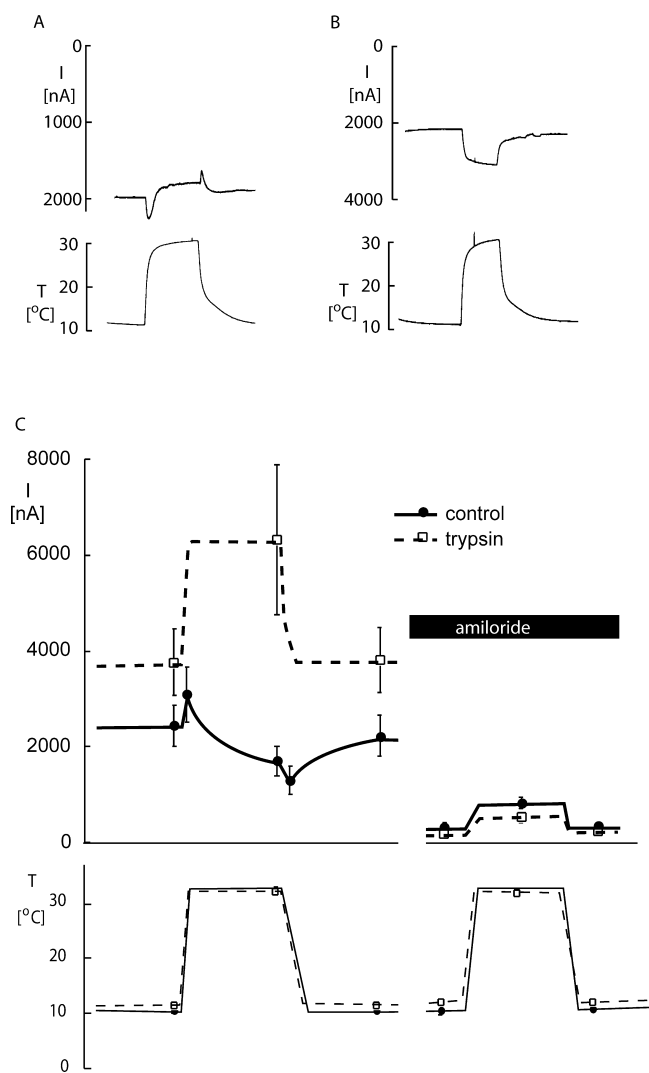


FIGURE 6. Effect of temperature on the steady-state amiloride-sensitive current. A shows current and temperature recordings illustrating the biphasic effect of a sudden rise or decrease of temperature in a control oocyte expressing hENaC. B shows the effect of the same change of temperature on an oocyte previously exposed for 10 min to 2  $\mu\text{g/ml}$  trypsin. In this case, the response was monophasic with an increase in current amplitude with higher temperature. C is a scheme showing the summary of measurement on 11 control oocytes and 7 oocytes exposed to trypsin. The thick continuous line (control) and dashed line (trypsin) indicate the mean values of the amiloride-sensitive current recorded at the points indicated by the trace. The right part of the figure shows the effect of temperature change in the presence of 10  $\mu\text{M}$  amiloride. These values were similar in the control and trypsin-treated group. The left part of the figure shows the mean values of the amiloride-sensitive current (i.e., the current values after subtraction of the values recorded in the presence of amiloride). The lower part of the figure shows the mean values of the recorded temperature. Some error bars are not visible because they are smaller than the symbols.

With *Xenopus* ENaC, the inactivation and activation rate constants seem to be faster and thus were more difficult to quantify due to the limited speed of our solution ex-

change, and we think that the values that we obtained were not reliable. With rat ENaC, the current relaxation had a time course similar to that obtained with human ENaC, but a smaller amplitude. Kinetic parameters obtained by model fitting of measurements performed by 1–100 mM  $\text{Na}^+$  concentration jump at room temperature ( $n = 8$ ) indicated a faster activation constant ( $k_a = 0.33 \pm 0.03 \text{ s}^{-1}$ ), and a slower inactivation rate ( $k_i = 0.089 \pm 0.013 \text{ s}^{-1}$ ) than that observed with human ENaC. The rundown factor had a value similar to that observed in the human ( $k_{down} = 0.0024 \pm 0.003 \text{ s}^{-1}$ ). As shown in the example shown in Fig. 1 C, the higher  $k_a$  and the slower  $k_i$  result in a steady-state equilibrium poised to the active state of the channel and thus a smaller amplitude of self inhibition with rat ENaC than with human ENaC.

#### DISCUSSION

Our experiments with fast solution exchanges allowed us to study self inhibition of human ENaC expressed in *Xenopus* oocyte. The solution exchange rate (with a  $t_{1/2}$  of  $\sim 1$  s) that we could obtain around the oocytes were not much faster than the rate of the onset of self inhibition allowing only for a limited precision in the determination of the kinetic parameters that were estimated by model fitting. Self inhibition occurred with kinetics and an amplitude that were similar to those described for the  $\text{Na}^+$  current measured in amphibian epithelia (Fuchs et al., 1977; Lindemann, 1984). This simple observation suggests that self inhibition is a property of the ENaC protein itself, composed of the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, and does not require additional components, such as a distinct “sodium receptor” protein. However, we cannot exclude that endogenous components, naturally present at the surface of the oocyte, could also play a role in this regulatory mechanism. The possibility of observing self inhibition in an artificial expression system will allow further characterization molecular mechanisms responsible for this phenomenon and to define the nature of the  $\text{Na}^+$  sensing site associated with ENaC.

Very similar results were observed when the current relaxation was observed when the starting condition was a 1 mM  $\text{Na}^+$  solution or when it was a 100 mM solution containing 10  $\mu\text{M}$  amiloride. This observation indicates that immediately after removal of amiloride the channel is in a state analogous to that of the channel exposed to a low  $\text{Na}^+$  solution. Two hypothesis could explain this fact. First, amiloride could prevent extracellular  $\text{Na}^+$  ions from reaching the  $\text{Na}^+$ -sensing site, which would suggest a sensing site located close to the channel pore. Second, amiloride binding may stabilize a high open probability conformation by acting on the “gating” mechanism rather than on the  $\text{Na}^+$  “sensing” mechanism of the channel. The fact

that amiloride did not prevent trypsin from abolishing self inhibition suggests that the  $\text{Na}^+$ -sensing site is still accessible in the presence of amiloride and favors the second hypothesis. There are several indications that, in addition to their pore blocking property, amiloride and some of its analogs may also activate ENaC or related channels. First, amiloride-related guanidinium compounds such as benz-thiazolyl-guanidinium (BTG) or BIG have been shown to increase the  $\text{Na}^+$  current by preventing self inhibition in amphibian epithelia (Zeiske and Lindemann, 1974; Lindemann and Voute, 1976; Fuchs et al., 1977; Garty and Benos, 1988). Second, amiloride has a biphasic effect (agonist and then antagonist) on the cation conductance of the larval frog skin (Hillyard and Van Driessche, 1989). Our results suggest that amiloride also has an agonist effect on human ENaC, but that this effect is usually masked by the high affinity antagonist effect due to pore block and can be revealed only by fast amiloride removal.

To confirm that the current decline observed in the present experiments was analogous to the self inhibition reported with amphibian tissue, we have also tested compounds known to interfere with this process. Two of these compounds, BIG and novobiocin, could not be studied because they produced an amiloride-like inhibition of the amiloride-sensitive current; the effect of BIG seems to be species dependent; it was an activator in frog skin and an inhibitor in the toad urinary bladder (Li and Lindemann, 1983). To our knowledge the effects of these two chemicals have been reported only on amphibian tissues. A significant effect of *p*-CMB, and other organic mercurials, has, however, been demonstrated on mammalian colon epithelium (Luger and Turnheim, 1981). This effect was interpreted as due to the interaction of the organic mercurial with a sulphydryl group of the channel protein. We observed a nearly complete reduction of the self inhibition in the presence of 0.5 mM *p*-CMB. This effect was rapidly reversible (within seconds) by simple washing off *p*-CMB, without reducing the agent, and it is thus improbable that this effect is due to reaction of *p*-CMB with a sulphydryl group of the protein.

Our experiments with  $\text{Na}^+$  concentrations ranging from 4 to 100 mM indicate that the  $\text{Na}^+$  sensing site responsible for self inhibition has a low affinity, with a  $k_M$  of  $\sim 100$  mM or above. The distinct values of the  $\text{Na}^+$  concentration dependence of the steady-state current and of the maximal current (before self inhibition) are those expected from the hypothesis of a  $\sim 30$  mM  $k_M$  for the open channel conductance and an apparently lower  $k_M$  of  $\sim 10$  mM for the steady-state macroscopic current due to self inhibition acting on the open probability at high  $\text{Na}^+$  concentrations.

The current generated by the movement of ions in solution is expected to increase with absolute temperature (Hille, 1992). In the case of the flow of ions across an open channel, the precise relation between the temperature and the current depends on the activation energy of complex processes that allow ion permeation (ion dehydration, binding, dissociation). An apparently paradoxical effect of temperature on the current carried by ENaC was recently reported by Askwith et al. (2001), who showed a cold-induced stimulation of current carried by this channel. These authors also studied the acid-sensing ion channels (ASICs), which are rapidly desensitizing cation channels activated by extracellular acid pH. They showed that the rate of ASIC desensitization was highly temperature dependent. In the present work, we demonstrate that the  $\text{Na}^+$  self inhibition phenomenon is similarly temperature dependent.

Our results can be well explained by the existence of two main conformations of the channel, one with a high open probability ( $P_o$ ) and another one with a low  $P_o$ , as suggested by Palmer and coworkers (Palmer and Frindt, 1996; Palmer et al., 1998). According to this model, when exposed to a low extracellular  $\text{Na}^+$  concentration, or immediately after dissociation of amiloride from its binding site, ENaC is found predominantly in the high  $P_o$  mode. When exposed to a higher extracellular  $\text{Na}^+$  concentration, an  $\text{Na}^+$  self inhibition process is activated and the channel relaxes to a new equilibrium between the high  $P_o$  and the low  $P_o$  modes. At low temperature the equilibrium is strongly poised toward the active state and, conversely, the low  $P_o$  state is favored at high temperature, because the rate of the conversion from the high  $P_o$  to the low  $P_o$  mode (the inactivation rate,  $k_i$ ) is highly temperature dependent, whereas rate of the reverse reaction (the activation rate,  $k_a$ ) is poorly temperature dependent. This model can account for the observation of a higher current carried by human ENaC at low temperature reported by Askwith et al. (2001) and confirmed in the present work (see Fig. 6). In fact, our results also show the expected increase in conductance with temperature, but this increase can only be observed during a short time after a fast solution exchange, before it is masked by self inhibition, or when self inhibition has been abolished by protease treatment (see below). The fast component was not apparent in the data of Askwith et al. (2001), but could be observed in our experiments thanks to the fast rate of temperature change as documented in the example of Fig. 6.

Concerning the effects of temperature change on the macroscopic amiloride-sensitive current, we interpret our observations of a biphasic response as the results of two opposing phenomenon. First, the initial increase in current with raising temperature is due to the increase

in ion mobility with temperature that tends to enlarge the single channel conductance due to higher ion mobility. Second, the slower decrease in current is due to the fact that the inactivation rate  $k_i$  increases more than the activation rate  $k_a$  (see Fig. 2) with rising temperature, probably resulting in a lower open probability at high temperature. This interpretation is confirmed by the observation of the effect of temperature after trypsin treatment. After abolishment of the self inhibition process by protease treatment, only the increase in current amplitude due to increased ion mobility at high temperature can be seen. Technical difficulties in obtaining single channel current recording in a large range of well controlled temperatures has prevented us, thus far, from confirming this interpretation by measurements of open probability and single channel conductance.

#### *Protease Treatment Abolishes Na<sup>+</sup> Self Inhibition*

Treatment with a low concentration of extracellular trypsin increased the steady-state current and abolished the current relaxation after an Na<sup>+</sup> concentration increase. This suggests that the proteolytic action and/or the binding of trypsin results in a modification of ENaC interfering with the self inhibition process. This effect seems to be analogous to what has been reported for several compounds that have been shown to abolish self inhibition in amphibian and mammalian epithelia (Lindemann and Voute, 1976; Luger and Turnheim, 1981; Li and Lindemann, 1983; Li et al., 1986; Garty and Benos, 1988) and to the effects of *p*-CMB observed in the present study. The observation that the S<sub>520</sub>K mutation in the  $\beta$  subunit of ENaC (the mutation homologous to the degenerin activating mutation) resulted in a loss of the temperature sensitivity (Askwith et al., 2001) suggest the hypothesis that this mutation also prevents or slows down the inactivation process.

The overall open probability of ENaC calculated from the ratio of the whole membrane current to the density of the ENaC protein expressed at the oocyte surface has been estimated to be very low (Firsov et al., 1996). Self inhibition could be one of the factors responsible for this low open probability. If one can extrapolates the results obtained with hENaC expressed in *Xenopus* oocyte to the situation of the human distal nephron, one can calculate (from the  $k_i$  and  $k_a$  Arrhenius plots) that, at high extracellular Na<sup>+</sup> concentration, only ~10% of ENaC would be in the high open probability configuration at 37°C. Therefore, removing self inhibition by the effect of an apical membrane protease could result in an ~10-fold increase in ENaC open probability and thus could be a potentially significant regulatory mechanism.

Our results propose a general model of gating for members of the ENaC/degenerin channel family. Fol-

lowing "activation" by amiloride or exposure to a low Na<sup>+</sup> solution in the case of ENaC, or by acid exposure in the case of ASIC, both types of channel undergo a temperature-dependent inactivation process. In the case of ENaC, this inactivation is clearly dependent on the presence of a high extracellular Na<sup>+</sup> concentration, and is responsible for the phenomenon described as self inhibition. This process has a time course of a few seconds and is highly temperature dependent. The slow time course and the large value of the activation energy suggest that this process does not only reflect the binding of Na<sup>+</sup> ions, but includes a large conformational change in the protein, possibly an energy consuming event, that may be initiated by the binding of Na<sup>+</sup> ion(s) to an extracellular binding site.

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