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ION CHANNELS, TRANSPORTERS

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Dual effect of temperature on the human epithelial Na^+ channel

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Abstract The amiloride-sensitive epithelial sodium channel (ENaC) is the rate-limiting step for sodium reabsorption in the distal segments of the nephron, in the colon and in the airways. Its activity is regulated by intracellular and extracellular factors but the mechanisms of this regulation are not yet completely understood. Recently, we have shown that the fast regulation of ENaC by the extracellular [Na^+], a phenomenon termed self-inhibition, is temperature dependent. In the present study we examined the effects of temperature on the single-channel properties of ENaC. Single-channel recordings from excised patches showed that the channel open probability (P_o, estimated from the number of open channels $N{\cdot}P_o$, where N is the total number of channels) increased on average two- to threefold while the single-channel conductance decreased by about half when the temperature of the perfusion solution was lowered from ~30 to ~15°C. The effects of temperature on the single-channel conductance and P_o explain the changes of the macroscopic current that can be observed upon temperature changes and, in particular, the paradoxical effect of temperature on the current carried by ENaC.

Keywords ENaC · Open probability · Unitary current · Temperature

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Introduction

The epithelial sodium channel (ENaC) is a heteromeric channel consisting of three subunits α, β and γ, localized in the apical membrane of epithelial cells [7]. Transport across the apical membrane through ENaC is the rate-limiting step in sodium reabsorption by the epithelial cells of the distal nephron, the distal colon and in airways. ENaC thereby plays a key role in the regulation of sodium balance, extracellular fluid volume and blood pressure by the kidney and in the controlled fluid reabsorption in the airways [9]. ENaC activity is regulated by several intracellular and extracellular factors. Two different types of regulation can be distinguished: the control of the channel density and the control of channel open probability (P_o). The role of the regulation of channel density is well established as the main mechanism for the classical hormonal control of sodium reabsorption, even though modulation of P_o may also be important [13]. The control of P_o of ENaC is, however, not well understood, for either hormonal or other types of regulation. Some studies have suggested that membrane potential and extracellular sodium control the activity of ENaC by modulating its P_o [11, 12]. Recently, we have shown that the inactivation of ENaC by the extracellular [Na^+] is an intrinsic property of this channel, is highly temperature dependent and can be abolished by treatment with extracellular proteases [5], suggesting the existence of two main conformations of the channel: one with a high P_o and one with a low P_o, as proposed by Palmer and collaborators [11, 12]. In addition, the temperature dependence of the amiloride-sensitive current induced by the expression of human ENaC in *Xenopus* oocytes appeared paradoxical: the steady-state amiloride-sensitive current at cold temperatures was larger than that at high temperatures. A similar effect has been reported by Askwith et al. [1], who also reported that the ENaC-related brain sodium channel (BNaC) exhibits a similar temperature dependence with slower inactivation at low temperature.

In the present study, we used the patch-clamp technique to study the effects of temperature on the single-channel

properties of human ENaC expressed in *Xenopus* oocytes. Our results show opposite effects of temperature on P_o and on single-channel conductance and allow us understand the complex effect of temperature on macroscopic amiloride-sensitive currents. In particular, the apparently paradoxical increase of the Na^+ current observed at low temperature can be explained by an increased P_o of ENaC.

Materials and methods

Isolation of *Xenopus* oocytes, injection and expression of human α-, β- and γ-ENaC

Oocytes were collected from anaesthetized (MS 222, 2 g/l; Sandoz, Basel, Switzerland) female *Xenopus laevis* and prepared as described previously [3]. Defolliculated stage V–VI oocytes were injected with equal amounts (0.2–0.5 ng) of cDNA encoding each of the α-, β- and γ-subunits of human ENaC (hENaC) in a total volume of 50 nl. The hENaC subunit cDNAs, generously provided by P. Barbry, were subcloned into the pBSK vector, linearized by NOT I and capped cRNA was synthesized in vitro by SP6 polymerase. The injected oocytes were incubated for 1–2 days in a low-Na^+ solution (in mM): NaCl 10, KCl 2.0, $CaCl_2$ 0.7, $MgCl_2$ 0.8, *N*-methyl-D-glucamine (NMDG)-Cl 80, NMDG-HEPES 10.0, pH 7.4. The low [Na^+] was chosen to prevent excessive Na^+ loading during the time needed for ENaC expression.

Ion current measurement in whole oocytes

The amiloride-sensitive Na^+ current was measured as previously described [2, 4] using the two-electrode voltage-clamp technique and a Dagan TEV voltage-clamp apparatus (Dagan, Minneapolis, Minn., USA) at a holding potential of −60 mV. The perfusion chamber, with a cross-section of 3×3 mm and a length of 35 mm was perfused by gravity at 12–15 ml/min. The composition of the control experimental solution was (in mM): Na-gluconate 100, NMDG-HEPES 10, $MgCl_2$ 0.8, $CaCl_2$ 0.4, $BaCl_2$ 5, TEA-Cl (tetraethyl-ammonium-Cl) 10, pH 7.4. Barium, TEA and a low [Cl^-] were used to reduce the background membrane conductance. To achieve rapid amiloride removal, amiloride was added by injection of a 300 μM solution directly into 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 final amiloride concentration of 10 μM.

The current signal was filtered at 20 Hz using the internal filter of the voltage-clamp apparatus, and continuously recorded on a paper chart. The current elicited by amiloride removal was recorded for 30- to 50-s episodes using a Digidata 1322 A acquisition system (Axon Instruments, Union City, Calif., USA) for analysis.

The experiments were carried out at two different temperatures ~30°C and ~15°C. The temperature in the bath was measured using a digital thermometer (TTX 1090, Ebro, Ingolstadt, Germany), the sensing tip of which was placed in the flow of the solution 5–10 mm downstream from the oocyte.

Single-channel recordings

Before patch-clamp experiments the oocytes were placed for 3–5 min at room temperature in a hypertonic medium (475 mOsm) with the following composition (in mM): K-aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, HEPES Na 10, pH 7.4. The vitelline membrane could then be removed manually from the cell using fine forceps [10]. The oocytes were then transferred to the recording chamber immediately.

Single-channel currents were measured in the excised outside-out patch configuration of the patch-clamp according to [8]. Patch pipettes were made of borosilicate glass (WPI, Sarasota, Fla., USA) and pulled in two stages with a PP-83 vertical puller (Narishige, Japan) and fire-polished. They had a resistance of 10–20 MΩ. The pipette solution contained (in mM): 75 CsF, 17 NMDG, 10 EGTA and 10 HEPES, pH 7.4, titrated with HEPES acid. The composition of the extracellular solution was as given above. The effect of 1 μM amiloride in the bath solution was used to identify the presence of ENaC in the membrane patch. By convention, negative (downwards) single-channel currents correspond to Na^+ flux from the extracellular to the intracellular side of the membrane and the intracellular potential corresponds to the pipette potential (V_{pip}). Extracellular solutions (with or without amiloride) flowed under gravity through water-jacketed inflow lines and the temperature of the flowing solutions was controlled by adjusting the temperature of the water in the water jacket. The temperature of solutions flowing into the measurement chamber was measured using the TTX 1090 digital thermometer with the thermistor placed about 3 mm from the solution outflow. The temperature could not be measured at the same time as current recording but was checked before and after the measurements by placing the temperature probe in the flowing solution at the position previously occupied by the patch pipette. The measured temperature was 29±1°C in the warm solution and 15 ±1°C in the cold solution. Solutions were exchanged by moving the set of perfusion pipettes manually, so exposing the patch pipette to the flow of extracellular solution.

Single-channel currents were recorded with a List LM EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) and stored on a digital tape recorder (DTR 1200 Biologic, France). Current signals were filtered at 200 Hz with an 8-pole Bessel filter (Frequency Devices, 902, USA) and digitized at 1 kHz using a Digidata 1200 interface analogue-to-digital converter and AxoScope software (Axon Instruments). Single-channel current data were analysed using PClamp 8 software (Axon Instruments). The number of open channels (the product of P_o and the total number of channels N) was calculated as $N \cdot P_o = I_{Na}/i_{Na}$, where i_{Na} is the unitary current measured as the peak-to-peak interval in the current amplitude histogram and I_{Na} the current due to ENaC. I_{Na} was measured in 0.5- to 1-min recordings as the difference between the mean total current and the current in the absence of any open channels (the latter defined from recording in the presence of 1 μM amiloride).

Results

To understand the mechanism of the complex effect of temperature on current carried by ENaC channels we compared the effects of cold (~15°C) and warm (~30°C) temperatures on the macroscopic amiloride-sensitive current and the single-channel properties using *Xenopus* oocytes expressing human ENaC.

Effect of temperature on whole-cell amiloride-sensitive current

Figure 1 (upper panel) illustrates the effects of temperature on the macroscopic amiloride-sensitive current. These results are very similar to those obtained earlier [5]. At the high temperature (28°C), after fast removal of amiloride, the sodium current increased to a peak followed by relaxation with a time course of a few seconds to a steady-state value. This fast, secondary decline of the Na^+ current is due to sodium self-inhibition in the presence of a high extracellular [Na^+]. A slower run-down of the current is

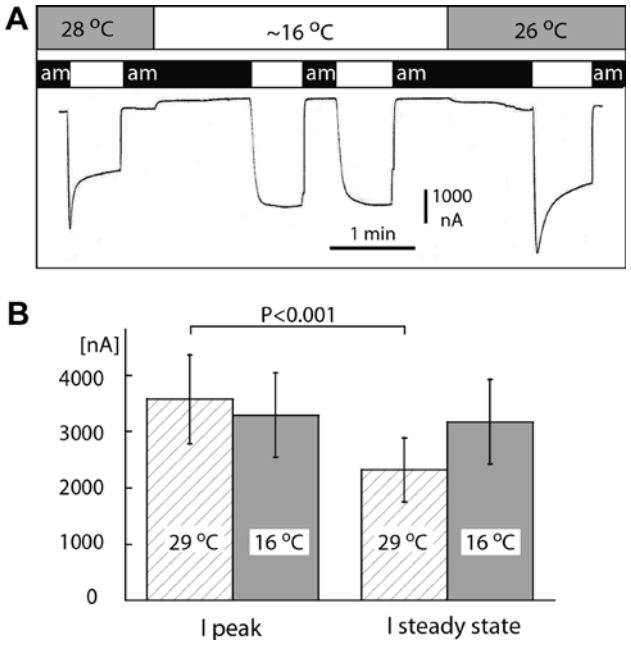

Fig. 1A, B Effect of temperature on human epithelial Na^+ channel (hENaC)-mediated amiloride-sensitive current. **A** Original whole-oocyte current recorded at −60 mV holding potential, in a warm (28°C) and a cold (16°C) solution 100 mM Na^+ solution without or with 10 μM amiloride (*am*). **B** Mean values of peak (I_{peak}) and steady-state ($I_{steady\ state}$) amiloride-sensitive current measured at warm (28.9±0.3°C, *n*=22) and cold (15.8±0.3°C, *n*=21) temperatures. In the cold there was no obvious "peak" so that the maximal current amplitude was used as the peak current

probably due to feed-back inhibition resulting from the increase of intracellular [Na^+] [5]. The mean values of the ratio of peak current to steady-state current (current measured 30 s after the removal of amiloride) was 1.61 ±0.05 (*n*=22). In contrast, when the temperature of the perfusion solution was around 15°C, amiloride removal elicited a large inwards current, but there was no significant secondary decline in the current amplitude and, 30 s after amiloride removal, the current stabilized at a value 37% larger (*n*=22) than the steady-state value observed at 29°C (the mean values obtained under these conditions are shown in Fig. 1, lower panel).

Effect of temperature on the number of open channels

In the patch-clamp experiments, the identity of the recorded channels was verified at each temperature by reversible block with 1 μM amiloride. As shown in Fig. 2, the number of open channels was clearly larger under cold than under warm conditions. The identity of the channel was verified in each case by the effect of 1 μM amiloride, which decreased P_o to a low value, allowing the current level corresponding to the absence of opened ENaC to be determined. The two traces in Fig. 2 also illustrate the larger value of single-channel conductance at high than at low temperature. For the experiment shown in Fig. 2 the mean $N{\cdot}P_o$ during the 40- to 50-s period without amiloride was 3.23 and 0.84 in the cold and warm solutions, respectively. The unitary current was 0.26 and 0.52 pA in the cold and warm solutions, respectively.

Raising the temperature from ~15 ° to ~30°C in the presence of amiloride increased the baseline inwards current, i.e. the current level with all ENaC blocked. An

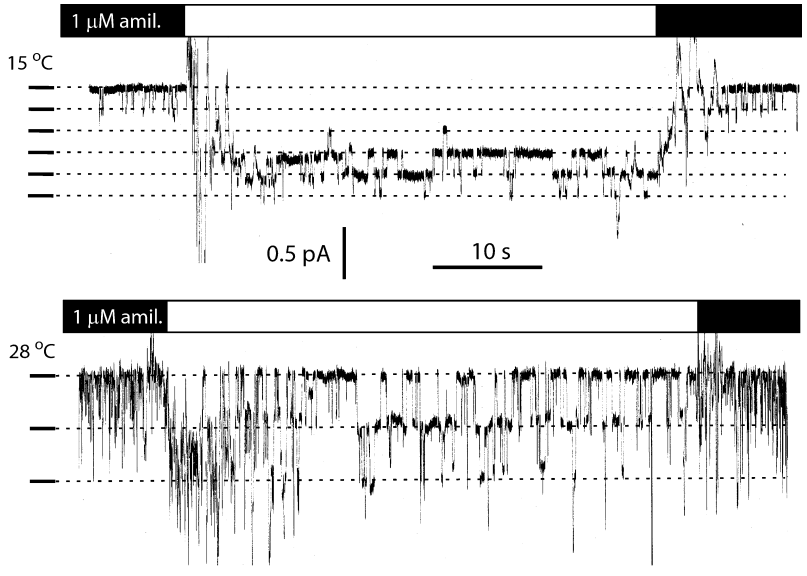

Fig. 2 Effect of temperature on amiloride-sensitive single-channel activity. Original patch-clamp current recordings demonstrating ENaC activity in out-side out configuration. Both recordings are from the same patch and the *top* and *bottom* traces show current recorded at 15°C and 28°C, respectively. The extracellular solution contained 100 mM Na^+ and the potential was −120 mV. A *downwards* deflection indicates current flowing into the pipette. The *x*-axis (time) and the *y*-axis (current) scales are the same for both traces. Amiloride (1 μM) was present at the beginning of the recording, was then removed and added again at the end. This manoeuvre was performed to identity ENaC channels and to determine the current level corresponding to the closed state of all ENaC in the patch. This closed channel level, indicated by the *0 level* (*first dashed lines*), can be clearly identified even if there are artefacts at the time of the solution exchange

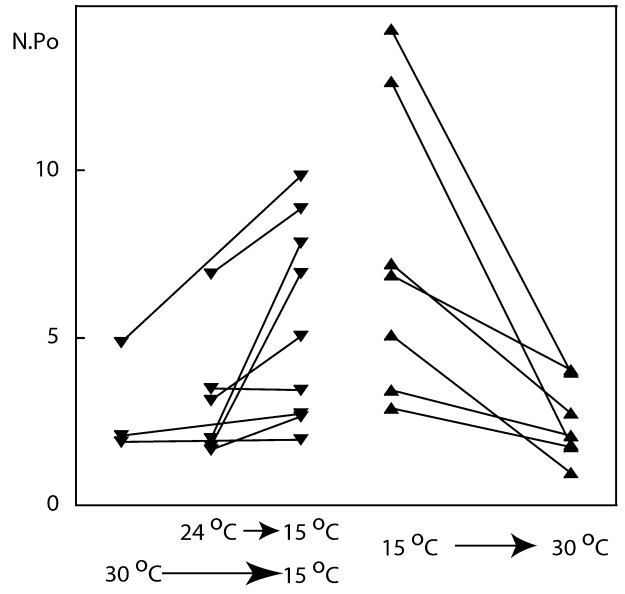

Fig. 3 Effect of temperature on the numbers of open ENaC in an excised patch. The data are values of $N \cdot P_o$ (where N is the total number of channels and P_o the open probability, and $N \cdot P_o$ is calculated as the ratio between the macroscopic and single-channel currents, see text) from pairs of current recordings, (each of 30–60 s duration) in presence of 100 mM Na^+ at warm and cold temperatures. The data at the *left* are pairs of measurements made first at the high (~24 or ~30°C) and then at the cold (~15°C) temperature; the data at the *right* were obtained in the reverse order

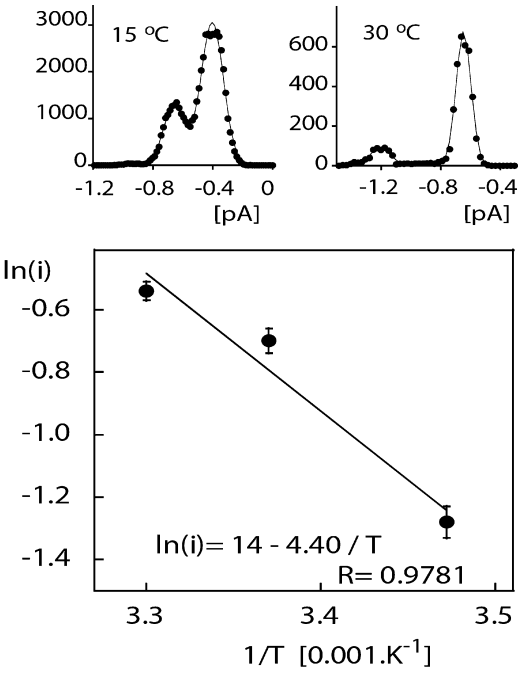

Fig. 4 Effect of temperature on single-channel conductance. The *upper panels* show amplitude histograms of recordings at high (*right*) and low (*left*) temperatures. The *continuous line* is the best fit to the Gaussian distribution function. The unitary currents were 0.25 pA at 15°C and 0.56 pA at 30°C in these sample cases. The lower panel shows an Arrhenius plot of the relationship between unitary current and temperature (15±1, 24±1 and 30±2°C). The *three points* represent the mean of 16 pairs of measurements at −120 mV grouped for the three temperatures. The differences between the values at 15 and 30°C and between 15 and 24°C are significant (P<0.001 in both cases). The *solid line* is the regression line, from which the activation energy was calculated (see text)

increase of the baseline amiloride-insensitive current is also apparent in the macroscopic current recording in the presence of amiloride (see Fig. 1 and [5]). This may be due to the activation by high temperature of endogenous channels or other ion-conductive pathways in the oocyte membrane. This increase of the amiloride-insensitive current was proportionally larger for the microscopic current (about 1–5 pA compared the ENaC-mediated current of 1–2 pA depending on the number of channels present in the patch) than in the whole-oocyte current, where it was only a small percentage of the amiloride-sensitive current. The large amiloride-insensitive current activated by raising the temperature may be rather due to reduced seal resistance induced by the temperature change.

Although it was difficult to maintain stable patches during temperature changes, we were able to obtain 16 paired recordings in which current was recorded for at least 30 s at high and low temperature without amiloride and for which the zero-channel-open current was recorded in the presence of amiloride at both temperatures. In 9 of these recordings the exposure to the warm temperature preceded the exposure to the cold and in the other 7 recordings vice versa. The analysis of these recordings is presented in Figs. 3 and 4.

The number of open channels ($N \cdot P_o$) increased in all but one case when the temperature was changed from warm to cold (Fig. 3, left) and decreased in all cases when the temperature was increased (Fig. 3, right). When the values of both experimental sequences are pooled the mean $N \cdot P_o$ was 2.8±0.3 at 24–30°C and 6.4±0.9 at 15°C (n=16, P<0.001, paired t-test).

Effect of temperature on the single-channel current

Temperature had also an effect on the single-channel current. The current traces in Fig. 2 show that the single-channel current (i_{Na}) was about twofold larger at 28°C than at 15°C. The mean single-channel current was 0.59 ±0.016 (n=9) and 0.27±0.016 pA (n=9) at high and low temperature, respectively. Figure 4 also shows an Arrhenius plot of the mean single-channel currents at the three temperatures. The data were obtained from 16 pairs of measurements of the unitary current: in 6 of these pairs of measurements the current was measured first at 24 and then at 15°C, in 7 cases first at 15 and then at 30°C and in 3 cases at 30 and then at 15°C. For both directions of change the difference between the high and the low temperature was significant (P<0.001, paired t-test). The activation energy (E_a) was estimated from the slope of the regression line (s_{Arr}) of the Arrhenius plot according to: $E_a = s_{Arr} \cdot R$, with R=8.314 J·mol^{-1} K^{-1}. E_a for the ENaC unitary current was 37 kJ·mol^{-1} under our conditions,

corresponding to a relative change for a 10°C temperature increase (Q_{10}) of 1.64.

Discussion

We have demonstrated recently that human ENaC expressed in *Xenopus* oocytes also shows the phenomenon of self-inhibition described earlier in urinary epithelia [5, 6]. From these studies we hypothesised that extracellular sodium inhibits the ENaC-mediated current through a highly temperature-dependent conformational change affecting most probably the channel P_o. However, the effects of temperature were complex with an immediate increase in Na^+ current after a rapid rise in temperature followed by a secondary decrease. In addition, the secondary decrease was abolished by prior protease treatment. In the present study we examined the effects of temperature on the single-channel properties of ENaC and these results provide a coherent explanation for the complex effect of temperature on the macroscopic amiloride-sensitive current.

We first showed that ENaC unitary current changed as expected with temperature, increasing with rising temperature, with an E_a of about 37 kJ·mol^{-1}, which is similar to observations on other cation channels [14]. This increased unitary current at high temperature explains the initial rise in current after a rapid temperature increase and also the steady current increase at high temperature observed when self-inhibition has been abolished by protease treatment [5]. In fact, the twofold increase of the unitary current on warming from 15 to 30°C that we observe in the present study can well explain the change of macroscopic amiloride-sensitive current (a 1.7-fold increase) seen on warming from 11 to 32°C after trypsin treatment (see Fig. 6 in [5]).

Second, P_o for ENaC increased significantly, a 2.3-fold change, on cooling by ~15°C. Kinetic analysis of the macroscopic ENaC-mediated current indicated that this increase resulted from the high temperature sensitivity of the inactivation rate that is responsible for the self-inhibition phenomenon. Our present data confirm that this effect of temperature occurs essentially through a rapid change of the P_o of ENaC. It is very unlikely that the rapidly reversible change in $N \cdot P_o$ that can be recorded over a few seconds in an excised patch could be due to a modification of the number of channels N present in the membrane and therefore we can safely attribute the $N \cdot P_o$ change to a modification of P_o. This is also confirmed by the obvious qualitative change of the opening duration in several patches, even though we could not obtain precise gating kinetic parameters because of the presence of multiple channels in all the patches from which we could record currents at both temperatures.

The decrease of P_o with increasing temperatures appears thus as the mechanism responsible for the paradoxical reduction of Na^+ current at high temperature [1, 5]. The combined effect of cooling from 30 to 15°C, namely a 2.1-fold decrease in unitary current and a 2.55-fold increase in P_o should result in a 1.2-fold (2.6/2.1=1.2) increase of the steady state macroscopic current, in reasonable agreement with the 1.37-fold increase of steady-state current seen for a similar change of temperature in whole-oocyte current experiments (see Fig. 1).

In summary, we have shown that the dual effect of temperature on ENaC unitary current and on the open probability can explain the complex effects observed in whole-cell current and, in particular, the seemingly paradoxical increase in ENaC-mediated current when temperature is reduced.

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References

1. Askwith CC, Benson CJ, Welsh MJ, Snyder PM (2001) DEG/ENaC ion channels involved in sensory transduction are modulated by cold temperature. Proc Natl Acad Sci USA 98:6459–6463
2. Canessa CM, Horisberger J-D, Rossier BC (1993) Functional cloning of the epithelial sodium channel: relation with genes involved in neurodegeneration. Nature 361:467–470
3. Canessa CM, Schild L, Buell G, Thorens B, Gautshi Y, Horisberger J-D, Rossier BC (1994) The amiloride-sensitive epithelial sodium channel is made of three homologous subunits. Nature 367:463–467
4. Chraïbi A, Vallet V, Firsov D, Kharoubi-Hess S, Horisberger J-D (1998) Protease modulation of the activity of the epithelial sodium channel expressed in *Xenopus* oocyte. J Gen Physiol 111:127–138
5. Chraïbi A, Horisberger J-D (2002) Na self inhibition of human epithelial Na channel: temperature dependence and effect of extracellular proteases. J Gen Physiol 120:133–145
6. Garty H, Benos DJ (1988) Characteristics and regulatory mechanisms of the amiloride-blockable Na^+ channel. Physiol Rev 68:309–372
7. Garty H, Palmer LG (1997) Epithelial sodium channels—function, structure, and regulation. Physiol Rev 77:359–396
8. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85–100
9. Horisberger J-D (1998) Amiloride-sensitive Na channels. Curr Opin Cell Biol 10:443–449
10. Methfessel C, Witzemann V, Takahashi T, Mishina M, Numa S, Sakmann B (1986) Patch clamp measurements on Xenopus laevis oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. Pflugers Arch 407:577–588
11. Palmer LG, Frindt G (1996) Gating of Na channels in the rat cortical collecting tubule—effects of voltage and membrane stretch. J Gen Physiol 107:35–45
12. Palmer LG, Sackin H, Frindt G (1998) Regulation of Na^+ channels by luminal Na^+ in rat cortical collecting tubule. J Physiol (Lond) 509:151–162
13. Rossier BC (2002) Hormonal regulation of the epithelial sodium channel ENaC: N or P_o? J Gen Physiol 120:67–70
14. Sesti F, Nizzari M, Torre V (1996) Effect of changing temperature on the ionic permeation through the cyclic GMP-gated channel from vertebrate photoreceptors. Biophys J 70:2616–2639