

# Rat ENaC expressed in *Xenopus laevis* oocytes is activated by cAMP and blocked by $\text{Ni}^{2+}$

Andrei Segal, Dana Cucu, Willy Van Driessche, Wolf-Michael Weber\*

Laboratory of Physiology, KU Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

Received 18 January 2002; revised 13 February 2002; accepted 13 February 2002

First published online 26 February 2002

Edited by Maurice Montal

**Abstract** We used oocytes of the South African clawed toad *Xenopus laevis* to express the three subunits of the epithelial  $\text{Na}^+$  channel from rat distal colon (rENaC). We combined conventional dual-microelectrode voltage-clamp with continuous capacitance ( $C_m$ ) measurements and noise analysis to evaluate the effects of cAMP and  $\text{Ni}^{2+}$  on rENaC. Control oocytes or rENaC-expressing oocytes exhibited no spontaneous fluctuations in current. However, in rENaC-expressing oocytes amiloride induced a marked plateau-shaped rise of the power density spectra. Recordings using four different concentrations of amiloride revealed that the blocker–channel interactions were of the first order. A cocktail of the membrane permeant cAMP analogue chlorophenylthio-cAMP and IBMX (cAMP cocktail) increased amiloride-sensitive current ( $I_{\text{ami}}$ ) and conductance ( $G_{\text{ami}}$ ). Furthermore,  $C_m$  was also increased following cAMP application, indicating an increase in plasma membrane surface area. Noise analysis showed that cAMP increased the number of active channels in the oocyte membrane while single-channel current decreased. From these data we conclude that cAMP triggered exocytotic delivery of preformed rENaCs to the plasma membrane.  $\text{Ni}^{2+}$  (2.5 mM) inhibited about 60% of the rENaC current and conductance while  $C_m$  remained unaffected. Noise analysis revealed that this inhibition could be attributed to a decrease in the apparent channel density, while single-channel current did not change significantly. These observations argue for direct effects of  $\text{Ni}^{2+}$  on channel activity rather than induction of endocytotic removal of active channels from the plasma membrane. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Amiloride-induced current fluctuation; Epithelial  $\text{Na}^+$  channel; Exocytosis; Membrane capacitance; Noise analysis

## 1. Introduction

$\text{Na}^+$  absorbing tight epithelia are characterized by the presence of a channel that mediates the uptake of  $\text{Na}^+$  from the luminal side into the cells using a gradient that is maintained by the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase. These epithelial  $\text{Na}^+$  channels (ENaCs) can be found in many vertebrate and invertebrate tissues where they serve diverse functions that range from taste transduction to  $\text{Na}^+$  reabsorption in the kidney [13]. This  $\text{Na}^+$  channel is characterized by its high sensitivity to the  $\text{K}^+$ -sparing diuretic amiloride ( $K_{1/2} \sim 0.1$

$\mu\text{M}$ ), has been cloned in 1993 and was termed ENaC [7,8]. ENaC has a high affinity for  $\text{Na}^+$  over other monovalent cations (e.g. 100:1 for  $\text{Na}^+:\text{K}^+$ ), exhibits a very low single-channel conductance of around 3–5 pS and shows extremely slow gating in the range of seconds. The channel is composed of three homologous subunits termed  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits, respectively; yet, the exact stoichiometry of the subunit composition is still controversial. Each of the subunits that share 35% homology at the amino acid level possesses two trans-membrane domains linked by a large extracellular loop. In the last few years since the cloning of ENaC a whole new family of structurally related ion channels termed the ENaC-Deg family was discovered [1].

Epithelial expression of ENaC under physiological conditions is quite low and there are few tissues that exhibit sufficient  $\text{Na}^+$  absorption to allow satisfactory study of the channel. Besides frog skin, A6 cells, originally derived from *Xenopus laevis* distal kidney, possess high  $\text{Na}^+$  absorption. Therefore, these cells are widely used to investigate thoroughly the features of ENaC. In A6 cells cAMP activates  $\text{Na}^+$  absorption by ENaC probably due to exocytotic delivery and functional insertion of preformed channel molecules into the plasma membrane [2]. Activation of amiloride-sensitive  $\text{Na}^+$  absorption by cAMP was also shown in the skin of the medical leech *Hirudo medicinalis* [29]. However, when ENaCs derived from many different tissues were expressed in *X. laevis* oocytes, no activation by cAMP could be detected except in the case of ENaC derived from guinea pig distal colon [18] and a chimerical ENaC composed of the  $\alpha$ -subunit of guinea pig distal colon and the rat  $\beta$ - and  $\gamma$ -subunits [9,22].

It has been shown in frog skin that divalent cations such as  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Ni}^{2+}$  cause an increase in active  $\text{Na}^+$  transport [14]. A6 cells were also used to investigate the effects of  $\text{Ni}^{2+}$  and other divalent cations on ENaC. It could be demonstrated that  $\text{Ni}^{2+}$  directly activates ENaC-mediated currents [26].

In the present study we expressed ENaC from rat distal colon (rENaC) in oocytes of the South African clawed toad *X. laevis*. These cells endogenously exhibit only a negligible amiloride-sensitive conductance [32] that is tiny compared to the currents obtained following heterologous expression of rENaC. Using an experimental design aimed to minimize intracellular  $\text{Na}^+$  accumulation through active rENaCs, we demonstrate that increasing intracellular cAMP concentration activates amiloride-sensitive rENaC current and conductance by triggering exocytotic delivery of preformed channel molecules to the plasma membrane. In contrast to results from A6 cells [26]  $\text{Ni}^{2+}$  inhibits whole-cell rENaC current. As revealed

\*Corresponding author. Fax: (32)-16-345991.

E-mail address: michael.weber@med.kuleuven.ac.be (W.-M. Weber).

by noise analysis  $\text{Ni}^{2+}$  reduced the number of active channels in a way that is not dependent on endocytosis based on the observation that membrane capacitance did not decrease. These data demonstrate that the combination of capacitance measurements and noise analysis is a suitable tool to further improve the usefulness of the *X. laevis* oocyte expression system.

Some of the results were presented as a poster at the annual meeting of the German Physiological Society [24].

## 2. Materials and methods

### 2.1. Oocyte preparation

Animals were purchased from African Xenopus Facility (Knysna, South Africa) and oocytes were isolated as previously described [34]. Oocytes were defolliculated by incubation in collagenase (1 mg/ml; Serva, Mannheim, Germany) for 2 h and subsequently washing with  $\text{Ca}^{2+}$ -free ORI (oocyte Ringer, composition see below) for 10 min. Healthy looking oocytes were collected and stored in ORI. For injection of cRNA standard protocols were used [30]. cRNA was prepared from cDNA as described in detail elsewhere [3] and oocytes were injected with 23 nl of solution containing a 1:1:1:3 ratio of  $\alpha$ -ENaC: $\beta$ -ENaC: $\gamma$ -ENaC:water (final cRNA concentration 0.25  $\mu\text{g}/\mu\text{l}$ ). After cRNA injection, oocytes were incubated in ESR (ENaC storage solution, composition see below) and could be measured after 24 h, with amiloride-sensitive currents being stable for at least 5 days.

### 2.2. Electrophysiology

All measurements were performed under voltage-clamp conditions using the standard two-microelectrode technique as described in detail elsewhere [33]. Briefly, oocytes were placed in a small Plexiglas chamber (1 ml volume) and were continuously perfused with a flow rate of about 2 ml/min, except for short periods of time when electrical noise was recorded. Two different types of electrodes have been used: one typical (about 1 M $\Omega$  resistance) for capacitance measurements, and a low-resistance electrode (200 k $\Omega$ ) for noise analysis, both filled with 3 M KCl. In order to prevent KCl from leaking out of the low-resistance electrode, the tip was filled with an agarose solution [3]. Two Ag/AgCl pellets were used as bath electrodes. Electrodes were connected to an oocyte voltage-clamp amplifier (OC-725C, Warner Instruments, Hamden, USA), and filtered holding currents were further processed digitally. Flow of positive charge (i.e.  $\text{Na}^+$ ) from the bathing solution to the oocyte cytosol is conventionally termed inward current and is plotted downwards in all graphs. Capacitance measurements were performed using the multiple sine wave method, as described in detail recently [30].

### 2.3. Noise analysis

We used the technique of noise analysis performed on oocytes by means of conventional dual-microelectrode voltage-clamp that was introduced in a recent report [25]. Briefly, ion channels in biological membranes fluctuate between different conductance states. This process can be spontaneous, i.e. triggered by factors such as voltage and/or temperature, or can be induced by the interaction of intra- or extracellular effectors with the channel. The fluctuations in current through these gated channels (i.e. electrical noise) give rise to a Lorentzian component in the power density spectrum with a corner frequency that reflects the rate of the open-close reaction. The Lorentzian plateau is proportional to the amount of current flow through the gated channels. Noise analysis provides direct information about the kinetic behavior of the channel, the blocker-receptor interaction and allows the calculation of single-channel parameters (e.g. single-channel current) and channel density in the oocyte plasma membrane.

### 2.4. Solutions

The composition of ORI is (in mM): 90 NaCl, 1 KCl, 2  $\text{CaCl}_2$  and 5 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Merck, Darmstadt, Germany). The low-sodium solution (LNS) used in all experiments contains (in mM): 15 NaCl, 75 NMDG, 1 KCl, 2  $\text{CaCl}_2$  and 5 HEPES. The solution used to store the ENaC-injected oocytes (ESR) contained 10 NaCl, 80 NMDG, 1 KCl, 2  $\text{CaCl}_2$  and 5 HEPES. pH was always adjusted to 7.6, either with NaOH or HCl.

Unless otherwise mentioned, all noise analysis experiments were performed in LNS containing 0.5  $\mu\text{M}$  amiloride. If not stated otherwise, all substances were purchased from Sigma (Deisenhofen, Germany). In the experiments on the effects of cAMP we used a mixture ('cAMP cocktail') of the membrane permeant 8-(4-chlorophenylthio)-cAMP (100  $\mu\text{M}$ ) and the phosphodiesterase inhibitor IBMX (1 mM). Oocytes were clamped to 20 mV to minimize feedback inhibition of ENaC by accumulating  $[\text{Na}^+]_i$ .

### 2.5. Statistics

Where applicable, data are expressed as arithmetic means  $\pm$  S.E.M.;  $n$  is the number of oocytes and  $N$  is the number of donor animals. Statistical analysis was made by *t*-test where appropriate, significant difference was assumed at  $P < 0.001$  and is marked in the figures with two asterisks.

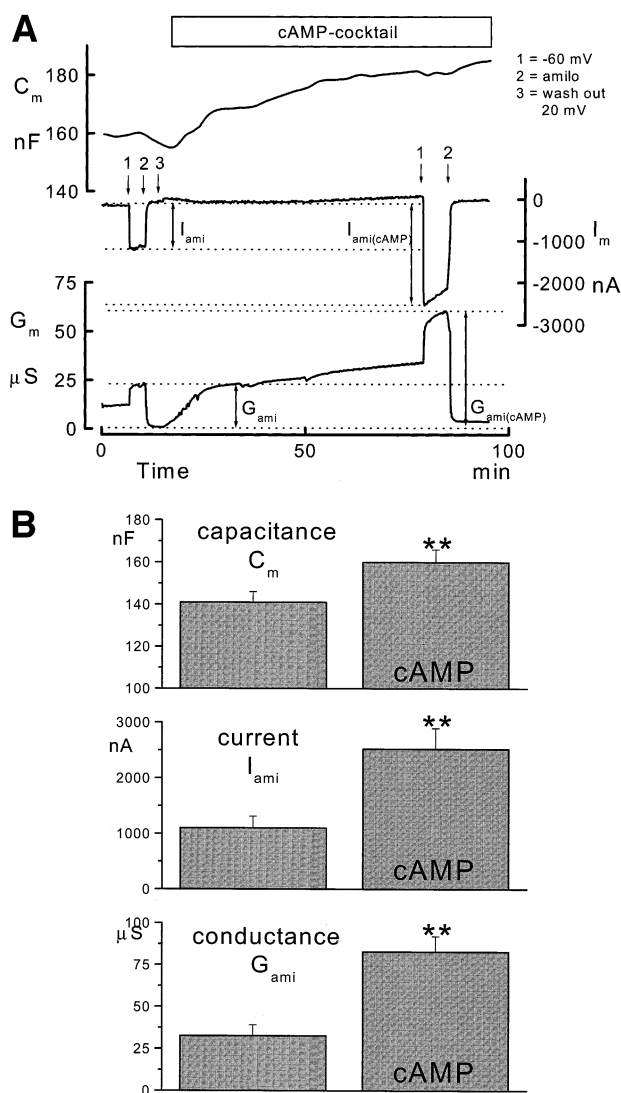


Fig. 1. rENaC activation by cAMP cocktail. A: Long term exposure to cAMP cocktail causes a slow but marked increase in current ( $I_m$ ; middle trace), conductance ( $G_m$ ; lower trace) and capacitance ( $C_m$ ; upper trace) over time. The oocyte was clamped to 20 mV and holding potential was only switched to  $-60$  mV to determine the amiloride-sensitive parameters at marker 1. Amiloride (10  $\mu\text{M}$ ) was added at marker 2 and subsequent washout is shown by marker 3. The difference between the dotted lines represents amiloride-sensitive current and conductance before ( $I_{ami}$ ,  $G_{ami}$ ) and after cAMP cocktail application ( $I_{ami(cAMP)}$ ,  $G_{ami(cAMP)}$ ), respectively. Please note the marked increase in both parameters after cAMP cocktail treatment. B: Pooled data derived from 20 rENaC-expressing oocytes ( $N = 9$ ). \*\* indicates statistic significance at  $P < 0.001$ .

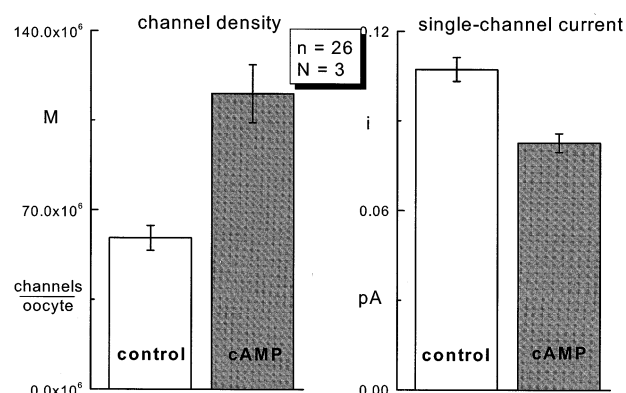


Fig. 2. Data from noise analysis. cAMP cocktail doubled the number ( $M$ ) of active rENaC channels in the oocyte membrane (left panel), while the single-channel current ( $i$ ) was slightly reduced (right panel). The experiments were performed at  $V_m = -60$  mV.

### 3. Results

#### 3.1. Experimental protocol

To minimize intracellular  $\text{Na}^+$  accumulation all experiments were performed in low- $\text{Na}^+$  solutions (15 mM) and membrane voltage-clamped to 20 mV. This  $\text{Na}^+$  concentration is close to the normal intracellular  $\text{Na}^+$  concentration of the oocytes [28] as also demonstrated by the slightly positive reversal potential of rENaC-expressing oocytes determined by applying current–voltage relationships ( $I$ – $V$  curves). Only for measurements of amiloride-sensitive rENaC current ( $I_{\text{ami}}$ ) and conductance ( $G_{\text{ami}}$ ) the oocyte membrane was shortly clamped to  $-60$  mV. Analysis of blocker-induced fluctuations in current using four different amiloride concentrations (0.25, 0.5, 1.0 and 1.5  $\mu\text{M}$ ) was also performed at this membrane voltage. This technique has been shown recently to be perfectly suited to investigate properties of rENaC heterologously expressed in *X. laevis* oocytes [25]. rENaC-expressing oocytes were strongly depolarized ( $V_m = 7.2 \pm 1.6$  mV;  $n = 104$ ,  $N = 16$ ) compared to control oocytes ( $V_m = -41.5 \pm 1.6$  mV;  $n = 42$ ,  $N = 14$ ). Amiloride-sensitive currents ( $I_{\text{ami}}$ ) mediated by rENaC amounted on average  $3.8 \pm 0.3$   $\mu\text{A}$  ( $n = 119$ ,  $N = 16$ ) and initial membrane conductance was increased from  $\sim 1$   $\mu\text{S}$  in control oocytes to 112  $\mu\text{S}$  in rENaC-expressing oocytes ( $n = 102$ ,  $N = 16$ ).

#### 3.2. Effects of cAMP on rENaC-expressing oocytes

IBMX is commonly used as an inhibitor of cellular phosphodiesterases and is applied together with membrane permeable cAMP analogues to prevent fast degradation of cAMP [23]. This cAMP–IBMX ‘cocktail’ has been successfully used to trigger cellular processes mediated by the secondary messenger cAMP, such as activation of ion channels [18] and protein kinase A (PKA)-dependent exocytotic delivery of functional ion channels to the plasma membrane [30]. In our hands, oocytes expressing rENaC superfused for 1 h with cAMP cocktail in LNS showed a slow, but significant increase in amiloride-sensitive current ( $I_{\text{ami}}$ ) and conductance ( $G_{\text{ami}}$ ). cAMP cocktail more than doubled  $I_{\text{ami}}$  in these series of experiments from  $1103 \pm 206$  nA to  $2521 \pm 373$  nA. cAMP cocktail-stimulated increases in  $G_{\text{ami}}$  were even larger and  $G_{\text{ami}}$  nearly tripled from  $32.5 \pm 6.5$   $\mu\text{S}$  to  $96.9 \pm 9.1$   $\mu\text{S}$  ( $n = 20$ ,  $N = 9$ ). Fig. 1A shows a typical experiment. Simultaneously

membrane surface area measured as membrane capacitance ( $C_m$ ) increased by  $\sim 13\%$  or  $18.6 \pm 1.9$  nF, indicating the insertion of new membrane material that contained additional rENaC molecules (Fig. 1A). Interestingly, rENaC-expressing oocytes exhibited a significant lower  $C_m$  of  $141 \pm 5$  nF compared with non-injected control oocytes (i.e.  $189 \pm 4$  nF). Orientating experiments with a few oocytes ( $n = 4$ ,  $N = 2$ ) showed that the specific blocker of the PKA KT5720 completely inhibited the cAMP cocktail-induced increase in  $C_m$ , while  $I_m$  and  $G_m$  increased only by  $25 \pm 6\%$  and  $40 \pm 5\%$ , respectively. From these experiments we conclude that cAMP cocktail activated rENaC already present in the plasma membrane, while new insertion of preformed ENaC via exocytosis was completely inhibited by the PKA blocker KT5720.

In all these experiments we switched only for a few minutes to  $-60$  mV for the determination of the amiloride-sensitive parameters. This protocol was necessary to detect the slow cAMP cocktail-induced increases in the parameters and helped to prevent channel run-down induced by massive

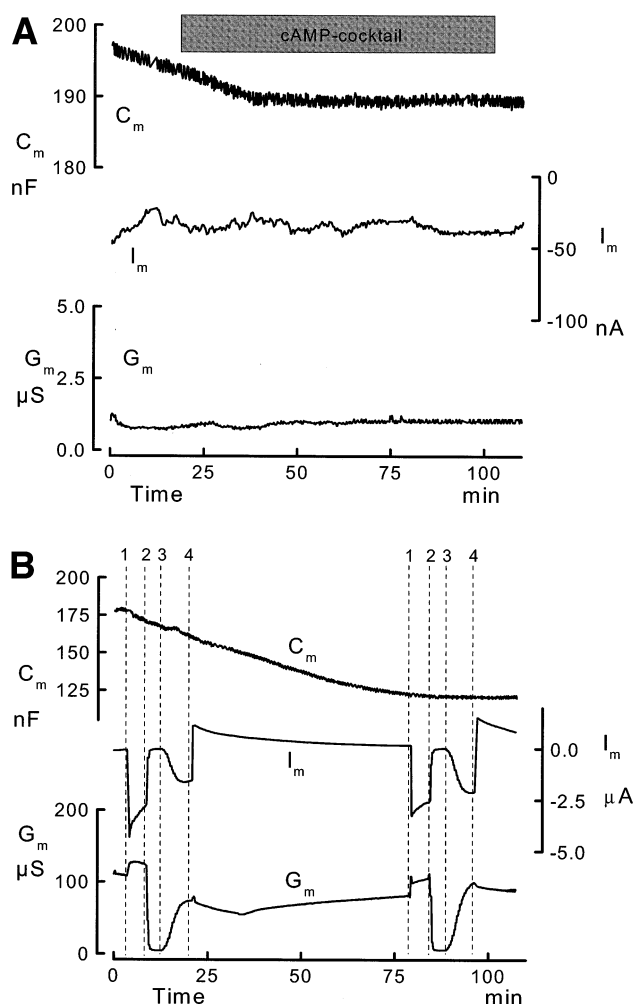


Fig. 3. Control experiments. A: Lack of effects of cAMP cocktail on non-injected oocytes in long lasting experiments. After an initial decrease  $C_m$  remains stable (upper trace).  $I_m$  and  $G_m$  do not change during the whole experiment. Shown is a typical result representative of 14 oocytes from four donors. B: Amiloride-sensitive  $I_m$  and  $G_m$  of rENaC-expressing oocytes ( $n = 9$ ,  $N = 3$ ) do not change with time in the absence of cAMP cocktail. Again,  $C_m$  remains stable after a short initial decrease. Marker 1:  $-60$  mV, marker 2: amiloride (100  $\mu\text{M}$ ), marker 3: washout, marker 4: 20 mV.



$\text{Na}^+$  influx that would have masked the cAMP effects. Fig. 1B summarizes the effects of cAMP on  $C_m$  and the amiloride-sensitive portions of  $I_{ami}$  and  $G_{ami}$ .

The observed increase in  $G_m$  (Fig. 1A, marker 1) when stepping from 20 mV to  $-60$  mV is due to the opening of ENaC. This change in  $G_m$  is also apparent in  $I$ - $V$  curves as a change in slope conductance ( $g_s$ ):  $g_s$  at  $-60$  mV (i.e.  $770 \mu\text{S}$ ) is nearly doubled as compared to  $410 \mu\text{S}$  at 20 mV (Fig. 4A).

### 3.3. Noise analysis of rENaC

Analysis of amiloride-induced current fluctuations revealed that the increase in macroscopic amiloride-sensitive ENaC current could be fully attributed to the increase in channel density. cAMP cocktail nearly doubled the number of active ENaCs from 59 to 115 million channels per oocyte ( $n=26$ ,  $N=3$ ; Fig. 2). Therefore, data revealed by means of noise analysis and capacitance measurements are in perfect accordance, demonstrating exocytotic insertion of preformed ENaC molecules into the plasma membrane. Yet, single-channel current was slightly decreased (Fig. 2, right panel).

### 3.4. Control experiments

In non-injected control oocytes, the cAMP cocktail in the presence of high  $\text{Na}^+$  (90 mM) or low  $\text{Na}^+$  (20 mM) did not affect  $I_m$ ,  $G_m$  and  $C_m$ , demonstrating that no conductances were stimulated. Only some oocytes ( $\sim 10\%$ ) responded to cAMP cocktail with a small increase in  $C_m$  ( $\sim 1\%$ ) as also described earlier [30]. These changes in  $C_m$  are probably due to the cAMP-stimulated insertion of small vesicles with high

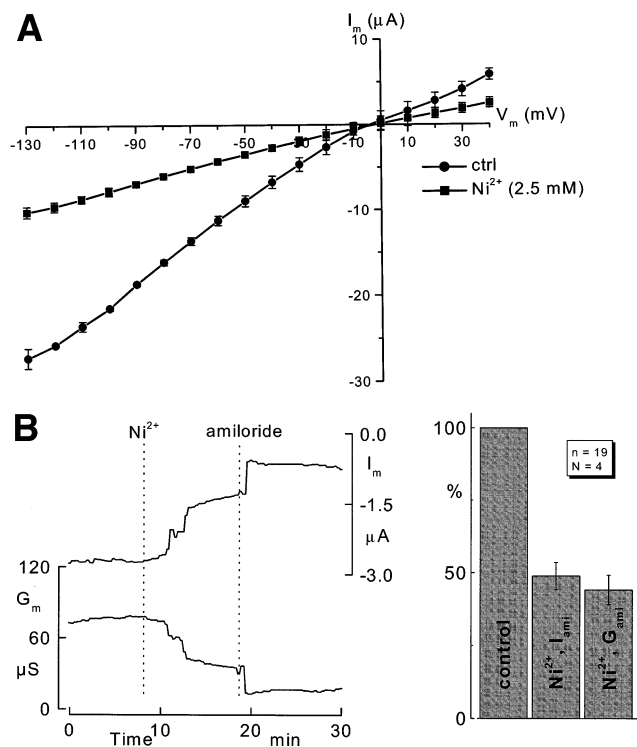


Fig. 4. rENaC inhibition by  $\text{Ni}^{2+}$ . A:  $I$ - $V$  relationship in the absence (dots) and presence (squares) of 2.5 mM  $\text{Ni}^{2+}$ . rENaC current is inhibited over the whole voltage range. Shown are the means of eight oocytes from three donors. B: rENaC current (upper trace) and conductance (lower trace) of a typical experiment before and after  $\text{Ni}^{2+}$  (2.5 mM) application (left panel). The right panel gives pooled data from 19 oocytes derived from four donors.

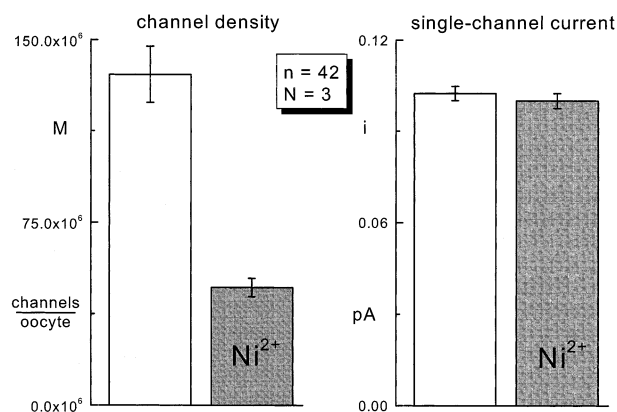


Fig. 5. Data from noise analysis.  $\text{Ni}^{2+}$  drastically reduced the number ( $M$ ) of active channels by directly inactivating rENaCs (left panel), while single-channel current ( $i$ ) was not affected. Shown are pooled data from 42 oocytes from three animals.

protein density as described recently by Schillers et al. [21]. However, in 90% of all experiments ( $n=14$ ,  $N=4$ ) we saw an initial small decrease in  $C_m$ , while  $I_m$  and  $G_m$  remained stable (Fig. 3A). To ensure that the reported effects are not an artefact due to the relatively long duration of the experimental protocol, we performed another set of measurements ( $n=9$ ,  $N=3$ ) on ENaC-expressing oocytes with precisely the same time course in the absence of cAMP cocktail (Fig. 3B). Again, the measured parameters did not change.

### 3.5. Effects of $\text{Ni}^{2+}$ on rENaC

$\text{Ni}^{2+}$  has been described to have various effects on ion channels [19]. In a cell line originally derived from *X. laevis* kidney (A6 cells)  $\text{Ni}^{2+}$  reportedly activated ENaC [26]. However, in ENaC-expressing oocytes a 2.5 mM  $\text{NiCl}_2$  solution in LNS produced a reversible inhibition of the  $\text{Na}^+$  current as revealed by  $I$ - $V$  relationships in the absence and in the presence of  $\text{Ni}^{2+}$  (Fig. 4A).  $I_{ami}$  was inhibited to  $49 \pm 5\%$  and the amiloride-sensitive ENaC conductance was inhibited to  $44 \pm 5\%$  of its initial value (Fig. 4B;  $n=19$ ,  $N=4$ ). Noise analysis revealed that  $\text{Ni}^{2+}$  had no effect on single-channel current, while the apparent channel density significantly decreased from  $135.0 \pm 11.6$  to  $48.3 \pm 3.8$  million channels per oocyte (Fig. 5). The inhibition by  $\text{Ni}^{2+}$  was reversible although washout took at least 15 min. Therefore, it seems probable that the observed decrease in apparent channel density could be due to a stabilization of a long time closed state. Capacitance measurements showed no effect of  $\text{Ni}^{2+}$  on membrane surface area, indicating that  $\text{Ni}^{2+}$  directly inactivates ENaC rather than inducing endocytotic removal of ENaC from the plasma membrane. However, from our present data we cannot exclude an endocytotic process if the membrane is rapidly recycled or if the membrane area per channel ratio is small.

## 4. Discussion

### 4.1. rENaC activation by cAMP

cAMP increases the activity of ENaC in epithelial tissues as reported from whole tissue measurements using frog skin [12], leech skin [31] and *X. laevis* kidney cells (A6) [2,6]. There are several possible mechanisms that could be responsible for the cAMP activation of ENaC: stimulation by cAMP could be

achieved by direct phosphorylation of the channel protein itself or by cAMP-stimulated recruitment of preformed channels and/or subunits from intracellular pools to the apical plasma membrane. Furthermore, cAMP could inhibit the endocytotic removal of active channels from the plasma membrane as reported for a human chloride secretory epithelial cell line [5]. Yet, several lines of evidence argue for a contribution of exocytotic processes in the cAMP stimulation of ENaC. Using analysis of amiloride-induced fluctuations in current across leech skin, Weber et al. [29,31] showed that cAMP increased the number of active channels in the apical membrane while single-channel current remained stable. More recently, Butterworth et al. [6] demonstrated that cAMP increases the number of conductive  $\text{Na}^+$  channels in A6 cells without changes in the open probability of individual channels. These data confirm that cAMP promotes the insertion of preformed ENaC molecules into the plasma membrane probably via a PKA-dependent pathway.

Close investigations on ENaC are severely hampered by the channel's low abundance in native, hormone-unstimulated tissues. Therefore, many groups made use of the *X. laevis* oocyte expression system to heterologously express ENaC derived from several tissues and species (reviewed in [16]). With the exception of data on ENaC from guinea pig distal colon [18,22], there were no reports on cAMP stimulation of ENaCs expressed in oocytes. Particularly, it seemed not possible to stimulate rENaC expressed in oocytes [4,22]. The authors came to the conclusion that cAMP activation of rENaC needs an additional factor that is present in the original tissue but not in the oocytes. A more recent report using a chimeric ENaC from guinea pig distal colon ( $\alpha$ -subunit) and rat small intestine ( $\beta$ - and  $\gamma$ -subunits)[9] described activation by cptcAMP but not cAMP. The authors concluded that cAMP interacts with the extracellular part of the channel and excluded cAMP interactions with an intracellular site.

All these papers seem to be somewhat contradictory to what we report here. However, at a closer look there might be no discrepancies between the data reported in the above quoted papers and our findings. We will explain that more detailed in the following. (1) Stimulation of rENaC by cAMP cocktail is a quite slow process that takes far more than 30 min. Therefore, Chraïbi et al. probably could not see the cAMP cocktail effects since they applied cAMP cocktail only some minutes [9,22]. (2) The oocyte that expresses active rENaC accumulates  $\text{Na}^+$  so fast that the endogenous  $\text{Na}^+/\text{K}^+$ -ATPase cannot effectively recycle the bulk of  $\text{Na}^+$ . Intracellular  $\text{Na}^+$  accumulation in turn leads to ENaC self-inhibition as described for a wide variety of tissues [11] and/or channel run-down as described for rENaC in *X. laevis* oocytes [27]. Therefore, in contrast to other groups [4,9,22] we performed all experiments in low- $\text{Na}^+$  solutions as a first way to prevent that the intracellular  $\text{Na}^+$  exceeded the normal intracellular  $[\text{Na}^+]$  of around 20 mM. (3) To further prevent  $\text{Na}^+$  accumulation it is inevitable to reduce the electrochemical driving force for  $\text{Na}^+$  entry by clamping the membrane voltage as long as possible to values that minimize  $\text{Na}^+$  current through rENaC during the long lasting experiments. Thus, we kept the oocyte membrane voltage predominantly at 20 mV while other groups used clamp voltages of  $-60$  mV [9,22] or even  $-100$  mV [4]. In some initial control experiments we clamped the membrane voltage to  $-60$  mV and we were not able to detect cAMP cocktail stimulation of rENaC.  $\text{Na}^+$

entry, favored by the driving force created by the negative potential, is obviously concealing the activation of rENaC by cAMP cocktail.

Therefore, from our data it becomes clear that cAMP has an activating effect on rENaC. Moreover, we show that the mechanism of cAMP activation occurs through triggering the exocytotic delivery of preformed rENaC into the oocyte plasma membrane. This has been revealed by the combination of measuring membrane surface area and the number of active ENaCs in the plasma membrane. Furthermore, our data are obviously not in contrast with data from other groups using the oocyte expression system [4,9,22] but in good accordance with findings from native tissue: Butterworth et al. [6] revealed that forskolin stimulation of A6 cells triggered PKA-dependent exocytotic insertion of preformed ENaC molecules into the apical membrane. Recently, it was also shown that in alveolar epithelial cells cAMP triggers the induction of ENaC via upregulation of mRNAs encoding the  $\alpha$ -subunit in a time-dependent way [10]. The authors of this study further reported that detectable ENaC activation only occurred after 4 h of cAMP treatment and peaked after 8 h. Our data presented here confirm also observations that we obtained earlier using leech integument [31].

Injection of cRNA encoding rENaC leads to expression of large numbers of active channels in the range of  $\sim 6 \cdot 10^7$  rENaC molecules per oocyte as also reported recently [25]. The average increase in capacitance after cAMP allows calculating how many vesicles fused with the membrane. Moreover, from the combination of  $C_m$  measurements and noise analysis we can estimate the number of rENaC channels that were activated by cAMP cocktail. Given an average vesicle diameter of 120 nm in oocytes [35], about  $2.5 \cdot 10^7$  vesicles were inserted into the oocyte plasma membrane under the influence of cAMP cocktail. This corresponds with a fusion rate of about 10 000 vesicles per second, a value that is in good accordance with data of the  $\text{Na}^+/\text{glucose}$  cotransporter (SGLT1) expressed in oocytes [15]. Assuming that all rENaC molecules, the number of which was determined by noise analysis, were delivered via exocytosis to the oocyte membrane, we can calculate that each vesicle that fused with the oocyte membrane transported about 2.25 rENaC molecules to the plasma membrane. Thus, cAMP cocktail triggered the insertion of another  $\sim 6 \cdot 10^7$  rENaC molecules into the oocyte membrane. However, from those data it remains to be elucidated whether the results in oocytes can be directly transferred to other cell systems.

#### 4.2. rENaC inhibition by $\text{Ni}^{2+}$

The divalent cation  $\text{Ni}^{2+}$  is an essential trace element without pharmacological importance that has manifold effects on several cation channels and acts mainly as an inhibitor [19]. Yet, in frog skin,  $\text{Ni}^{2+}$  and other divalent cations stimulated active  $\text{Na}^+$  transport [14]. Also in *Xenopus* renal distal tubule cells (A6)  $\text{Ni}^{2+}$  caused direct activation of ENaC in a way that was independent of exocytotic insertion of preformed ENaC [26]. Measuring  $I_m$ ,  $G_m$  and  $C_m$  of rENaC-expressing oocytes revealed that  $\text{Ni}^{2+}$  inhibited about 60% of the rENaC current and conductance while membrane capacitance remained unaffected. Subsequent noise analysis demonstrated that this inhibition could be attributed to a loss of channel function, while single-channel current did not change significantly. These observations argue for effects of  $\text{Ni}^{2+}$  on channel ki-

netics rather than induction of endocytotic removal of active channels from the plasma membrane. At the moment, there is no explanation for these discrepancies between  $\text{Ni}^{2+}$  effects in A6 cells and on rENaC expressed in oocytes except that the regulation of the expressed rat ENaC in oocytes might be different from the *Xenopus* A6 ENaC in its native environment. Future investigations should reveal whether the observed effects of  $\text{Ni}^{2+}$  are targeting directly the channel's pore or are possibly due to influences of  $\text{Ni}^{2+}$  on the plasma membrane surface. Furthermore, from the data that we have now we cannot conclude whether the  $\text{Ni}^{2+}$  inhibition is typical for ENaCs expressed in *X. laevis* oocytes or specific for rENaC. Therefore, it would be quite interesting to express other ENaCs, including ENaC extracted from A6 cells, and compare the data with the native tissue.

#### 4.3. Final considerations

We describe the application of classical noise analysis achieved with conventional microelectrode techniques in *X. laevis* oocytes expressing rat ENaC. Furthermore, we demonstrate that the combination of the noise analysis with simultaneous, continuous measurements of  $I_m$ ,  $G_m$  and  $C_m$  yields new insights into physiological pathways of ion channel regulation. Using these techniques we show that cAMP stimulates the exocytotic machinery of the oocytes leading to transport and functional insertion of preformed rat ENaC molecules from intracellular pools to the oocyte membrane, thereby significantly increasing the plasma membrane surface area and the number of active channels in the membrane. Additionally, we demonstrate that  $\text{Ni}^{2+}$  inhibits rENaC. For the future it will be interesting to compare the results presented here with ENaC mutants that have been used over the past years by different groups [17,20] and with an ENaC from human airway epithelia that was recently cloned by our group.

**Acknowledgements:** We thank Prof. B. Rossier (Lausanne, Switzerland) for the kind gift of rat cDNA that we used in this work and Diane Hermans for cRNA synthesis. This project was supported by research grants from the 'Fonds voor wetenschappelijk onderzoek – Vlaanderen' (G.0179.99), GOA 99/07, the Interuniversity Poles of Attraction Program – Belgian State, Prime Minister's Office – Federal Office for Scientific, Technical and Cultural Affairs IUAP P4/23, the bilateral program BIL96/23 of the Flemish government, providing the support of A.S. and the 'Alphonse and Jean Forton Foundation – Koning Boudewijn Stichting, Nr. 2000 13 R7115B0' granted to W.-M.W.

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