Expression of ENaC and other Transport Proteins in *Xenopus* Oocytes is Modulated by Intracellular Na^+

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Key Words

CFTR • ENaC • Intracellular sodium • Protein expression • Plasma membrane insertion

Abstract

The expression of the epithelial Na^+ channel (ENaC) is tissue-specific and dependent on a variety of mediators and interacting proteins. Here we examined the role of intracellular Na^+ ($[\text{Na}^+]_i$) as a modulator of the expression of rat ENaC in *Xenopus laevis* oocytes. We manipulated $[\text{Na}^+]_i$ of ENaC-expressing oocytes in the range of 0–20 mM by incubating in extracellular solutions of different $[\text{Na}^+]_o$. Electrophysiological, protein biochemical and fluorescence optical methods were used to determine the effects of different $[\text{Na}^+]_i$ on ENaC expression and membrane abundance. In voltage-clamp experiments we found that amiloride-sensitive ENaC current (I_{ami}) and conductance (G_{ami}) peak at a $[\text{Na}^+]_i$ of ~10 mM Na^+ , but were significantly reduced in 5 mM and 20 mM $[\text{Na}^+]_i$. Fluorescence intensity of EGFP-ENaC-expressing oocytes also followed a bell-shaped curve with a maximum at ~10 mM $[\text{Na}^+]_i$. In Western blot experiments with specific anti-ENaC antibodies the highest protein expression was found in ENaC-expressing oocytes with $[\text{Na}^+]_i$ of

10–15 mM. Since ENaC is also highly permeable for Li^+ , we incubated ENaC-expressing oocytes in different Li^+ concentrations and found a peak of I_{ami} and G_{ami} with 5 mM Li^+ . The influence of $[\text{Na}^+]_i$ on the expression is not ENaC-specific, since expression of a Cl^- channel (CFTR) and a Na^+ /glucose cotransporter (SGLT1) showed the same dependence on $[\text{Na}^+]_i$. We conclude that specific concentrations of Na^+ and Li^+ influence the expression and abundance of ENaC and other transport proteins in the plasma membrane in *Xenopus laevis* oocytes. Furthermore, we suggest the existence of a general mechanism dependent on monovalent cations that optimizes the expression of membrane proteins.

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Introduction

The epithelial Na^+ channel (ENaC) is the main regulator of apical Na^+ entry in epithelial cells and its physiological role has been extensively studied in many different epithelia [1]. The large electrochemical gradient across the apical membrane provides the driving force for Na^+ entry into the cell through ENaC, while at the

basolateral membrane Na^+ is actively transported out of the cell by the Na^+/K^+ -ATPase. Beside its substantial contribution to the maintenance of electrolyte homeostasis, ENaC function is a key factor in keeping the composition and the volume of the luminal fluid lining the respiratory epithelium constant [1, 2]. The ENaC genes were cloned from various species such as human, rat, mouse, cow and *Xenopus laevis*, and the functional channel in the airways and gastrointestinal tract of adult animals has been shown to be predominantly composed of three homologous subunits termed α , β and γ -ENaC [3-5]. The channel is constitutively active and manifests slow kinetics with open and close dwell times in the range of seconds at room temperature. It is permeable only to small monovalent cations (Na^+ , Li^+ , H^+), with a selectivity ratio for Na^+ over K^+ larger than 500. The single channel conductance begins to saturate at Na^+ concentrations above 100 mM with a K_m of 20-50 mM (extracellular) and measures 4-5 pS for Na^+ and 9-10 pS for Li^+ in symmetrical solutions with 100-150 mM of the respective ion. Amiloride is a specific ENaC blocker that acts in submicromolar concentrations by a substantial reduction in mean channel open time without changes in single channel conductance [2].

The regulation of ENaC is complex and mediated by the classical hormonal pathway via aldosterone and by different proteins and extracellular factors interacting directly or indirectly with the channel. Thus, the regulation of ENaC is highly tissue-specific and occurs predominantly through mechanisms that control ENaC expression and transition into the plasma membrane [2]. There are several mechanisms being responsible for the insertion of ENaC from intracellular pools into the plasma membrane or influence the open probability and activity of already inserted channels. In this context ions such as Na^+ or Ca^{2+} are also discussed as possible candidates for ENaC regulation. However, it is not clear if such regulatory mechanisms influence trafficking, insertion or activity of the channel.

It was shown that the cell itself can control the Na^+ absorption via mechanisms called self-inhibition and feedback inhibition. Self-inhibition means that extracellular Na^+ itself can modify the activity of channels independent of changes in intracellular ion composition [6]. Feedback inhibition is a down-regulation of ENaC due to sodium transport across the apical membrane, which leads to an increase of intracellular Na^+ concentration [7]. Feedback inhibition has been investigated in many studies, which have firstly established a clear concentration dependence of feedback inhibition (dramatic decrease of ENaC

function at $[\text{Na}^+]_i$ around 60-70 mM), and secondly, some studies indicate that an increase in intracellular Na^+ decreases most likely both, ENaC expression at the cell surface and channel open probability [8, 9].

In this study we used the *Xenopus laevis* oocyte expression system for the heterologous expression of rat ENaC and other transport proteins. In the last few years we examined factors that are possible candidates for the expression and the control of the functional insertion of ENaC into the oocyte plasma membrane. The aim of the present work was to study the influences of low and high $[\text{Na}^+]_i$ on the ENaC expression. To point out if this effect reflects a more general mechanism we also analyzed the influences on the expression of another two transport proteins, the cystic fibrosis transmembrane conductance regulator (CFTR) and the human $\text{Na}^+/\text{glucose}$ cotransporter SGLT1. Our data indicate that expression and abundance of those transport proteins in the oocyte plasma membrane follow a bell-shaped curve with a peak at 10-15 mM $[\text{Na}^+]_i$. The same dependence on $[\text{Na}^+]_i$ was observed for CFTR and SGLT1. Therefore, we postulate that there is an optimum of Na^+ concentration for the expression of ENaC and other transport proteins and that intracellular Na^+ itself has an effect on the translational and/or posttranslational processes at least in *Xenopus* oocytes, which certainly has nothing to do with Na^+ feedback inhibition. From the data we obtained with CFTR we conclude that protein synthesis rather than degradation is influenced by $[\text{Na}^+]_i$. Parts of the work were published in abstract form.

Materials and Methods

Oocyte preparation and injection of cRNA/cDNA

Oocytes were taken from mature female *Xenopus laevis* (purchased from the African Xenopus Facility, Knysna, RSA and Nasco, USA) and isolated as described in previous publications of the three involved laboratories [10, 11]. Briefly, oocyte clusters were surgically removed from the ovaries of the frogs, which were anesthetized with tricain and stored in oocyte Ringer (ORi) containing: NaCl 90 mM, KCl 2 mM, Hepes 10 mM, CaCl_2 1.8 mM; pH 7.4. Defolliculation of the oocytes was performed by incubation in collagenase solution (1 mg/ml; Serva, Mannheim, Germany) for 60-90 min followed by 10 min in Ca^{2+} -free ORi to remove follicle cells. Healthy looking stage V-VI oocytes were selected and injected with up to 50 nl cRNA into the cytoplasm (α -EGFP-ENaC, β -ENaC, γ -ENaC, 12 ng each subunit) or 12 nl cDNA into the nucleus of the oocytes (α -EGFP-ENaC, β -ENaC, γ -ENaC, 0.25 ng each subunit), respectively. Previous studies in our group showed that both methods led to identical functional expression of ENaC.

All three rat ENaC subunits were inserted into the pcDNA3 vector, and cRNA for cytoplasmic injection was synthesized by using the Cap Scribe T7 *in vitro* transcription kit (Roche, Grenzach-Wyhlen, Germany). The EGFP-ENaC construct was designed by inserting α -rENaC pcDNA3 at the N-terminus into an EGFP vector (Clontech, Mountain View, CA 94043, USA). CFTR (23 ng/oocyte) was either injected alone or co-injected with ENaC to allow manipulation of $[\text{Na}^+]_i$. SGLT1-injected oocytes were stored in ORI.

ENaC-injected oocytes were stored up to 5 days in ENaC storage solution (ESR: NaCl 10 mM, NMDGCl 80 mM, KCl 3 mM, CaCl_2 2 mM, Pyruvate 2.5 mM, Hepes 5 mM; pH 7.6) or ESR containing different sodium or lithium concentrations (0 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM).

Determination of intracellular Na^+ concentrations

We used two methods for the determination of the intracellular Na^+ concentration $[\text{Na}^+]_i$ in ENaC expressing oocytes: ion-selective microelectrodes and measuring the Na^+ current reversal potential, which was used to solve Nernst's equation. For the Na^+ -selective microelectrode method electrodes were pulled from filamentous borosilicate glass. The silanized pipettes were then filled with a membrane cocktail selective to Na^+ (Sodium Ionopore II-Cocktail A, Fluka Chemie GmbH, CH-9471 Buchs) and back-filled with 100 mM NaCl solution. A chlorinated Ag wire was inserted to complete the Ag/AgCl semicell. The resulting electrodes were calibrated by using a series of 7 reciprocal NaCl/KCl solutions ($[\text{Na}^+] + [\text{K}^+] = 100 \text{ mM}$). A setup consisting of two intra-oocyte electrodes (one Na^+ -selective and one normal to measure membrane potential), a bath reference and a differential amplifier were used to record the potential difference across the ion-selective membrane at the electrode tip.

For the alternate method we determined the Na^+ equilibrium.

$$E_{\text{revNa}^+} = RT/F \ln ([\text{Na}^+]_0/[\text{Na}^+]_i) \quad (\text{eq. 1})$$

With $RT/F = 25.69 \text{ mV}$ (at 25°C):

$$[\text{Na}^+]_i = [\text{Na}^+]_0 * [1/\exp(E_{\text{revNa}^+}/25.69)] \quad (\text{eq. 2})$$

Two electrode voltage clamp measurements and electrophysiology protocols

All measurements were performed under voltage-clamp conditions using the standard two-microelectrode technique as described in detail elsewhere [10, 12]. Briefly, oocytes were placed in a small plexiglas chamber (1 ml volume) and were continuously perfused. Oocytes were impaled with two microelectrodes filled with 3 M KCl exhibiting a resistance around 1 M Ω . Two Ag/AgCl pellets were used as bath electrodes. Electrodes were connected to an oocyte voltage-clamp amplifier (OC-725C, Warner Instruments, Hamden, USA). Filtered holding currents were digitally processed. Except where explicitly stated, all measurements were performed in a low- Na^+ solution (LNS: NaCl 15 mM, NMDGCl 75 mM, KCl 3 mM, CaCl_2 2 mM, Pyruvate 2.5 mM, Hepes 5 mM; pH 7.6) to minimize loading the oocytes with Na^+ .

ENaC-expressing oocytes were always clamped to zero current (current-clamp) to minimize Na^+ influx. We used a

software-driven current-clamp on top of the traditional two-microelectrode voltage-clamp technique, which was adjusting the clamp potential every 10 s to keep the clamp current below 100 nA (absolute value). This was in order to make sure that there were no significant changes in the intracellular ion concentrations that could have resulted in the activation of endogenous regulatory mechanisms such as feedback inhibition and/or channel rundown. Conductance (G_m) was also continuously measured and allowed us to monitor the (in-) activation processes of the investigated channels in real-time. By convention, inward currents are considered negative, and the membrane potential is referenced to the extracellular medium. Currents (I_m) were estimated by means of current-voltage relationships (IV plots): the clamp potential was briefly (350 ms) switched from the initial value to a range of potentials from -100 to +30 mV and back, with a 1500 ms pause between steps. All I_m values reported here represent the current value at -60 mV as it was derived from the IV plot.

CFTR was activated by addition of a cAMP-cocktail containing the cell permeable cAMP analogue 8-(4-chlorophenylthio) cAMP (100 μM) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1 mM). SGLT1 was stimulated with the non-metabolizable glucose analogue α -methylglucopyranoside (AMG, 5 mM).

EGFP fluorescence imaging

Images of ENaC (α -EGFP-ENaC, β -ENaC, γ -ENaC) expressing oocytes were taken with a confocal fluorescence microscope (Zeiss LSM 510 META, Jena, Germany) equipped with a filter for green fluorescence protein detection (488 nm excitation, 509 nm emission filter). Oocytes were placed in a special glass chamber, and the animal pole images were analyzed. The abundance of ENaC at the plasma membrane or nearby regions was determined by measuring the fluorescence intensity in 5-10 randomly selected membrane regions with a tool provided within the LSM-specific software. The background fluorescence was measured with non-injected oocytes and subtracted from fluorescence measured with EGFP-expressing oocytes. Images of EGFP-CFTR expressing oocytes were taken using the same technical procedures.

Protein biochemistry

Membrane proteins from ENaC-expressing oocytes were isolated by using Triton X-100. For Western blotting about 10 or 20 μg protein was submitted to SDS-PAGE (7.5 % acrylamide) and transferred to a PVDF (polyvinylidene fluoride) membrane. Non-specific binding sites were blocked 4 h by 5 % nonfat dry milk in Tris-buffered saline/Tween (TBST) (10 mM Tris HCl, pH 7.4; 140 mM NaCl; 0.3 % Tween 20). The epithelial sodium channel α -subunit and γ -subunit were detected with a rabbit anti-ENaC alpha antibody and a rabbit anti-ENaC gamma antibody (Dianova, Hamburg, Germany) with concentrations of 1:2500 diluted in 5 % nonfat dry milk/TBST at 4°C overnight. The antigens correspond to amino acid residues 20-42 from human ENaC α -subunit and 630-649 from human ENaC γ -subunit, respectively. The β -subunit was detected with a rabbit anti-ENaC beta antibody corresponding to amino acid residues 271-460 (Santa Cruz, California, USA) with a

concentration of 1:500 diluted in 1 % nonfat dry milk/TBST overnight at 4°C. All antibodies are highly specific for rat ENaC and no cross-reactions were documented. After washing in TBST the membrane was incubated for 1 h at room temperature with the goat anti-rabbit IgGs conjugated with alkaline phosphatase (Santa Cruz, California, USA) diluted 1:10000 in 5 % nonfat dry milk/TBST for the alpha- and gamma-ENaC subunits and in 1% nonfat dry milk/TBST for the beta-ENaC subunit. The membrane was washed again in TBST, and detection was carried out with NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). The transferred membranes were digitally scanned, and protein bands of 4 (α -ENaC) and 3 (β - and γ -ENaC) different blots for each ENaC subunit were semi-quantitatively analyzed by densitometry using ImageJ analysis software 1.36 [13]. Because of the different background staining of the membranes densitometric values are given within one blot as percent of intensity.

Statistics

All results are expressed as means $\pm$ S.E.M. of n observations (n gives the number of oocytes, N the number of the donors). Sets of data were compared with student's t test. Differences were considered statistically significant when $P \leq 0.05$ (marked *) or $P \leq 0.005$ (marked **). All statistical tests were performed using Origin version 7.5 for Windows (OriginLab Corporation, Northampton, USA).

Results

Influence of $[Na^+]_i$ on ENaC expression

A) Experimental design and basic parameters of ENaC expressing oocytes. During incubation after ENaC-mRNA microinjection and in all experiments with ENaC we used a low Na^+ concentration (i.e. 10 and 15 mM, respectively) to prevent intracellular Na^+ accumulation and subsequent ENaC run-down [14]. This extracellular Na^+ concentration is close to the average intracellular Na^+ concentration of the oocytes as reported by various authors (for a review see [15]). Furthermore, we prevented intracellular accumulation of Na^+ via the expressed ENaC by clamping the current to 0 nA (the software-driven current-clamp mode, see *Materials and Methods* above) at the beginning of the experiments (Fig. 1A). Then we briefly voltage-clamped to -60 mV to assess the amiloride-sensitive current (I_{ami}) and to perform IV curves in absence and presence of amiloride. Difference curves reveal the amiloride sensitive ENaC current and were used for the determination of the reversal potential (Fig. 1B).

Control experiments revealed that H_2O -injected oocytes and non-injected oocytes showed no differences in membrane potential (V_m), current (I_m), conductance (G_m), and capacitance (C_m). Therefore, the term

Fig. 1. Typical amiloride sensitive membrane current and conductance of an ENaC expressing oocyte. A) The oocytes were kept in ESR and current was clamped to 0, then switched to -60 mV. Amiloride (50 μM , ami) blocked I_m and G_m completely. B) Difference curves in presence and absence of amiloride yield pure amiloride sensitive ENaC currents (I_{ami}). Traces are representative of 117 oocytes from 14 donors.

“controls” refers to non-injected oocytes. ENaC mRNA-injected oocytes expressed rat ENaC within 20 hours and could be used for at least the next 5 days. Oocytes injected with mRNA coding for ENaC always had a strongly depolarized V_m due to the high Na^+ permeability via the heterologously expressed channel ($V_m = 6.3 \pm 1.5$ mV in ENaC-expressing oocytes, $n = 102$, $N = 13$ versus -47.0 ± 2.0 mV in control oocytes, $n = 59$, $N = 13$). ENaC expressing oocytes exhibited a high initial transmembrane conductance (G_m) that amounted 113.5 ± 10.6 μS ($n = 100$, $N = 13$) as compared with approximately 1 μS

Fig. 2. $[\text{Na}^+]_i$ of ENaC-expressing oocytes is dependent on $[\text{Na}^+]_o$. We calculated intracellular sodium concentrations $[\text{Na}^+]_i$ from the Nernst equation using the equilibrium potential of IV relationships of ENaC-expressing oocytes. The extracellular sodium concentrations $[\text{Na}^+]_o$ correspond to different sodium concentrations of LNS. All calculated concentrations are significantly different. $[\text{Na}^+]_i$ is in good agreement with $[\text{Na}^+]_o$.

in control oocytes (at -60 mV). The application of amiloride (50 μM) immediately led to drastic decline of current and conductance down to values characteristic for non-injected control oocytes. The amiloride-sensitive current (I_{ami}) at $V_m = -60$ mV mediated via the expressed ENaC averaged at 3.8 ± 0.3 μA ($n = 117$, $N = 14$) and was distinct from the endogenous amiloride-sensitive current that amounted to some 30 nA and could be found in around 30 % of the oocytes [16]. Amiloride restored membrane potential to physiologically normal negative values. The membrane capacitance (C_m) of the oocyte plasma membrane did not change during expression of up to 3×10^8 functional copies of ENaC per oocyte (data from noise analysis) when oocytes were stored in low Na^+ Ringer (ESR): in ENaC-expressing oocytes we observed $C_m = 164 \pm 3$ nF and in control oocytes we measured $C_m = 158 \pm 6$ nF ($n = 29$, $N = 5$) [17].

B) Determination of intracellular Na^+ concentration. Previous observations in our group revealed a strong dependence of the expression of transport proteins (ENaC, CFTR, SGLT1) on $[\text{Na}^+]_i$ in *Xenopus laevis* oocytes [18, 19]. Therefore, in the present detailed study on ENaC expression we were interested in methods to determine exactly $[\text{Na}^+]_i$. For that purpose we used ion-selective electrodes and compared these results with those obtained by calculation $[\text{Na}^+]_i$ using the ENaC reversal potential and solving Nernst's equation. Both methods yielded very consistent results: calculating the Na^+ concentration turned out a value of 13.98 ± 0.87 mM ($n = 102$, $N = 13$) for $[\text{Na}^+]_i$ of ENaC-expressing oocytes incubated in ESR (10 mM Na^+); measurements in LNS (15 mM Na^+) using ion-selective microelectrodes gave $[\text{Na}^+]_i = 13.88 \pm 0.46$ mM ($n = 23$, $N = 3$). This comparison shows clearly that both methods are equivalent for ENaC-expressing oocytes and, therefore, for all subsequent determinations of $[\text{Na}^+]_i$ we used the more convenient method using the reversal potential and resolving Nernst's equation.

Fig. 2 shows the dependence of $[\text{Na}^+]_i$ of ENaC expressing oocytes on the extracellular Na^+ concentration $[\text{Na}^+]_o$. Oocytes incubated in 2 mM, 5 mM, 10 mM, 15 mM and 20 mM $[\text{Na}^+]_o$ exhibited $[\text{Na}^+]_i$ of $1.8 \pm$

0.3 mM, 6.5 ± 1 mM, 11.8 ± 1.5 mM, 14.8 ± 2.8 mM and 24 ± 5 mM $[\text{Na}^+]_i$, respectively. In each case, $[\text{Na}^+]_i$ is very close to $[\text{Na}^+]_o$ demonstrating that in ENaC-expressing oocytes $[\text{Na}^+]_o$ and $[\text{Na}^+]_i$ are in equilibrium.

C) I_{ami} and G_{ami} of ENaC-expressing oocytes are modulated by Na^+ and Li^+ . ENaC expressing oocytes showed stable amiloride-sensitive current (I_{ami}) and conductance (G_{ami}) 24 h after injection. Yet, the level of I_{ami} and G_{ami} is strongly dependent on the $[\text{Na}^+]_i$. Fig. 3A shows the ENaC current of oocytes incubated in different sodium solutions (2 mM to 20 mM). The peak of amiloride-sensitive current and conductance was reached when ENaC injected oocytes were incubated in 10 mM (average 2.3 μA ; $n = 36$, $N = 8$) compared to incubation in 2 mM, 5 mM, 15 mM and 20 mM sodium solution (average 1.3 μA). I_{ami} is significantly increased with incubation of the injected oocytes in 10 mM Na^+ . Similar dependence was observed for G_{ami} of ENaC-expressing oocytes; G_{ami} significantly increased with incubation in 10 mM Na^+ (average 52 μS ; $n = 36$, $N = 8$) as compared to 2 mM, 5 mM, 15 mM and 20 mM Na^+ (average 30 μS) (Fig. 3B). These results indicate a strong dependence of I_{ami} and G_{ami} on $[\text{Na}^+]_i$ in *Xenopus* oocytes. The dependence of I_{ami} and G_{ami} on the $[\text{Na}^+]_i$ follows a bell-shaped curve with a maximum between 10 and 15 mM $[\text{Na}^+]_i$. As shown in figure 3C the boosting effects of $[\text{Na}^+]_i$ can be abolished by inhibiting ENaC with amiloride (50 μM).

Li^+ , which is also well conducted by ENaC, had basically the same effect as Na^+ . When we incubated ENaC injected oocytes in different Li^+ concentrations (Li^+ was substituted for Na^+) we found a significant increase in I_{ami} and G_{ami} with incubation in 5 mM Li^+ as compared to 2 mM, 10 mM and 15 mM Li^+ solution.

Fig. 3. ENaC current and conductance depends on $[Na^+]_i$. A) Amiloride-sensitive currents are monitored three days after injection of α -, β -, γ -ENaC cRNA into oocytes and heterologous expression of functional channels in the plasma membrane. With incubation in 10 mM Na^+ I_{ami} is significantly increased compared to 2 mM, 5 mM, 15 mM and 20 mM $[Na^+]_i$. B) After three days of incubation an increase from 2 mM to 10 mM $[Na^+]_i$ causes an increase in amiloride-sensitive conductance of ENaC-expressing oocytes. A further increase to 15 mM and 20 mM $[Na^+]_i$ leads to decreased G_{ami} values. C) When ENaC is inhibited by amiloride (50 μ M) the boosting effect of 10 mM $[Na^+]_i$ is abolished.

Fig. 4. Amiloride-sensitive current I_{ami} [nA] and conductance G_{ami} [μ S] subject to different Li^+ concentrations. A) ENaC-expressing oocytes reveal an increased I_{ami} when incubated three days in solution containing 5 mM Li^+ . The amiloride-sensitive current is controlled by $[Li^+]$ and increases in a time dependent manner from day 1 to day 3. B) The amiloride-sensitive conductance of ENaC-expressing oocytes also changes in a $[Li^+]$ dependent manner. The peak is reached at day 3 after injection of α -, β -, γ -ENaC cRNA when oocytes are incubated in 5 mM Li^+ containing solution compared to 2, 10 and 15 mM.

Furthermore, the levels of I_{ami} and G_{ami} were time dependent and had a maximum at day 3 after injection and incubation in 5 mM Li^+ (Fig. 4A, B).

D) Effects of $[Na^+]_i$ on ENaC surface expression. The protocols used in the above experiments were specifically designed to minimize unwanted Na^+ influx during electrophysiological measurements, which could result in Na^+ -dependent ENaC inactivation (run-down, self-inhibition). Although it is straight forward to reduce $[Na^+]_i$ by incubating ENaC expressing oocytes in Na^+ -

free solutions, it may complicate the interpretation of electrophysiological parameters measured in different conditions. The I_{ami} , G_{ami} (measured at -60 mV) and E_{rev} (reversal potential) are directly affected by changes of electrochemical gradient and intracellular ionic concentrations regardless of ENaC channel activity. Therefore, to verify that the observed increases of I_{ami} and G_{ami} are due to changes in ENaC expression level we performed a set of experiments as follows: (i) Half of the injected oocytes were incubated in ESR for 3 days, then I_{ami} and its reversal potential ($E_{rev_Na^+}$, thus also $[Na^+]_i$) were measured, yielding these values: $I_{ami} = 4.64 \pm 0.66 \mu A$; $E_{rev_Na^+} = +9.43 \pm 1.03$ mV; $[Na^+]_i = 7.07 \pm 0.26$ mM ($n = 27$, $N = 2$). (ii) The remaining oocytes were incubated for 3 days in Na^+ -free solution and were only moved to ESR at the beginning of the experiment. The clamp potential was set to +9.5 mV (i.e. the average $E_{rev_Na^+}$ from the previous point) and repeatedly pulsed to -60 mV for two minutes until the current reached zero. This pulsing protocol (two to five pulses) allowed the oocytes to rapidly accumulate Na^+ to an amount similar to that of ESR-incubated cells. Then the parameters of interest were measured as usual with the results being: $I_{ami} = 1.49 \pm 0.19 \mu A$; $E_{rev_Na^+} = +12.5 \pm 0.9$ mV and $[Na^+]_i = 6.21 \pm 0.2$ mM ($n = 27$, $N = 2$). The difference in $E_{rev_Na^+}$ and $[Na^+]_i$ for the two sets of oocytes was not statistically significant. Thus, the intracellular ionic conditions and the transmembrane electrochemical gradient for Na^+ being the same, it can be concluded that the threefold increase in I_{ami} was entirely due to change in ENaC activity, which in turn was triggered by the low $[Na^+]_i$ achieved during the three days of Na^+ -free incubation (Fig. 5). These results again demonstrate that ENaC expression is up-regulated by elevated $[Na^+]_i$ during the three day incubation after oocyte injection. Because ENaC-mediated G_{ami} of oocytes is a product of ENaC surface density and single-channel open probability (P_o), the observed increase of G_{ami} could result in an increase of these two processes. However, previous studies found that ENaC exists in the plasma membrane in two states: either almost completely open (P_o close to 1) or inactive (P_o close to 0) [20]. Thus, the increase of G_{ami} could be simply interpreted as an increased number (density) of active ENaC in the oocyte surface membrane. To further confirm that increased ENaC expression and density at the plasma membrane is involved, we performed EGFP-ENaC fluorescence measurements in oocytes incubated in different extracellular Na^+ .

E) Fluorescence intensity of EGFP-ENaC is modulated by $[Na^+]_i$. For the fluorescence examination

Fig. 5. Effects of $[Na^+]_i$ on ENaC activity (I_{Na}). Some of the ENaC expressing oocytes were incubated in ESR (10 mM), the other ones in Na^+ -free solution. The lack of extracellular Na^+ and the ensuing low intracellular Na^+ resulted in a threefold decrease of the amiloride-sensitive current (I_{Na} , right panel) from $4.64 \pm 0.66 \mu A$. The left panel shows that the reversal potential of the sodium current ($E_{rev_Na^+}$) was not significantly different in the two groups, with averages of $+9.43 \pm 1.03$ mV and $+12.5 \pm 0.88$ mV. Therefore, the difference in I_{Na} stems from different ENaC expression, and not from changes in the electrochemical gradient ($N = 2$, $n = 2 \times 27$).

of Na^+ dependent expression of ENaC we injected α -EGFP-ENaC, β -ENaC, γ -ENaC cDNA into the nucleus of *Xenopus* oocytes and measured the fluorescence signal of the protein at the plasma membrane using an inverted confocal fluorescence microscope. We analyzed ENaC-injected oocytes incubated in 5 mM, 10 mM and 20 mM Na^+ solution. The expression of ENaC protein incubated in 10 mM Na^+ solution was significantly elevated as compared to 5 mM Na^+ . Oocytes incubated in 10 mM Na^+ exhibit a stronger EGFP-ENaC signal. These fluorescence measurements of EGFP-ENaC expression confirm and extend our electrophysiological data and show that expression and subsequent ENaC protein density is influenced by $[Na^+]_i$.

Furthermore, we analyzed the EGFP-tagged ENaC using confocal microscopy to detect EGFP-ENaC more closely at the plasma membrane. The EGFP signal was detected 3 days after injection in oocytes incubated in different Na^+ solutions (5, 10, 15 mM) and in control oocytes. Again, by means of this method we found increased ENaC protein density in the plasma membrane with incubation in 10 mM Na^+ solution as compared to 5 and 15 mM (Fig. 6A). Since we focused on the plasma membrane (see transmitted light images) the fluorescence signals that we observed derive from EGFP-ENaC

molecules located in the plasma membrane or residing in vesicles situated close to the oocyte membrane. Fig. 6B summarises our findings and shows that ENaC levels in 20 mM and 5 mM Na^+ incubation are quite similar and far lower than in 10 mM Na^+ .

F) Cellular amount of ENaC depends on $[\text{Na}^+]_i$. To determine whether the increased ENaC abundance in the plasma membrane is associated with an increase in total protein we carried out Western blotting with highly specific primary antibodies against α -, β - and γ -ENaC subunits and alkaline phosphatase conjugated secondary antibodies. The antibodies that we used did not recognize any endogenous protein in non-injected oocytes. For separating the membrane proteins we loaded either 10 μg or 20 μg protein onto the SDS gel. The Western blot showed a distinct band of approximately 100 kDa for the ENaC α -subunit (Fig. 7A), approximately

100 kDa for the β -subunit (Fig. 7B) and approximately 110 kDa for the γ -ENaC subunit (Fig. 7C); the molecular weights of all subunits are in broad agreement with the published size. Before protein preparation ENaC-mRNA injected oocytes were incubated for 3 days in solutions containing 5 mM, 10 mM, 15 mM or 20 mM Na^+ , respectively. We detected the highest amount of all ENaC subunits in oocytes that were incubated in 10 or 15 mM Na^+ , while oocytes incubated in 5 or 20 mM Na^+ exhibited significantly lower ENaC amounts (Fig. 7A-C). In total we carried out Western blots preferably for the alpha-ENaC subunit (N = 2, n = 4), for beta- and gamma- ENaC the oocytes were obtained only from one frog (N = 1, n = 4).

Densitometric analysis revealed that expression of α - and γ -ENaC in 5 mM Na^+ and 20 mM Na^+ is significantly lower as compared with 10 mM Na^+ and

Fig. 7. Dependence of ENaC expression on different Na^+ concentrations in oocytes. Membrane proteins were isolated from ENaC-expressing oocytes 3 days after injection and incubation in different Na^+ solutions (5, 10, 15 and 20 mM). Representative blots for all three ENaC subunits are shown ($p \leq 0.05 = *$, $p \leq 0.01 = **$). All blots were scanned and digital densitometry was carried out by using ImageJ software. The expression of all three ENaC subunits follows a bell-shaped curve with a peak between 10-15 mM Na^+ . A) The ENaC α -subunit was detected with a specific anti α -ENaC antibody. The ENaC α -subunit is significantly increased with incubation in 10-15 mM Na^+ ($N = 2$, $n = 4$). B) With a specific anti β -ENaC antibody a distinct band in the range of approximately 100 kDa was detected. We found a significant increase in ENaC β -subunit with incubation of the ENaC-expressing oocytes in 15 mM ($N = 1$, $n = 3$). C) ENaC γ -subunit was recognized by a highly specific anti ENaC γ -subunit antibody and possesses a molecular weight of approximately 100 kDa. Expression of the γ -subunit is also modulated by Na^+ and follows a bell-shaped curve with a maximum at 15 mM ($N = 1$, $n = 3$).

15 mM Na^+ (Fig. 7A, C). The β -subunit has a maximum of protein amount with incubation in 15 mM Na^+ , while 5 mM and 10 mM decreased the expression of the subunits (Fig. 7B). In all cases the amount of ENaC expression follows a bell-shaped curve and correlates well with fluorescence intensity measurements of EGFP-ENaC signals in the oocyte interior and its surface. Moreover, the results from Western blot experiments are also in a very good accordance with the electrophysiological data analysing ENaC current and conductance.

Influence of $[\text{Na}^+]_i$ on CFTR and SGLT1 expression

A) Effects of $[\text{Na}^+]_i$ on CFTR current. In a first series of experiments we injected CFTR and incubated

the oocytes in different Na^+ concentrations ranging from 0 mM to 90 mM Na^+ . $[\text{Na}^+]_i$ was manipulated using the endogenous Na^+ /glucose cotransporter of the oocytes [21]. CFTR injected oocytes were incubated in the presence of 2.5 mM AMG (α -methyl D-glucopyranoside; a glucose analogue that is exclusively transported by Na^+ dependent systems) and 20 μM ouabain to prevent Na^+ loss through the Na^+ /K⁺-ATPase. Measurements of $[\text{Na}^+]_i$ with Na^+ -selective electrodes showed that $[\text{Na}^+]_i$ increased up to 22 mM with 90 mM Na^+ outside.

After 3-4 days we clamped the oocytes to -60 mV and activated CFTR with cAMP-cocktail. The cAMP induced CFTR current (I_{CFTR}) was strongly dependent on the Na^+ concentration of the incubation solution. I_{CFTR} followed a bell-shaped curve with a peak current between

Fig. 8. Effects of $[\text{Na}^+]_i$ on CFTR expression. Oocytes were co-injected with ENaC and CFTR. Therefore, $[\text{Na}^+]_i$ is assumed to be close to $[\text{Na}^+]_o$ as demonstrated above. 3 days after injection we measured cAMP-cocktail induced CFTR current (I_{CFTR}). A) In CFTR-ENaC injected oocytes, I_{CFTR} depends strongly on $[\text{Na}^+]_o$. The effects range from $0.196 \pm 0.045 \mu\text{A}$ ($n = 13$, $N = 2$) in the absence of extracellular Na^+ to marked boosting ($16.22 \pm 3.1 \mu\text{A}$, $n = 14$, $N = 2$) with 15 mM $[\text{Na}^+]_o$. Intermediate values are $1.94 \pm 0.36 \mu\text{A}$ ($n = 13$, $N = 2$), $6.83 \pm 1.53 \mu\text{A}$ ($n = 14$, $N = 2$) and $16.72 \pm 3.31 \mu\text{A}$ ($n = 14$, $N = 2$) after incubation in 2, 5 and 10 mM $[\text{Na}^+]_o$, respectively. B) After 3 days of incubation in a Na^+ -free environment, 24 hours of exposure to 10 mM $[\text{Na}^+]_o$ are sufficient for the boosting effect to manifest itself in co-injected oocytes and to bring I_{CFTR} from $0.19 \pm 0.04 \mu\text{A}$ ($n = 13$, $N = 2$) to $6.55 \pm 1.19 \mu\text{A}$ ($n = 9$, $N = 2$).

10 and 15 mM $[\text{Na}^+]_i$. Higher Na^+ concentrations reduced cAMP induced I_{CFTR} (data not shown).

In the next set of experiments we co-expressed ENaC and CFTR. To prevent unwanted intra-oocyte Na^+ accumulation in cells that were expressing both CFTR and ENaC, oocytes were stored and measured in ESR containing different Na^+ concentrations, and we used the software-driven current-clamp mode of our setup throughout the experiment. cAMP-cocktail induced I_{CFTR} was determined in presence of amiloride (50 μM). Fig. 8A shows a direct correlation between CFTR expression and $[\text{Na}^+]_o$ measured as cAMP-induced increase in CFTR current I_{CFTR} (in presence of ENaC $[\text{Na}^+]_o$ and $[\text{Na}^+]_i$ are nearly identical, please see above). Again, maximal expression is seen between 10 and 15 mM $[\text{Na}^+]_i$.

We were also interested to see whether Na^+ has to be present in the extracellular environment immediately after mRNA microinjection (i.e. in the early stages of heterologous protein synthesis), or whether its effect in conjunction with ENaC expression on CFTR activity is of a more acute nature. Hence, we performed a set of experiments in which co-injected oocytes were checked for cAMP-sensitive currents after a three-day incubation time in Na^+ -free medium. Then the same oocytes were moved to a medium with 10 mM Na^+ and measured again for CFTR currents after 24 h. The results depicted in Fig. 8B clearly suggest that a one-day exposure to Na^+ -containing medium is sufficient for the boosting effect to manifest itself and to bring the average CFTR current from the mere 0.2 μA characteristic for zero- Na^+ incubation to values in excess of 6.5 μA .

B) Fluorescence intensity of EGFP-CFTR. For quantitative determination of CFTR in the plasma membrane we injected EGFP-CFTR. Oocytes were

Fig. 9. Na^+ dependent expression of CFTR. 3 days after injection of EGFP-CFTR mRNA a significant increase in fluorescence intensity is detected in oocytes, which have been incubated in 10 or 15 mM Na^+ , respectively. Incubation in 5 or 20 mM Na^+ resulted in significant lower fluorescence intensity.

subsequently incubated in different Na^+ concentrations ranging from 5 to 20 mM. Using an inverted fluorescence microscope we measured the fluorescence signal of the protein at the plasma membrane and nearby regions. Three days after injection the fluorescence signal of EGFP-CFTR showed a maximum between 10 and 15 mM and significant lower fluorescence could be detected at 5 and 20 mM Na^+ , respectively (Fig. 9). Thus, $[\text{Na}^+]_i$ influences CFTR current via modulation of CFTR expression on the protein level as already described for ENaC.

C) Effects of $[\text{Na}^+]_i$ on SGLT1. We continued our investigation into the effects that $[\text{Na}^+]_i$ might have on the expression of transport systems with a protein that does not form an ion channel but a cotransporter: the human Na^+ /glucose cotransporter SGLT1 [22].

Fig. 10. Na⁺ dependent expression of the human sodium glucose cotransporter (SGLT1). Oocytes were injected with ENaC and SGLT1 and subsequently incubated in 0 and 15 mM Na⁺. 3 days after injection currents produced by SGLT1 (I_{SGLT1}) were measured. Expression of SGLT1 is drastically increased following incubation at 15 mM as compared with 0 mM Na⁺. All measurements were performed in presence of 50 μM amiloride.

The transport is electrogenic, thus in the presence of extracellular Na⁺ and glucose (or AMG, K_{1/2} ~ 2.5 mM) an inward Na⁺ current can be recorded at -60 mV. For the following set of experiments, the SGLT1 current (I_{SGLT1}) is expressed as the difference in clamp current produced by adding 5 mM AMG to the perfusion medium. Oocytes were incubated in 0 and 15 mM Na⁺ containing ESR, respectively, and were measured after 3 days. Since ENaC was co-expressed to have access on [Na⁺]_i, all the solutions used during measurement contained 50 μM amiloride.

AMG-induced I_{SGLT1} of oocytes incubated in 0 mM Na⁺ averaged at 31.0 ± 3.9 nA (Fig. 10). Yet, I_{SGLT1} of oocytes incubated in 15 mM Na⁺ was significantly elevated to 260.2 ± 18.8 nA (n = 26, N = 4). Again, the highest expression of the cotransporter SGLT1 was observed at 15 mM Na⁺, thereby confirming the results obtained with the ion channels ENaC and CFTR.

Discussion

In this study we demonstrate for the first time that in *Xenopus laevis* oocytes translational or posttranslational processes are modulated by distinct [Na⁺]_i. We investigated the effects of different [Na⁺]_i on ENaC, CFTR and SGLT1 with three different methods: (i) with electrophysiological techniques such as conventional voltage-clamp protocols we recorded currents and conductances, (ii) with measurements of

EGFP-labelled ENaC and CFTR we detected expression of the channels and (iii) with the help of protein biochemistry we detected the total ENaC amount in dependence of different [Na⁺]_i.

From our results we hypothesized that Na⁺ could be a factor that is involved in the control of protein expression, holding true at least for transport proteins expressed in *Xenopus laevis* oocytes. To demonstrate that it is really Na⁺ and not another ion having the influences on protein expression we varied the Cl⁻ concentration of the incubation media and measured CFTR current. No influence of Cl⁻ on the expression of CFTR could be observed. Because Cl⁻ is the only mobile anion in the cytoplasm, its concentration cannot change considerably without a direct effect on intracellular ionic strength and osmolality and subsequent changes in cell volume. From the same assertion follows that the sum of the intracellular concentrations of the two main cations (Na⁺ and K⁺) must be relatively constant - an assumption which also dictated the protocol for the calibration of the Na⁺-selective microelectrodes. At [Na⁺]_i around 5 mM - where the correlation of the observed effects with cation concentration is most striking - an alteration of e.g. 1 mM represents a relative change for Na⁺ of 20%, versus only approximately 1% for K⁺.

As a prerequisite for our investigations we made sure that we had always strict control over [Na⁺]_i. For that purpose we applied two methods for the determination of [Na⁺]_i: ion-selective microelectrodes and measurements of the Na⁺ current reversal potential that was used to solve Nernst's equation. Both methods yielded identical values for [Na⁺]_i. Values for [Na⁺]_i of non-injected oocytes in the literature vary remarkably ranging from 6 mM Na⁺ to 23 mM Na⁺ (for a compilation please see [15]). In our hands, both methods for determining [Na⁺]_i yielded a value of 7.5 mM intracellular Na⁺ (N = 3, n = 18). By varying [Na⁺]_i from close to 0 mM up to 20 mM Na⁺ in ENaC expressing oocytes we are looking at both decreased and increased [Na⁺]_i as compared to the normal physiological conditions.

We first examined the influence of [Na⁺]_i on ENaC expression and abundance in the plasma membrane. All methods that we used yielded the same consistent result: total ENaC expression, its plasma membrane-associated abundance and functional activity in the plasma membrane have a maximum between 10 mM and 15 mM of [Na⁺]_i when [Na⁺]_i was clamped to values between 10 and 15 mM Na⁺ after ENaC-mRNA injection. Lower (5 mM) and higher (≥ 20 mM) [Na⁺]_i had the opposite effect and decrease ENaC expression.

Densitometric analyses of Western blots with total membrane proteins isolated from ENaC-expressing oocytes and with specific antibodies against α -, β - and γ -ENaC revealed the strongest ENaC expression in oocytes incubated in 10 mM and 15 mM. Those results clearly show that at $[\text{Na}^+]_i$ in the range of 10 mM ENaC expression reaches a maximum, which demonstrates that $[\text{Na}^+]_i$ influences the expression of ENaC and thus could modulate its abundance at the plasma membrane. Measurements of EGFP-labeled ENaC in regions of the oocyte membrane also showed that the abundance of ENaC - not the activity - is modulated by $[\text{Na}^+]_i$ and follows again a bell-shaped curve with a maximum of fluorescence intensity with 10 mM $[\text{Na}^+]_i$. We found the boosting effect of $[\text{Na}^+]_i$ on the functional expression. Therefore, we postulate that stimulation of protein expression by Na^+ occurs on the translational/posttranslational level. Furthermore, protein trafficking could be the target leading to increased exocytotic delivery and functional insertion of ENaC molecules into the oocyte plasma membrane. The effects of $[\text{Na}^+]_i$ on the expression and abundance of ENaC are not ENaC-specific. In fact, we obtained similar results by expression of other transport proteins such as CFTR and SGLT1. In the case of CFTR we could examine the effects of $[\text{Na}^+]_i$ on protein expression in a broader concentration range (0 - 90 mM extracellular Na^+), which is not possible with ENaC expressing oocytes due to Na^+ overcharge of the cells. We found a strong dependence of CFTR expression on $[\text{Na}^+]_i$ with a maximum between 10 mM and 15 mM Na^+ . Higher and lower concentrations decrease CFTR expression drastically. These findings are in good accordance with the results obtained with ENaC. In order to have a more precise control over $[\text{Na}^+]_i$ we co-expressed ENaC with CFTR. In presence of ENaC $[\text{Na}^+]_i$ is approximating $[\text{Na}^+]_o$ in the range from 0 to 20 mM Na^+ as demonstrated by our measurements of $[\text{Na}^+]_i$ in dependence of different $[\text{Na}^+]_o$. Again, expression of CFTR is clearly dependent on $[\text{Na}^+]_i$ with a maximum between 10 and 15 mM Na^+ . This reliance of CFTR expression on $[\text{Na}^+]_i$ is independent of ENaC as demonstrated by the experiments when CFTR was expressed alone. Moreover, manipulating $[\text{Na}^+]_i$ using activation of the endogenous Na^+ /glucose cotransporter found in *Xenopus* oocytes [21] yielded the same results.

In another approach we incubated ENaC-CFTR injected oocytes for 3 days in a solution without Na^+ . The oocytes were voltage-clamped every day and the cAMP-dependent CFTR current was assessed, which remained minute over this period. Then we transferred

the oocyte to a solution containing 10 mM Na^+ and measured the same oocytes again: CFTR current increased more than 300 times within 24 hours. From these particular experiments we conclude that, at least in oocytes $[\text{Na}^+]_i$ has strong influence on protein *de novo* synthesis and that Na^+ is a modulator of translational and/or posttranslational events. Furthermore, these results show also that protein degradation is not involved in the observed effects of Na^+ . Further evidence that protein synthesis rather than protein degradation is involved in the observed regulatory influences of $[\text{Na}^+]_i$ in *Xenopus laevis* oocytes comes from our experiments using EGFP-labelled ENaC or CFTR: in both cases it is obvious that an optimal $[\text{Na}^+]_i$ boosts the expression of the respective protein measured as increase in fluorescence intensity in the oocyte interior and its surface.

To further confirm and extend our data on influences of $[\text{Na}^+]_i$ on protein expression we choose a transport protein that is totally different from the ion channels that we investigated before. The human intestinal Na^+ /glucose cotransporter SGLT1 is an apically located secondary active transport protein that mediates glucose uptake relying on a Na^+ gradient established by the basolateral Na^+ / K^+ -ATPase [22]. Expression of this cotransporter was also dependent on $[\text{Na}^+]_i$ and SGLT1 produced more than 10 times higher currents with $[\text{Na}^+]_i$ of 10 mM Na^+ as compared with $[\text{Na}^+]_i$ around 0 mM. We clearly showed that $[\text{Na}^+]_i$ influenced the protein level of ENaC and CFTR. Although we do not have similar data on SGLT1 it is more than tempting to speculate that $[\text{Na}^+]_i$ influences SGLT1 protein level rather than SGLT1 current.

To sum up, these data obtained with three different transport proteins suggest that $[\text{Na}^+]_i$ strongly influences protein expression in *Xenopus laevis* oocytes. In the case of ENaC we can certainly exclude a mechanism involving Na^+ feedback inhibition: Feedback inhibition is rapid (within a few minutes), affects channel open probability and decreases the number of active channels in the plasma membrane. The effects of Na^+ on protein expression that we describe here are slow (take some days), do not affect channel open probability but decrease the number of active channels above a Na^+ concentration of about 15 mM. Moreover, Na^+ feedback inhibition occurs in a complete different concentration range [8]. Furthermore, Na^+ feedback inhibition has certainly no effects on the increased expression of CFTR and SGLT1 as we showed here.

Except for a few reports [23] Na^+ has never been directly associated with the protein expression and

Fig. 11. Possible mechanisms underlying the dual role of intracellular Na^+ concentration on CFTR expression. Intracellular Na^+ concentration ($[\text{Na}^+]_i$) was controlled via heterogeneously expressed ENaC or the oocyte endogenous Na^+ /glucose cotransporter (OGCT), leading to the observed optimal $[\text{Na}^+]_i$ for protein expression of 10 - 15 mM. This $[\text{Na}^+]_i$ elevation could activate exocytotic delivery and insertion of transport proteins from cytosolic pools to the oocyte membrane (①). Elevation of $[\text{Na}^+]_i$ could also activate aminoacyl t-RNA synthetases, leading to stimulation of protein synthesis and subsequent increased protein expression in the oocyte plasma membrane (②). As deduced from the observed time course we propose that both mechanisms are stimulated simultaneously. The same mechanisms are thought to stimulate the observed increased expression of ENaC and SGLT1.

trafficking machinery. Yet, in a very recent paper the properties of monovalent cations as second messenger were reviewed [24]. The authors describe the influence of e.g. sodium as an activator of gene expression. Furthermore, it has been shown that changes in $[\text{Na}^+]_i$ are detected by a putative Na^+ -sensor leading to stimulation of anti-apoptotic protein expression [25]. Although the authors did not identify all $[\text{Na}^+]_i$ sensible gene products that they observed, it could be possible that some of these proteins are related to translational and posttranslational events. From these data the authors conclude that the engagement of Na^+ in cellular regulatory events allows Na^+ to be considered as a second messenger. Yet, to our knowledge there are no studies on that matter in *Xenopus laevis* oocytes. Since ENaC is

also highly selective for Li^+ as a substrate we studied in parallel the effect of incubation in different Li^+ concentrations on ENaC expression. We found a strong influence of $[\text{Li}^+]_i$ similar to $[\text{Na}^+]_i$ with a peak of I_{ami} and G_{ami} when oocytes were incubated in 5 mM Li^+ , which is very interesting in this context. It could be that Li^+ accumulated to higher concentrations within the oocytes since Li^+ is not removed by the Na^+/K^+ -ATPase.

There are some possible mechanisms that could explain the observed boosting effects of $[\text{Na}^+]_i$ and $[\text{Li}^+]_i$ on protein expression. Since Na^+ on its own is not recognized as a classical regulatory ion, it is tempting to discuss the involvement of the intracellular Ca^{2+} concentration or pH and their change via Na^+ -dependent exchangers. It was reported that an increase in cytosolic

Ca^{2+} concentration from 1 nM to 1 μM was accompanied by a decrease in channel activity, while 10 μM Ca^{2+} had a pronounced inhibitory effect [26]. In this system an increase in $[\text{Na}^+]_i$ might decrease the driving force available to the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger and lead to an increase in $[\text{Ca}^{2+}]_i$, followed by an inhibitory effect of intracellular Ca^{2+} on ENaC activity. Yet, oocytes do not express $\text{Na}^+/\text{Ca}^{2+}$ -exchangers in such an amount that could significantly alter $[\text{Ca}^{2+}]_i$ in intra-oocyte Na^+ concentrations around 10 mM [27] where the influence of $[\text{Na}^+]_i$ on protein expression is the highest. In the presence of a Na^+/H^+ -exchanger an increase in $[\text{Na}^+]_i$ would result in intracellular acidification. Again, there are not enough Na^+/H^+ -exchangers in the oocyte plasma membrane that would modify intracellular pH significantly at $[\text{Na}^+]_i$ around 10 mM [28]. Furthermore, it was reported that only mechanically defolliculated oocytes, i.e. without collagenase treatment, exhibit some Na^+/H^+ exchange activity [29]. From all these arguments we do not expect that at the peak value of protein expression with $[\text{Na}^+]_i$ of 10 mM changes in $[\text{Ca}^{2+}]_i$ or $[\text{H}^+]_i$ will occur that could be responsible for the observed influence of $[\text{Na}^+]_i$ on the rate of protein expression. Similar observations were made for c-Fos in smooth muscle cells [23], and was attributed to a $[\text{Na}^+]_i$ -sensitive $[\text{Ca}^{2+}]_i$ -independent transcription factor(s) distinct from factors interacting with known response elements of this gene promoter. Therefore, we do not expect that endogenous $\text{Na}^+/\text{Ca}^{2+}$ or Na^+/H^+ exchanger of the oocytes are involved in the stimulation of protein expression that we see in dependence on $[\text{Na}^+]_i$.

Some hypotheses involve Na^+ as a putative regulator of protein expression: at first it could be that Na^+ is somehow interfering with the protein translating machinery as we have shown to be the case for ENaC expression. Other possible explanations would involve effects of $[\text{Na}^+]_i$ on protein maturation and trafficking to the plasma membrane or on the kinetics of the investigated channels and transporters. Indeed, it could be shown that Na^+ has an important part in controlling secretory activity of nerve terminals and that increasing $[\text{Na}^+]_i$ resulted in stimulation of transmitter secretion [30]. Many years ago it was published that distinct sodium concentrations influence the synthesis of brain proteins in a cell-free system [31]. The authors found that the optimal ionic concentrations for protein synthesis (i.e. 15 mM Na^+) are similar to those found inside the resting cell. Since 10–15 mM $[\text{Na}^+]_i$ are physiological for oocytes those findings are in perfect agreement with our data. Also many years ago Schmidt-Nielsen [32] showed that enzymes involved in protein

synthesis are quite sensitive to the intracellular potassium concentration and that an optimal $[\text{K}^+]_i$ appears to be necessary for proper protein synthesis. Changes in very narrow ranges have strong influences on DNA, RNA and protein synthesis. It is tempting to speculate that changes in $[\text{Na}^+]_i$ might evoke small alterations of $[\text{Na}^+]_i$ or effect the intracellular $\text{Na}^+:\text{K}^+$ ratio thereby effecting protein expression. Yet, from the present data we cannot totally rule out a possible contribution of K^+ , we will address this question in future studies.

Another important topic that emerges from our study is the question about possible protein-protein interactions between CFTR and ENaC when expressed in *Xenopus laevis* oocytes. The interactions between CFTR and ENaC are still a debated topic. In our hands, CFTR expression and/or activation never had a measurable effect on ENaC activity. The issue of CFTR modulation by ENaC has emerged more recently and the causalities involved are not clear. We have shown in this study that $[\text{Na}^+]_i$ is an important factor and that it extends to other heterologously expressed ion transport systems. However, at this point we cannot comment on an additional, specific interaction between ENaC and CFTR as the research is only in its incipient stages. Moreover, it could be that the oocyte expression system for studying CFTR-ENaC interactions might be not suited: First, parts of the complex macromolecular signalling complexes necessary for proper regulation of the putative interplay between CFTR and ENaC might be lacking in oocytes. Secondly, recent results showed that ENaC subunit composition and stoichiometry is strongly tissue specific [33, 34]. Therefore, co-expression of ENaC e.g. from rat intestine (as in most of all expression studies) together with human lung CFTR in oocytes might not result in correct data on protein-protein interaction *in vivo*, e.g. in human respiratory epithelia where ENaC and CFTR are constitutively expressed.

Conclusions and perspectives

In conclusion, our data clearly show that intracellular Na^+ influences the expression level of transport proteins such as ENaC, CFTR, and SGLT1, thereby elevating the amount of the respective protein in the oocyte plasma membrane. The exact mechanism is not understood yet, however, we postulate an influence on translation/posttranslational events or protein trafficking to the plasma membrane. Elevation of $[\text{Na}^+]_i$ could stimulate endogenous Ca^{2+} -dependent protein kinases A that subsequently initiate exocytotic delivery of transport proteins from intracellular stores to the oocyte plasma

membrane as described for CFTR following stimulation by cAMP [11, 35]. Furthermore, elevated $[Na^+]_i$ could stimulate transfer reactions of aminoacyl t-RNA synthetases as reported some years ago [36]. These transfer reactions in turn insure *de novo* protein synthesis, protein trafficking to and subsequent functional insertion into the oocyte plasma membrane. From the accelerated time course of the expression of all three proteins examined in this study we propose that both mechanisms are initiated simultaneously. We combined both mechanisms in a model that we propose as a first explanation of the stimulating effects of $[Na^+]_i$ on protein expression (Fig. 11).

The results of this study allow initial speculations on a potentially very interesting contribution of Na^+ to protein expression. This raises at least two questions: Is there an intracellular Na^+ sensor and if, what is its molecular nature? Which systems are involved in the downstream signal transduction of elevating $[Na^+]_i$? It will be very appealing to investigate whether other cells show this influence of $[Na^+]_i$ on protein expression, too.

Moreover, it remains studying whether these mechanisms are specific for transport proteins or a more common pathway of cells to regulate protein expression. We will address these questions in forthcoming experiments and hope that other groups using the oocyte expression system will contribute to this story.

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