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Hypotonic Regulation of Mouse Epithelial Sodium Channel in *Xenopus laevis* Oocytes

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Abstract The regulation of the epithelial Na^+ channel (ENaC) during cell swelling is relevant in cellular processes in which cell volume changes occur, i.e., migration, proliferation and cell absorption. Its sensitivity to hypotonically induced swelling was investigated in the *Xenopus* oocyte expression system with the injection of the three subunits of mouse ENaC. We used voltage-clamp techniques to study the amiloride-sensitive Na^+ currents ($I\text{Na}_{(\text{amil})}$) and video microscopic methodologies to assess oocyte volume changes. Under conditions of mild swelling (25 % reduced hypotonicity) inward current amplitude decreased rapidly over 1.5 min. In contrast, there was no change in current amplitude of H_2O-injected oocytes to the osmotic insult. $I\text{Na}_{(\text{amil})}$ kinetics analysis revealed a decrease in the slower inactivation time constant during the hypotonic stimuli. Currents from ENaC-injected oocytes were not sensitive to external Cl^- reduction. Neither short- nor long-term cytochalasin D treatment affected the observed response. Oocytes expressing a DEG mutant β-ENaC subunit (β-S518K) with an open probability of 1 had reduced $I\text{Na}_{(\text{amil})}$ hypotonic response compared to oocytes injected with wild-type ENaC subunits. Finally, during the hypotonic response ENaC-injected oocytes did not show a cell volume difference compared with water-injected oocytes. On this basis we suggest that hypotonicity-dependent ENaC inhibition is principally mediated through an effect on open probability of channels in the membrane.

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Introduction

Cell motion mechanisms are related to cytoskeletal reorganization, where cortical actin filaments are involved in the generation of locomotive structures and the interaction with other signaling and structural proteins (Mitchison and Cramer 1996). Ion channels and transporters actively participate in this process, being regulated by cytoskeletal components and cell volume (Mills and Mandel 1994; Schwab et al. 2005). Na^+ currents mediated by sodium channels and epithelial sodium channels sensitive to amiloride (ENaC) are involved in cell migration and proliferation, which is well documented in epithelial and vascular cells (Chifflet et al. 2005; Grifoni et al. 2006), liver cells (Bondarava et al. 2009) and tumor cells (Sparks et al. 1983; Rooj et al. 2012).

The ENaC belongs to a protein family which includes mechanoreceptors that possess a mechanical signal-translating function (Carattino et al. 2004; Drummond et al. 2004). Among other factors, ENaC is regulated by the actin cytoskeleton (Cantiello et al. 1991; Karpushev et al. 2010a) with the ENaC carboxyl terminus interacting with F-actin (Mazzochi et al. 2006). Our group reported the role of xShroom1, an actin binding protein, in the regulation of ENaC expressed in *Xenopus laevis* oocytes (Assef et al. 2011) and the participation of these channels in the migration of a placental cell line (Del Mónaco et al. 2009; Marino et al. 2013). The possible link between the role of ENaC in migration and its regulation by the actin cytoskeleton might be the activity changes of channels during cell volume modifications due to ion and water fluxes

during cell migration (Schwab et al. 2007; Hoffmann 2011).

We further pursued the investigation on the potential mechanisms in the relation between ENaC and volume regulation. To do this we examined the action of hypotonic stimuli upon the amiloride-sensitive Na^+ currents ($INa_{(amil)}$) in oocytes expressing the wild-type mouse ENaC (mENaC) and a DEG mutant (αβS518Kγ-ENaC) with an open probability (P_o) of nearly one. To establish the actin cytoskeleton's role in this channel regulation, we performed the same experiments in the presence of cytochalasin D. We also evaluated the cell volume changes during this osmotic challenge with video microscopic techniques. We observed that wild-type ENaC activity decreases rapidly in response to hypotonic stimuli whereas Na^+ channel mutants that exhibit high intrinsic P_o acutely inhibit the response. This response was not affected by actin cytoskeletal disruption. ENaC-injected oocytes did not show any difference in hypotonically induced volume changes compared with water-injected oocytes during the experiments. From our experiments, we suggest that this rapid hypotonicity-dependent ENaC activity reduction is principally mediated through an effect on the P_o of channels in the membrane.

Materials and Methods

Adult female *X. laevis* frogs were anesthetized with 0.3 % tricaine (MS-222) and the oocytes surgically removed from an abdominal incision. Oocytes were defolliculated using 1 mg/ml type IV collagenase (Sigma, St. Louis, MO, USA) for 1 h at 18 °C in agitation in OR-2 medium. After mechanical separation for 1 h with K-buffer, oocytes were placed in ND96 medium, containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5 (pH 7.4) supplemented with 1 mg/ml gentamicin (Gibco, Grand Island, NY, USA). Oocytes were injected with a Drummond injector (Drummond, Broomall, PA, USA) with 4 ng of α, β and γ mENaC (total volume 50 nl). At 16–36 h after ENaC injection we obtained steady $INa_{(amil)}$ in the range of microamperes, so all the experiments were done in this period.

We synthesized complementary RNAs for α, β and γ mouse wild-type and mutant ENaC subunits using the T3 mMessage mMachine kit (Ambion, Austin, TX, USA).

A standard two-electrode voltage clamp was performed using a Warner Oocyte Clamp OC 725C (Warner Instruments, Hamden, CT, USA) with a bath probe circuit. We acquired data through Clampex 8.0 (Axon Instruments, Union City, CA, USA) using a DigiData 1220A interface at 1 kHz and stored electronically on a PC hard disk. Micropipettes had resistances of 0.5–4 MΩ when filled with 3 M KCl. We clamped the bath with two chloride silver wires through 3 % agar bridges in 3 M KCl and positioned close to the acolyte. In the well with the acolyte, we estimated the bath-fluid resistance as the resistance between two electrodes (about 100–200 Ω). Without the bath probe this value is increased by a factor of 10 or 20. Thus, all the experiments were done using the bath-probe circuit to keep this resistance in series with the membrane and between electrodes as low as possible. We perfused the oocyte chamber with (1 ml/min) a peristaltic pump (Dynamax RP-1; Rainin Instrument, Woburn, MA, USA), with the solution ejected by a needle placed on top of the well containing the oocyte. Following the insertion of both microelectrodes, we waited for 5 min before starting the experiment. Then we ran two sets of records with a delay of 3 min to be sure that the currents were stable. After this, we applied amiloride and recorded the currents at 3 and 5 min, enough time to have a stable blocking effect.

For the current–voltage (*I–V*) relationships, we applied a series of 500 ms voltage steps from −100 to +60 mV in 20 mV increments. The currents were measured after 400–500 ms at a clamp potential of 0 mV. ENaC-mediated Na^+ currents were defined as the current difference measured in the absence versus the presence of 10 μM amiloride in the bath solution. To rule out the possibility of amiloride contamination in the perfusion system during the solution changes, most of the experiments were performed by dissolving amiloride stock solution in the syringe containing hypotonic medium during the course of the experiment after we observed that the hypotonic solution had produced the effect on the current.

To perform kinetic analysis of the currents, amiloride-sensitive currents were obtained by subtracting the currents remaining after 10 μM amiloride, which was added at the end of each experiment. The −100 mV voltage episodes were selected using pCLAMP. The data between the start (usually the first 20 ms after the initial voltage change was discarded) and end of the −100 mV voltage episodes were chosen for the double exponential fit. The current in each episode was fitted to a double exponential using the least squares. Inactivation kinetics were analyzed by fit analysis of double exponential factor of the general form

$$I = A1\exp(-t/\tau_1) + A2\exp(-t/\tau_2) + \text{offset}$$

where t is time, τ the associated inactivation time constant(s) and An the initial amplitude of each component (Patel et al. 2004).

Oocyte volume changes were obtained from video microscopic images. Oocytes were transferred to hypotonic solution after a period of 10 min of stabilization in isotonic medium. Changes in area were recorded by a webcam with a PC and Astra software (Phase Space Technology,

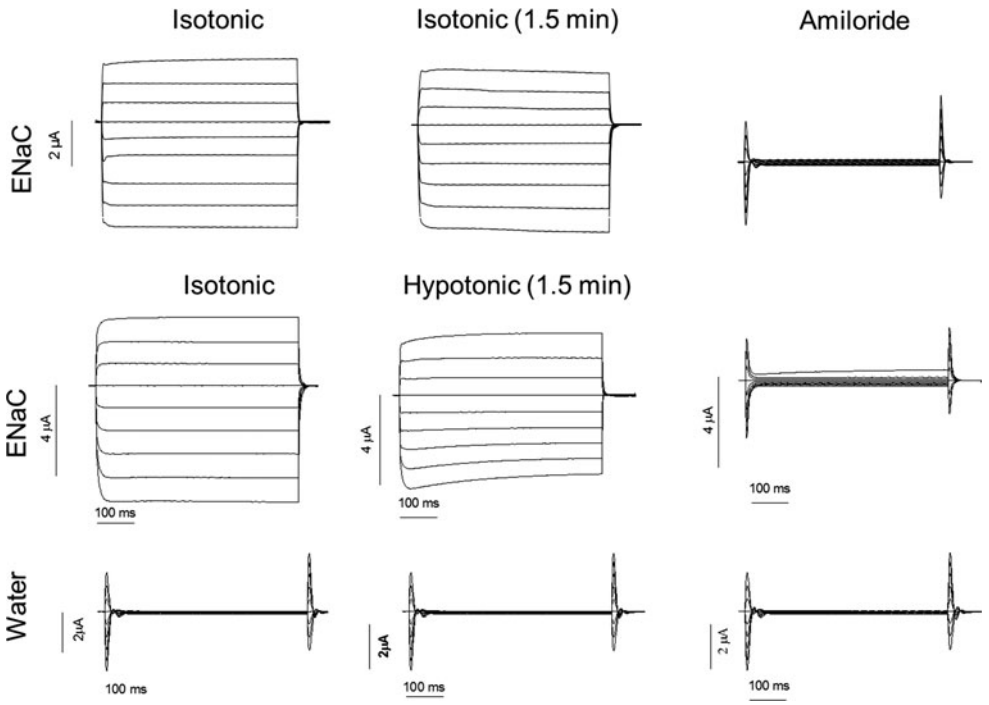

Fig. 1 Inhibition by hypotonic stimuli of $I\text{Na}_{(\text{amil})}$ oocytes expressing αβγ ENaC. Whole-cell currents were obtained by clamping the oocytes in 20 mV steps from –100 to 60 mV. As seen (*upper panel*) there were no appreciable changes in the currents in control ND72 isotonic solution. The *lower panel* shows representative recordings in an oocyte before (isotonic) and after addition of hypotonic stimuli (25 %) and after amiloride (10 μM). The decrease of current with hypo-osmolarity in the first 1.5 min of treatment is observed in ENaC-injected oocytes. In both cases these whole-cell currents were accompanied by a similar inhibition during 3–5 min of amiloride (10 μM) blockade. Water-injected oocytes did not show amiloride-sensitive currents, and no significant changes were observed during hyposmotic treatment

Rosebud, Australia). Images were analyzed with Image J software (Dorr et al. 2007) (NIH, Bethesda, MD, USA). The oocyte volume was calculated from surface area (A) using the formula $V = (4/3) \times A \times (A/\pi)^{0.5}$, and the relative cell volume (V_t/V_o, where V_o is volume at time zero and V is volume at time t) was calculated from the oocyte surface area at time zero (A_o) and at time t (A): $V_t/V_o = (A/A_o)^{1.5}$. Such a small change of volume would be an even smaller change of area.

Hypotonic solution has an NaCl concentration reduced to 72 mM (osmolarity 160 ± 10 mOsm/kg), and ND72 isotonic solution was prepared adding 50 mM of mannitol (osmolarity 210 ± 10 mOsm/kg) to ND72 hypotonic solution. Osmolarities were measured in a freeze point osmometer. Low-chloride experiments were performed with ND72 solution, replacing NaCl with Na-gluconate.

Data were expressed as the mean ± SE, where n equals the number of independent oocytes analyzed and N represents the number of frogs used in each experimental series. Statistical analysis was performed using the paired and unpaired t test where correspond. Differences were considered statistically significant when $p < 0.05$.

Results

Regulation of $I\text{Na}_{(\text{amil})}$ by Hypotonic Stimuli

In Fig. 1 (upper panel), we show superimposed current traces in which the potential was held at 0 mV and jumped to values between −100 and +60 mV for 500 ms. Following a period of stabilization in isotonic condition, oocytes were superfused with isotonic ND72 solution. It is evident that there is no time course inhibition and that the currents were abolished by 10 μM amiloride. In another set of experiments, after a stabilization period in isotonic ND72 solution, oocytes were exposed to hypotonic medium for 1.5 min. The middle panel in Fig. 1 shows the rapid inhibitory effect on ENaC currents during hypotonicity and the amiloride effect. In contrast, hypotonic stress in water-injected oocytes did not generate any amiloride-sensitive whole-cell currents and had no significant effects on whole-cell currents within the first 1.5 min after application of osmotic stress ($I\text{Na}_{(\text{amil})}$ at −100 mV: isotonic = 0.03 ± 0.01 μA vs. hypotonic 0.02 ± 0.01 μA, ns, $n = 5$, $N = 2$).

The data obtained from these experiments are summarized in Fig. 2a and indicate that the hypotonic bath

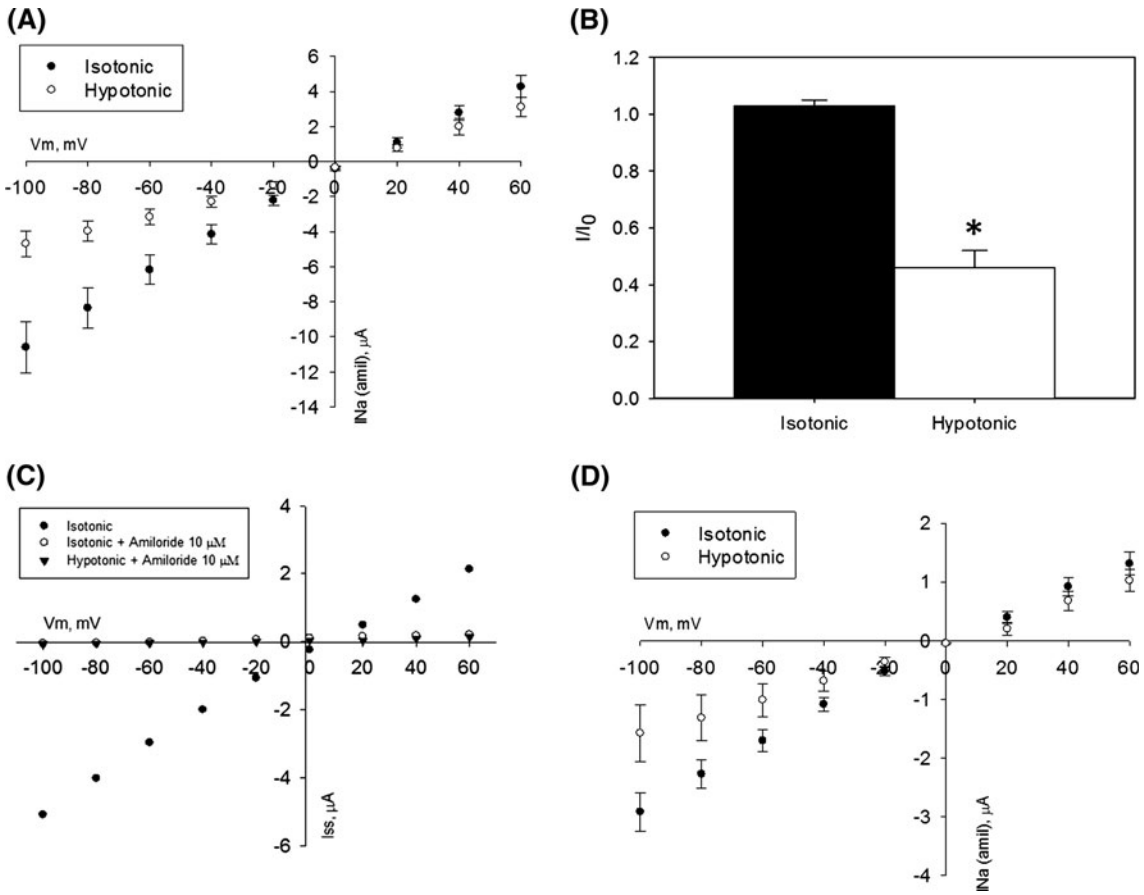

Fig. 2 Inhibition of ENaC inward sodium currents by hypotonic stimuli. **a** Current/voltage (*I–V*) relationship of $I\text{Na}_{(\text{amil})}$ in hypotonically treated oocytes before (*filled circles*) and after (*open circles*) 1.5 min perfusion with hypotonic solution (25 %) ($n = 13$, $N = 4$). $I\text{Na}_{(\text{amil})}$ was determined by superfusion with 10 µM amiloride. **b** Oocytes were perfused with ND72 isotonic alone (*black bar*, like those experiments shown in Fig. 1 upper panel) or with ND72 hypotonic (25 %) solution for 1.5 min (*white bar*). Amiloride (10 µM) was added 3–5 min after. Initial currents at $t = 0$ were -10.6 ± 1.5 µA (hypotonic group) and -4.2 ± 0.8 µA (isotonic group). I/I_0 represents the $I\text{Na}_{(\text{amil})}$ current recorded after the treatment (t) relative to time 0 in each experiment. Results are expressed as mean ± SE. *Asterisk* indicates differences in $I\text{Na}_{(\text{amil})}$ ($p < 0.01$ isotonic [$n = 5$, $N = 3$] vs. hypotonic treatment [$n = 12$, $N = 4$], unpaired t test). **c** Amiloride pretreatment blocks response to hypotonicity of ENaC-injected oocytes. Corresponding *I–V* curves (*closed circle*) in isotonic ND-72 medium, (*open circle*) inhibition of current in isotonic ND-72 in the presence of 10 µM amiloride isotonic, (*closed triangle*) in the continual presence of 10 µM amiloride. No changes in whole-cell currents were observed after hypotonic treatment in the continual presence of amiloride. Data represent recordings from three oocytes. **d** *I–V* relationship of $I\text{Na}_{(\text{amil})}$ in hypotonically treated oocytes before (*filled circles*) and after (*open circles*) 1.5 min perfusion with hypotonic (25 %) solution in low-chloride condition ($n = 5$, $N = 2$)

solution elicited a rapid decrease in $I\text{Na}_{(\text{amil})}$ inward currents. At –100 mV $I\text{Na}_{(\text{amil})}$ decreases from -10.6 ± 1.5 to -4.7 ± 0.8 µA ($n = 13$, $N = 4$, $p < 0.001$, paired t test). Amiloride-sensitive inward conductances, G_{inward} (from −100 to 0 mV), are reduced from 104 ± 15 to 45 ± 7 µS. However, G_{outward} (between 0 and +60 mV) only diminishes from 80 ± 11 to 55 ± 5 µS in hypotonicity.

To rule out the effect of time in the observed decay of currents, we compared the relative $I\text{Na}_{(\text{amil})}$ currents in isotonic condition (shown in Fig. 1 upper panel) with those oocytes challenged with hypotonic solution (shown in Fig. 1 lower panel). The $I\text{Na}_{(\text{amil})}$ relative current (I/I_0) was calculated in order to avoid the variability of expression between oocyte batches. Figure 2b shows the effect of the relative amiloride-sensitive current changes recorded at −100 mV of ENaC-injected oocytes in isotonic and hypotonic conditions: (I/I_0 isotonic $= 1.03 \pm 0.02$, $n = 5$, $N = 2$ vs. 0.46 ± 0.06, $n = 13$, $N = 4$, unpaired t test, $p < 0.001$). A similar response to that shown in Fig. 2a was observed with ENaC-injected oocytes when amiloride was perfused previous to hypotonic challenge (Fig. 2c).

From second-grade polynomial fitting of the *I–V* curves shown in Fig. 2, we obtained the reversal potential (ENa) before and after hyposmotic treatment. During the hypotonic stimuli ENa did not change (2.2 ± 0.2 to 2.6 ± 0.7 mV, $n = 12$, $N = 3$). This indicates a strong dependence on Na^+ movement and that no other ion permeates in the amiloride-sensitive pathway during

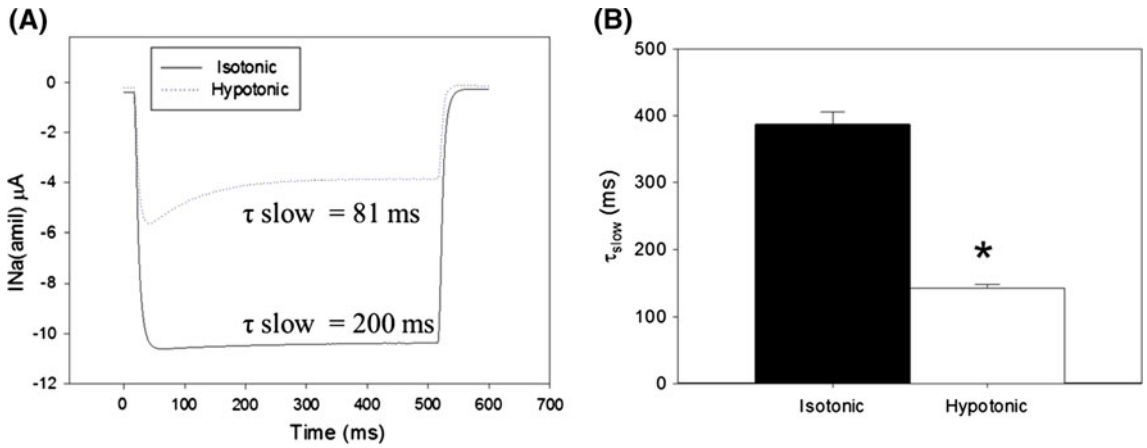

Fig. 3 Inactivation of $I\text{Na}_{(\text{amil})}$ currents during hypotonic stimuli. **a** Representative pulses of $I\text{Na}_{(\text{amil})}$ currents in isotonic and hypotonic conditions. Hypotonic and isotonic $I\text{Na}_{(\text{amil})}$ currents were compared by fitting the curves to a biexponential function (see "Materials and Methods" section). **b** Summary of tau slow component during hypotonic treatment; the tau slow parameter was obtained for both treatments. Tau slow decreased from 387 ± 19 ms in isotonic (*black bar*) stimuli to 143 ± 5 ms in hypotonic stimuli (*white bar*) ($n = 10$, $N = 4$). Results are expressed as mean ± SE

hypotonicity. With this value near 0 mV and an extracellular sodium concentration of 72 mM we can estimate an intracellular sodium concentration of about 70 mM. The ENa after exposure to amiloride was near −60 mV, indicating chloride/potassium endogenous oocyte currents.

The high intracellular sodium in these experiments is able to produce the slight inwardly rectifying behavior observed in the isotonic condition (Fig. 2a), although during the hypotonicity treatment there was an outward rectification. Although these rectifying properties of $I\text{Na}_{(\text{amil})}$ are unexpected, they have been observed by other authors in α-ENaC–injected oocytes (Fuller et al. 1995; Amuzescu et al. 2003; Ishikawa et al. 1998). It can also be explained by a voltage-dependent amiloride inhibition effect, as reported (Segal et al. 2002), showing that amiloride inhibition is increased at hyperpolarized membrane voltages.

Schreiber et al. (2003) reported a chloride-dependent activation of ENaC during hypotonicity. On this basis we examined the possible impact of osmotic stress on amiloride-sensitive Na^+ currents in the absence of extracellular Cl^- concentration, and we found that the reduction of extracellular Cl^- concentration did not affect the response to hypotonic stimuli (Fig. 2d).

We analyzed the kinetics of the $I\text{Na}_{(\text{amil})}$ currents in isotonic conditions, fitting the curves obtained at the largest hyperpolarized voltage used (−100 mV) to a double exponential function, which gives the time course of inactivation of the pulses (Fig. 3a). The slower time constant of inactivation (τ slow) of $I\text{Na}_{(\text{amil})}$ shows a decrease from 387 ± 19 ms during isotonic stimuli to 143 ± 5 ms in hypotonic stimuli (Fig. 3b). This implies that hypotonicity elicits not only an inhibition of the instantaneous current but also a strong but slower secondary inhibition at hyperpolarizing voltages. This supports the possibility that osmotic pressure modifies P_o.

Actin Cytoskeleton Role in ENaC Regulation by Hypotonicity

Hypotonicity-regulated channel activity is dependent on the actin cytoskeleton (Jorgensen et al. 2003; Galizia et al. 2012). Particularly, the ENaC carboxyl terminus and F-actin interact (Mazzochi et al. 2006), and the activity of these channels is dependent upon actin cytoskeletal integrity (Cantiello et al. 1991; Karpushev et al. 2010a). An acute disruption of the actin cytoskeleton enhances ENaC activity, but longer exposures to disrupting agents result in a decrease of ENaC activity (Karpushev et al. 2010a). In addition, hypotonic shocks decrease cellular F-actin (Jorgensen et al. 2003), and all these changes are a possible mechanism linking hypotonic stimuli to regulation of ion channel activity.

We then evaluated the short- (20 min) and long-term (2–5 h) effects of cytochalasin D (CytD, 6 µM) treatment on the hypotonicity-mediated decrease in ENaC activity. Neither 20 min nor 2–5 h of treatment with CytD changed the extent of inhibition of hypotonic amiloride-sensitive inward current (Fig. 4a, b). This observation is confirmed in Fig. 4c, d, where relative currents after hypotonic stimuli are plotted in the absence and presence of CytD for 20 min and 2–5 h. No significant changes were observed in the CytD-treated, water-injected oocytes (data not shown). This suggests that the actin cytoskeleton is not involved in the hypotonically mediated decrease of ENaC currents.

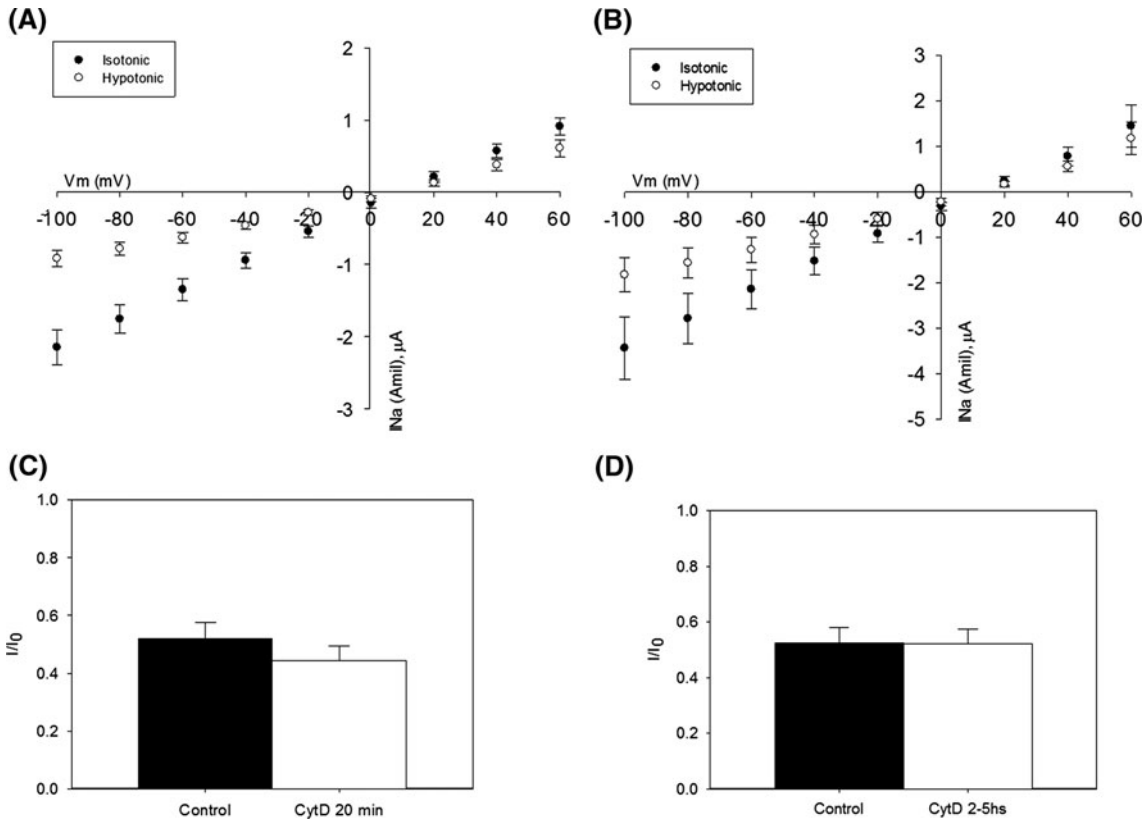

Fig. 4 Actin cytoskeletal depolymerization does not affect hypotonically mediated ENaC response. **a** Current–voltage (*I–V*) relationship of $INa_{(amil)}$ of CytD-treated oocytes (20 min) before (*filled circles*) and after (*open circles*) 1.5 min perfusion with hypotonic solution (25 %) ($n = 6$, $N = 3$). $INa_{(amil)}$ was determined by superfusion with 10 µM amiloride. **b** *I–V* of $INa_{(amil)}$ of CytD-treated oocytes (2–5 h) before (*filled circles*) and after (*open circles*) 1.5 min perfusion with hypotonic solution (25 %) ($n = 7$, $N = 3$). $INa_{(amil)}$ was determined by superfusion with 10 µM amiloride. **c** Relative $INa_{(amil)}$ (I/I_0) during hypotonic stimuli in the absence (*black bar*) and presence (*white bar*) of CytD (6 µM, 20 min). No differences were observed between these groups (CytD, $n = 6$, $N = 3$ vs. Ctrl, $n = 8$, $N = 3$) (NS, unpaired *t* test). **d** Relative $INa_{(amil)}$ (I/I_0) during hypotonic treatment in the absence (*black bar*) and presence (*white bar*) of CytD (6 µM, 2–5 h). No differences were observed between groups (CytD, $n = 7$, $N = 3$ vs. Ctrl, $n = 5$, $N = 3$) (NS, unpaired *t* test)

Open Probability Role in ENaC Regulation by Hypotonicity

The next step was to confirm whether hypotonicity reduces $INa_{(amil)}$ through changes in the number of active channels expressed in the plasma membrane or by modifying P_o. We used a DEG mutant β mENaC (β-S518K) with α- and γ-ENaC subunits, which have a P_o of nearly one (Snyder et al. 2000).

Figure 5a depicts a pool of experiments of αβ-S518Kγ-ENaC–expressing oocytes ($n = 5$) recorded before and after hypotonic (25 %) solution and after amiloride treatment. The *I–V* curve of $INa_{(amil)}$ with this mutant (Fig. 5b) indicates that $INa_{(amil)}$ is not affected by the hypotonic change, in contrast with the decrease observed in wild-type (WT) ENaC. Although the DEG mutant β mutant shows enhanced $INa_{(amil)}$ compared with WT-ENaC (Assef et al. 2011), in this pool of experiments we observed differences in channel expression between batches. In −100 mV, initial whole-cell amiloride-sensitive currents (time = 0 before addition of hypotonic medium) were -4.8 ± 2.5 (αβS518Kγ) and -4.3 ± 2.4 µA after hypotonic treatment.

Figure 5c shows the relative $INa_{(amil)}$ for the wild type or the DEG mutant treated with hypotonic solution. The decrease of the relative $INa_{(amil)}$ inward currents elicited by the osmotic stimuli in WT-ENaC (0.47 ± 0.06, $n = 12$, $N = 3$) was abolished in β-S518K–expressing oocytes (0.87 ± 0.03, $n = 5$, $N = 3$, $p < 0.01$, unpaired *t* test). These data demonstrate that gain-of-function mutations within a region that dramatically affect channel gating alter the channel's response to osmotic perturbation, suggesting a role of P_o in this inhibition.

ENaC-Injected Oocyte Volume Changes During Hypotonic Stimuli

Native oocytes respond to hypotonic swelling with a regulatory volume decrease (Hermoso et al. 2002), although other reports (Kelly et al.1995) found that heterologous Cl/anion exchange or mIK1 expression confers volume

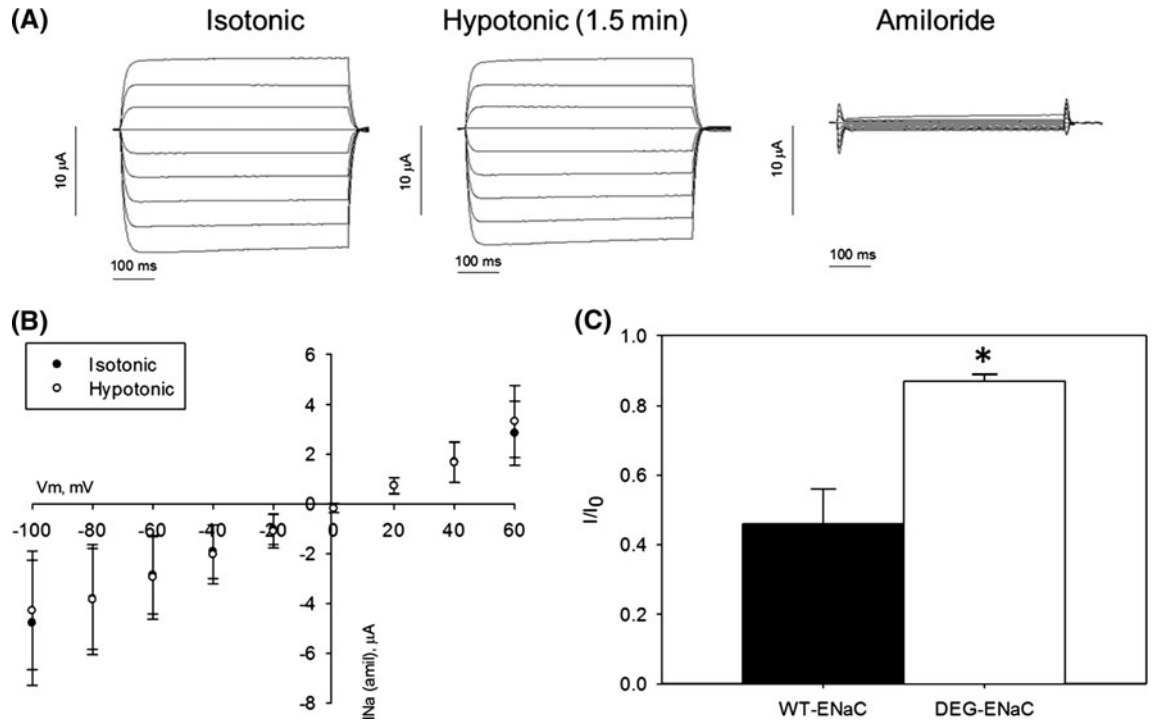

Fig. 5 Constitutively active channels do not decrease the response to hypotonic stimuli. **a** Currents in an oocyte expressing αβS518Kγ ENaC before (isotonic), after addition of hypotonic solution (1.5 min, 25 %) and after amiloride (10 µM). **b** Current–voltage (*I–V*) relationship of $I\text{Na}_{(\text{amil})}$ in hypotonically treated oocytes expressing αβS518Kγ ENaC before (*filled circles*) and after (*open circles*) perfusion with hypotonic solution ($n = 5, N = 3$). **c** Relative $I\text{Na}_{(\text{amil})}$ (I/I_0) responses to hypotonic stimuli of oocytes expressing the degenerin mutant (αβS518Kγ-ENaC). Inhibition percentage of currents recorded after 1.5 min of perfusion by hypotonicity in wild-type ENaC-expressing oocytes is also shown. Relative current in hypotonic stimuli in oocytes expressing αβS518Kγ-ENaC ($n = 5, N = 3$) was increased in comparison to wild-type ENaC-expressing oocyes ($n = 12$, $p < 0.05$, unpaired *t* test)

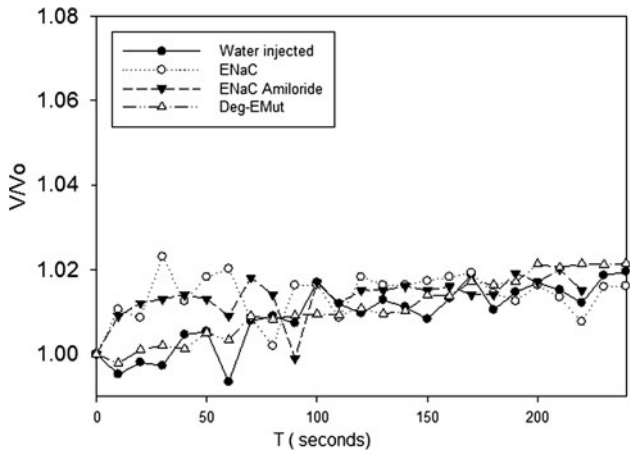

Fig. 6 ENaC-injected oocyte volume changes during mild hypotonic stimuli. Relative volume (V_t/V_o) responses to hypotonic stimuli (25 %) of water- and ENaC-injected oocytes. After 5–10 min of stabilization in ND72 isotonic solution, oocytes were transferred to hypotonic solution. During the first 200 s of osmotic challenge, WT-ENaC in the absence or presence of amiloride (10 µM), αβS518Kγ-ENaC– and water-injected oocytes showed the same increased swelling percentage. Each group corresponds to one representative experiment from one donor

regulation on *Xenopus* oocytes (Jiang et al. 1997; Vandorpe et al. 1998). ENaC regulation during osmotic changes was reported in rat hepatocytes (Böhmer and Wehner 2001; Plettenberg et al. 2008). Figure 6 shows representative experiments on the effect of −50 mOsM hypotonic stimuli on the volume in both water- and ENaC-injected oocytes. After 5–10 min of stabilization in ND72 isotonic medium, oocytes were transferred to hypotonic medium. Image captures were made every 10 s after the change (time 0). Figure 6 shows relative volume changes (V_t/V_o) during the osmotic challenge. As expected, in the first 200 s the water-injected oocytes swell slowly by less than 2 %, as reported (Grunnet et al. 2002). ENaC-expressing oocytes treated with or without amiloride (10 µM) or expressing the mutant β ENaC (β-S518K) showed a similar slow swelling response as water-injected oocytes.

Discussion

In this work we studied the regulation of ENaC by hypotonic stimuli in oocytes measuring $I\text{Na}_{(\text{amil})}$ as reported by Ji et al. (1998) with oocytes expressing rat ENaC. We

extend these results by using a DEG mutant β ENaC (β-S518K) along with wild-type α- and γ-mENaC subunits, with a P_o of nearly 1. We also analyzed the current inactivation kinetics during the osmotic stimuli and measured volume changes during hyposmotic changes and the possibility of an actin cytoskeleton role in this regulation. We showed that mENaC in control conditions shows no activation or a very slow activation with negative pulses. Although ENaC is not a voltage-gated channel, human ENaC (hENaC), *Xenopus* ENaC (xENaC) and rat ENaC (rENaC) show some degree of $I\text{Na}_{(\text{amil})}$ activation at hyperpolarized pulses (Amuzescu et al. 2003). In the cortical collecting tubule this activation is likely a direct effect of voltage on channel gating (Palmer and Frindt 1996). The reason why in our hands the mENaCs are not activated by strong hyperpolarization might be related to some different structure among ENaC species with different gating sensitivity to voltage.

The published reports about the role played by stretch of epithelial Na^+ channels have generated conflicting results (Ismailov et al. 1997; Achard et al. 1996; Awayda et al. 1995). Awayda and Subramanyam (1998) proposed that ENaC is insensitive to osmotic stimuli because no activation was detected; on the other hand, Ji et al. (1998) and Schreiber et al. (2003) indicated that ENaC activity was sensitive to hyposmotic stimuli, but they observed opposite responses. Ji et al. (1998) proposed that in *Xenopus* oocytes rENaC is sensitive to osmotic pressure in low external chloride, suggesting a role of ENaC in cell volume regulation. Our experiments show that the rapid hypotonicity reduction in $I\text{Na}_{(\text{amil})}$ is present in two different external chloride conditions in opposition to other published data supporting external chloride as a modulation factor of the hypotonicity-mediated $I\text{Na}_{(\text{amil})}$ response (Schreiber et al. 2003). The oocyte membrane has a small water permeability, which leads to very small changes in cell volume, although this change in volume can elicit tension changes due to increases in intracellular pressure (Kelly et al. 1995). We did not observe any additional sodium current contribution to volume swelling, as reported for another DEG/ENaC family channel, the acid-sensitive ion channel (ASIC1b) (Ugawa et al. 2008). However, a role of ENaC and ASIC channels in cell volume regulation was reported elsewhere (Böhmer and Wehner 2001). As occurs with other mechanical stimuli, the rapid $I\text{Na}_{(\text{amil})}$ reduction in response to a decrease in external osmolarity is more probably due to an external domain gating regulation than to a membrane tension–regulated mechanism. Volume changes performed with −50 mOsM hyposmotic gradients in the time window of the experiments presented here correspond to tension values around 2.7 mN/m (Ozu et al. 2013; Kelly et al. 1997), 0.5 mN/m being the tension required to obtain a 50 % aperture of mechanosensitive channels (see references in Ozu et al. 2013).

The possibility that osmotic stimuli regulate in some way the activity of functional ENaC channels in the membrane is supported by the results obtained with the DEG mutant and the inactivation of $I\text{Na}_{(\text{amil})}$ during hypotonicity. The DEG mutant (βS518Kγ- ENaC) increases the P_o of ENaC to nearly one (Snyder et al. 2000), and it was extensively used as a tool to discern between traffic and P_o in ENaC modulation (Carattino et al. 2004). We found that injection of this mutant produced a minute reduction in $I\text{Na}_{(\text{amil})}$ compared with the WT channels. The rapid decrease of currents during hypotonic stimuli with the WT-mENaC in the current is then explained by an inactivation of the channels, suggesting a P_o reduction. This phenomenon was observed in the potassium voltage-gated channel Shaker, in which membrane stretch produced a faster inactivation (Laitko and Morris 2006). Thus, the regulatory effect of hypotonicity upon $I\text{Na}_{(\text{amil})}$ is most probably due to a P_o modification, although we cannot exclude other factors, i.e., a change in the number of channels inserted in the plasma membrane. Although experimental evidence regarding actin cytoskeleton involvement on ENaC basal activity suggests the participation of actin microfilaments in the modulation of conductances, probably through an F-actin–ENaC physical interaction (Mazzochi et al. 2006; Cantiello et al. 1991; Karpushev et al. 2010a), this modulation mechanism seems not to be important during mechanical stresses, not disrupting the actin cytoskeleton by short- or long-term treatment as shown in this work (Fig. 4) and consistent with other reports (Awayda and Subramanyam 1998; Karpushev et al. 2010b). In additional experiments we observed no effects on colchicine-treated oocytes (data not shown), suggesting no involvement of a traffic mechanism during mechanical stress, also in agreement with published data (Morimoto et al. 2006).

Cell volume has an important role in many physiological processes, such as migration crucial in implantation and embryogenesis, immunity and inflammation, tissue regeneration, angiogenesis and metastasis (Schwab et al. 2007; Hoffmann 2011), and depends on many factors, among them the cytoskeletal regulation and ionic activity. Na^+ currents mediated by ENaC and other channels are involved in cell migration, well documented in epithelial and vascular cells (Kapoor et al. 2009; Chifflet et al. 2005) and in the proliferation of tumor cells (Sparks et al. 1983; Vila-Carriles et al. 2006). Perhaps the osmotic regulation of ENaC proteins has an important role in this process, although the mechanism underlying this change in activity must be established.

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