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ENaC Channels in Oocytes from *Xenopus laevis* and their Regulation by xShroom1 Protein

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Key Words
ENaC • Sodium currents • xShroom1 • Oocytes • Trypsin • Immunofluoresence

Abstract
Shroom is a family of related proteins linked to the actin cytoskeleton. xShroom1 is constitutively expressed in *X. oocytes* and is required for the expression of amiloride sensitive sodium channels (ENaC). Oocytes were injected with α, β, and γ mENaC and xShroom1 sense or antisense oligonucleotides. We used voltage clamp techniques to study the amiloride-sensitive Na^+ currents ($INa_{(amil)}$). We observed a marked reduction in $INa_{(amil)}$ in oocytes co-injected with xShroom1 antisense. Oocytes expressing a DEG mutant β-mENaC subunit (β-S518K) with an open probability of 1 had enhanced $INa_{(amil)}$ although these currents were also reduced when co-injected with xShroom1 antisense. Addition of low concentration (20 ng/ml) of trypsin which activates the membrane-resident ENaC channels led to a slow increase in $INa_{(amil)}$ in oocytes with xShroom1 sense but had no effect on the currents in oocytes coinjected with ENaC and xShroom1 antisense. The same results were obtained with higher concentrations of trypsin (2 µg/ml) exposed during 2.5 min. In addition, fluorescence positive staining of plasma membrane in the oocytes expressing α, β and γ mENaC and xShroom1 sense were observed but not in oocytes coinjected with ENaC and xShroom1 antisense oligonucleotides. On this basis, we suggest that xShroom1-dependent ENaC inhibition may be through the number of channels inserted in the membrane.

Introduction

Many of the functions of amiloride sensitive ion channels (ENaC) are through interactions with actin, actin-binding proteins, or scaffolding proteins [1-8]. Shroom is a family of proteins involved in the regulation and maintenance of cytoskeleton architecture by binding to actin. It has an important role in the morphogenesis of several different embryonic epithelial tissues and neuronal growth [9-12]. Hagens et al. [13] classified four different proteins (Shroom1 to Shroom4) in this family, showing similarity in their domains with a PDZ and two other

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domains named ASD1 and ASD2 (Apx/Shrm domains). The ASD2 domain seems to be the common denominator among family members.

Of a particular interest for us is xShroom1 (formerly knows as APX), a large protein formed by 1420 amino acids, 150kDa) which lacks the PDZ domain and is constitutively expressed in *Xenopus laevis* oocytes and necessary for the expression of ENaC in oocytes through an unknown mechanism [14]. In this investigation, we further pursued the investigation initiated by Zuckerman et al. [14]. To do this we examined the action of xShroom1 upon the amiloride-sensitive Na^+ currents ($INa_{(amil)}$) in oocytes expressing the wild type αβγ mENaC and a DEG mutant αβ-S518K-γ ENaC with an open probability (Po) of nearly 1 [15-16]. We tested trypsin in low concentration and high concentrations to activate the membrane-resident ENaC channels [17]. We also studied the fluorescence measurements of αENaC in the plasma membrane. From our experiments, we suggest that xShroom1-dependent ENaC inhibition is mediated through an effect on the number of channels inserted in the membrane.

Materials and Methods

Adult female *Xenopus laevis* frogs were anesthetized with 0.3% tricaine (MS-222) and the oocytes surgically removed from the abdominal incision. Oocytes were defolliculated incubating the oocytes with 1 mg/ml type IV collagenase for 40 minutes. The oocytes were placed in ND96 medium, containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, HEPES 5 (pH 7.4) supplemented with 10 μg/ml sodium penicillin and 10 μg/ml streptomycin sulfate.

Oocytes were injected with a Drummond injector (Drummond, Broomall, PA) with 2 ng of α, β, and γ mENaC and 25 ng of xShroom1 sense or antisense oligonucleotides (total volume 50 nl).

We synthesized complementary RNAs (cRNAs) for α, β, and γ mouse wild type and mutant ENaC subunits using the T3 mMessage mMachine kit (Ambion, Austin, TX). We used synthetic oligodeoxynucleotides complementary to nucleotides +455 to +479 of xShroom1 [14] (sense, 5'-GCA TTA AGC AGA ATC GCC CTA ACC AC-3'; antisense, 5'-GTG GTT AGG GCG ATT CTG CTT ATG C-3', Life Technologies, Inc).

A standard two-electrode voltage clamp was performed using a Warner Oocyte Clamp OC 725C (Warner Instruments, Hamden, CT) with a bath probe circuit. We acquired data through Clampex 8.0 (Axon Instruments, Union City, CA) using a DigiData 1220A interface at 1 kHz and stored electronically on a PC hard disk. Micropipettes had resistances of 1-3 MΩ when filled with 3 M KCl. We clamped the bath with two chlorided silver wires through 3 % agar bridges in 3 M KCl and positioned close to the oocyte. In the well with the oocyte, we estimated the bath-fluid resistance as the resistance between both 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 (0.6 ml/min) a peristaltic pump (Dynamax RP-1; Rainin Instrument, Woburn, MA) and 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 minutes before starting the experiment. Then we run two sets of records with a delay of 5 minutes to be sure that the currents were stable. After we applied amiloride and we recorded the currents at 3 and 5 minutes, 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 –160 to +40 mV in 20-mV increments. The currents were measured after 400 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. For the experiments with low concentration of trypsin, the oocyte was clamped at 0 mV and stimulated with a -100 mV pulse each 10 minutes first in control solution (30 minutes) and then during one hour in 20 ng/ml trypsin. We added 10 μM amiloride at the end of the run. With high concentration of trypsin (2 μg/ml) we exposed the oocytes to the drug for 2.5 minutes and we recorded the currents as usual. All the data are mean ± SE.

Immunofluorescence

Forty-eight hours after injection, oocytes were fixed for 1 h with 3 % paraformaldehyde in phosphate-buffered saline (PBS, mM: KCl 2.7, NaCl 137, KH_2PO_4 1.5, Na_2HPO_4 1.7; pH 7.6-8.0) and washed two times with PBS for 5 min. After that the oocytes were embedded in optimum cutting temperature compound (OCT, Sakura, Finetek Europe, Zoeterwoude, NL) and cut to 5 μm slices with a cryostat (Tissue-Tek, Ca). Sections were put in PBS for 5 min and then for 1 h in PBS (5% BSA, Sigma) and overnight at 4ºC with rabbit polyclonal antibody against human α-ENaC subunit (dilution of 1/50) (Santa Cruz Biotechnology, Santa Cruz CA). Sections were washed with PBS and incubated for 1 h at room temperature with anti-rabbit fluorescein isothiocynate (FITC)-conjugated secondary antibody at dilution 1/2500 (Jackson Immunoresearch Laboratories, Tuker, Ga). We mounted the samples over glass plates in PBS/glycerol 1:1 solution after washing with PBS. We visualized the sections with a fluorescence microscope. We analyzed the pictures with Image J 1.39 software.

Results

$INa_{(amil)}$ were detected 24-36 hs after the ENaC injection

After 24-36 hs we obtained steady $INa_{(amil)}$ in the range of microamperes so all the experiments were done during this period. The resting Vm of ENaC expressing oocytes were depolarized from -10 to + 10 mV, (-3 ± 2

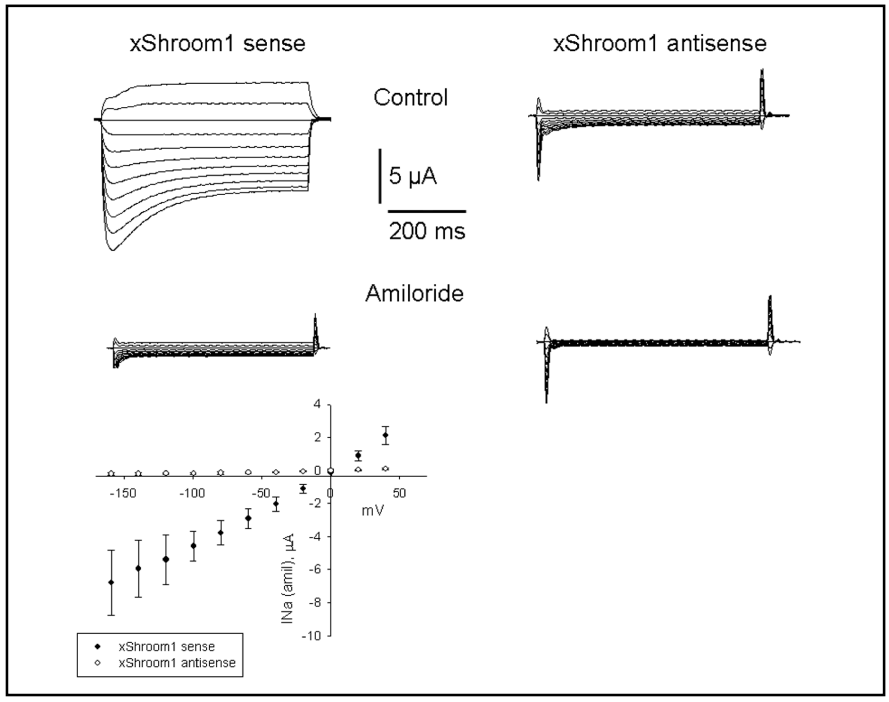

Fig. 1. After 24-36 h injection of the three ENaC subunits, $INa_{(amil)}$ was detected. In this and other figures control oocytes were those coinjected with ENaC subunits and the xShroom1 sense oligonucleotides. The figures show records from two oocytes, one coinjected with the xShroom1 sense and the other with the xShroom1 antisense oligonucleotides in control and in 10 µM amiloride. The traces were generated by pulses from -160 to + 40 mV from a holding potential of 0 mV. The IV plot shown below is the average results of $INa_{(amil)}$ obtained from 18 oocytes in each case. $INa_{(amil)}$ obtained subtracting the current remnant in amiloride from the control values.

mV, n=34) indicating that the expression of ENaC increased Na^+ permeability and depolarized the Vm. The Vm in oocytes injected with H_2O had a Vm of -43 ± 9 mV (n=22). $INa_{(amil)}$ was not observed in oocytes injected with H_2O (n = 16).

In Fig. 1 we show superimposed current traces in which the Vm was held at 0 mV and jumped to values of between -160 to +40 mV for 500 ms. Large inward currents (downward deflections) were observed. In some experiments at the most negative voltages there was a small increase in inward current over the last part of the pulse but in general, the currents were stable in most of the experiments. We observed outward currents due to a high intracellular Na^+ concentration. These currents were abolished by 10 µM amiloride and this is shown also in the figure. The currents were restored after washing out the applied amiloride although it took several minutes to get the recovery and in many cases that was incomplete, presumably due to a run down effect of ENaC channels. Similar effects were observed using a lower concentration of amiloride (2 µM) but the experiments were carried out with 10 µM amiloride to be sure that all ENaC channels in the wild type and in the mutant were blocked.

The right panel of Fig. 1 shows records from oocytes coinjected with xShroom1 antisense oligonucleotides. The currents were much smaller than in the oocytes injected with xShroom1 sense oligonucleotides. The $INa_{(amil)}$ calculated from the currents measured in the absence versus the presence of 10 µM amiloride in the bath solution for oocytes injected with either xShroom1 sense or

WT-ENaC – SENSE:	36 ± 10 µS
ANTISENSE: (n=18 from 6 frogs, p = 0.0017)	1.80 ± .50 µS
MUTANT β mENaC (β-S518K) – SENSE:	65 ± 9 µS
ANTISENSE: (n=14 from 5 frogs, p= 0.001)	2.0 ± 1.2 µS

Table 1. Conductances in different conditions. Data were obtained with pulses from -160 to 0 mV.

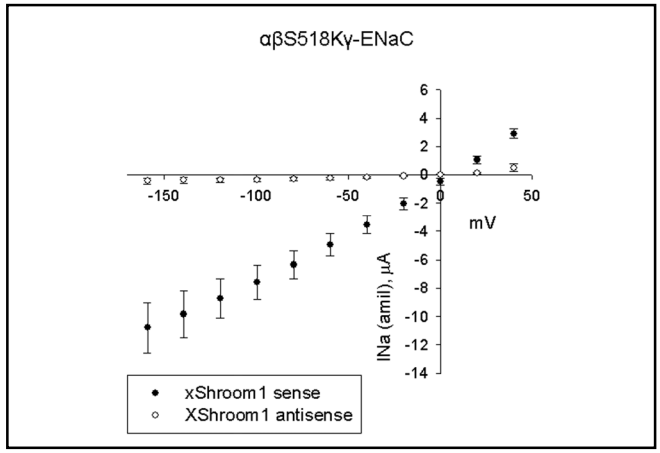

Fig. 2. IV plots showing average values of $INa_{(amil)}$ in oocytes co injected with the mutant αβS518Kγ-ENaC with either xShroom1 sense or antisense oligonucleotides (n= 14). Same protocol as in Fig. 1. It is evident the larger $INa_{(amil)}$ in the oocytes coinjected with the xShroom1 sense that in oocytes expressing the WT-ENaC (Fig.1) and the blocking effect of xShroom1 antisense upon the $INa_{(amil)}$.

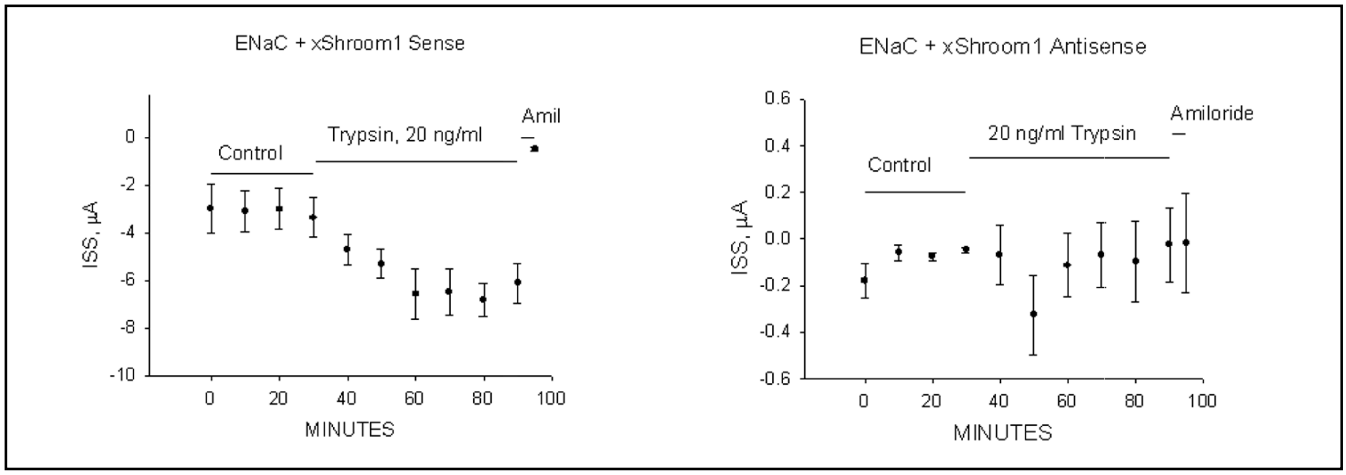

Fig. 3. Currents in the presence of trypsin. For these experiments the oocyte was clamped at 0 mV and stimulated with a -100 mV pulse each 10 minutes, first in control solution (30 minutes) and then during one hour in 20 ng/ml trypsin. At the end of the experiment 10 µM amiloride was added (n=14 in each case). The difference in current before and after trypsin in oocytes with xShroom1 sense was significant, p= 0.024, N= 14 from 5 frogs).

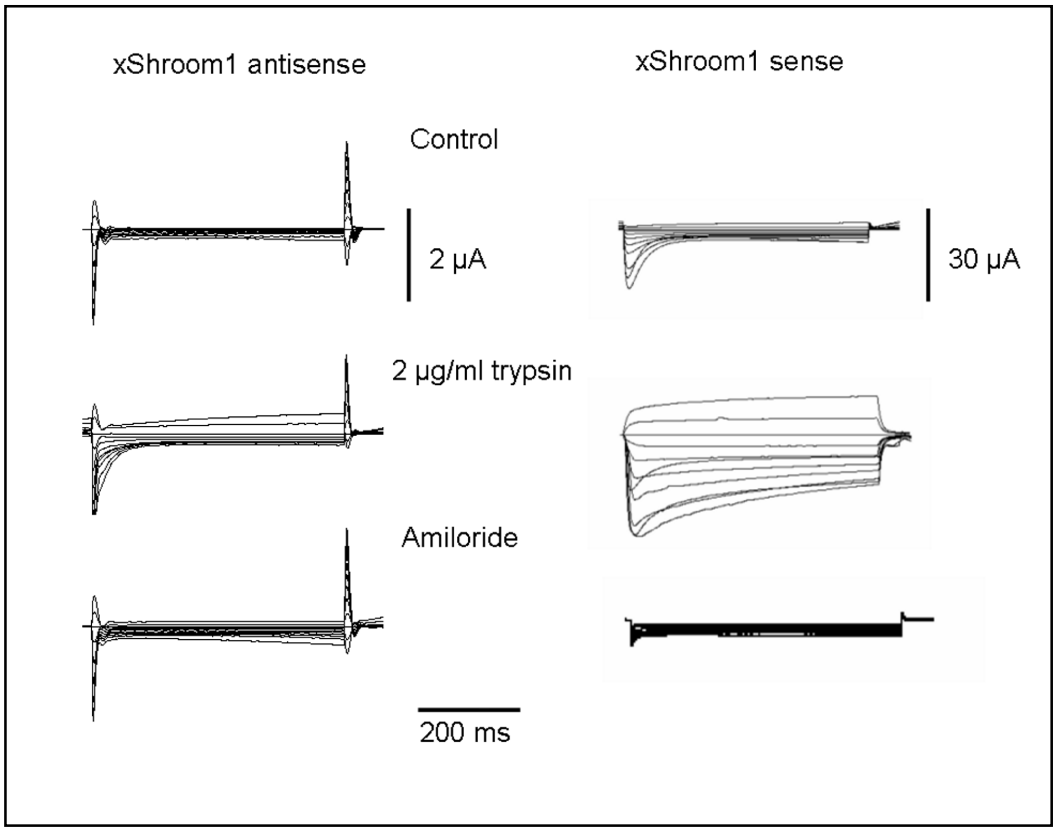

Fig. 4. Records from oocytes coinjected with the xShroom1 sense or antisense oligonucleotides in control, 2 µg/ml trypsin for 2.5 min and in 10 µM amiloride. The traces were generated by pulses from -160 to + 40 mV from a holding potential of 0 mV. Representative experiment from 9 oocytes (four donors) with xShroom1 antisense and 8 oocytes (four donors) with xShroom1 sense. Note different amplitude scales.

antisense oligonucleotides is shown at the bottom of the figure. The mean inward conductances measured between -160 to 0 mV were 36 and 1.80 µS for oocytes injected with xShroom1 sense or xShroom1 antisense respectively (Table 1).

$INa_{(amil)}$ through Δβ-ENaC

The next step was to see whether xShroom1 antisense inhibited the $INa_{(amil)}$ through effects on the number of active channels expressed in the plasma membrane or by enhancing kinetics properties such as

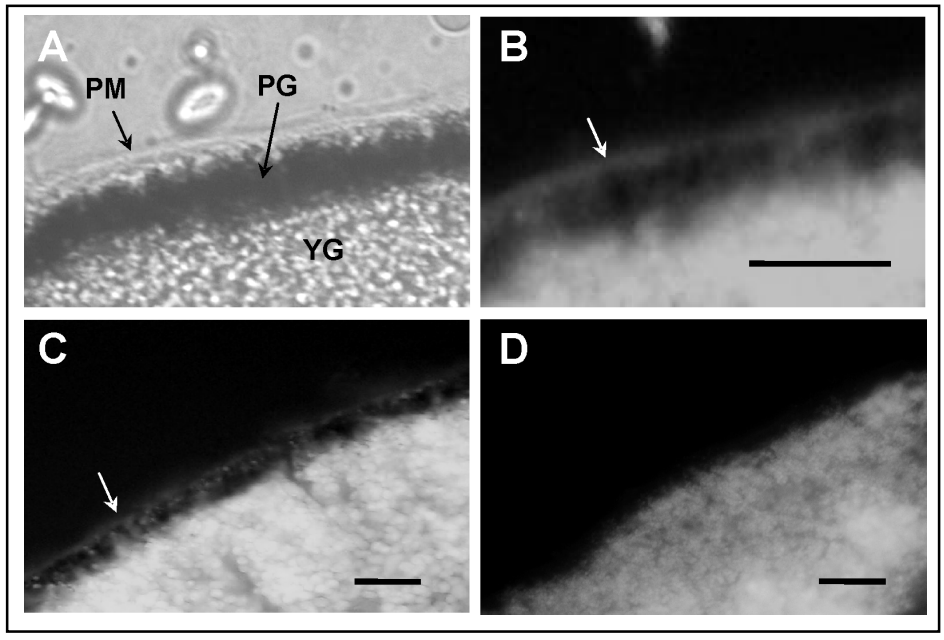

Fig. 5. Immunohistochemical localization of αENaC. (A) Representative bright-field photomicrograph of *Xenopus* oocyte thin section. Fluorescence positive staining of plasma membrane in the oocytes expressing α, β and γ mENaC alone (B) or coinjected with xShroom1 sense oligonucleotides (C) was shown. The staining is not observed in oocytes coinjected with ENaC and xShroom1 antisense oligonucleotides (D). This treatment leads to the disappearance of the exogenously expressed Na^+ channel. The data are representative of three experiments under each condition. PM: Plasma Membrane; PG: Pigmented Granules; YG: Yolk granules. Bar = 20 μm.

an increase in open probability (Po). We performed experiments in oocytes expressing a DEG mutant β mENaC (β-S518K) along with wild type α and γ mENaC subunits, which have a Po of nearly 1 (see above for references). Oocytes coinjected with either xShroom1 sense or antisense oligonucleotides were tested and the $INa_{(amil)}$ calculated. Fig. 2 shows the $INa_{(amil)}$ for oocytes with the wild type or the DEG mutant and coinjected with either xShroom1 sense or antisense oligonucleotides. As expected oocytes expressing the DEG mutant β mutant with xShroom1 sense had significantly enhanced $INa_{(amil)}$ to near twice that in controls. However, coinjection with xShroom1 antisense give similar results to those obtained with the WT ENaC. The mean inward conductances were 65 and about 2 μS for oocytes injected with xShroom1 sense or xShroom1 antisense respectively.

$INa_{(amil)}$ in oocytes treated with trypsin

Specific proteases activate ENaC channels by cleaving channel subunits at sites within their extracellular domains [18-19 for references]. Extracellularly applied trypsin in μg/ml concentration can activate near silent channels. On the other hand, Bengrine et al. [17] found that much lower concentration of trypsin in the range of ng/ml activates the membrane-resident ENaC channels, being this effect dependent on activation of G-proteins. Therefore, we examined the effects of low and high levels of trypsin on oocytes injected with xShroom1. Addition of 20 ng/ml of trypsin led to a slow increase in $INa_{(amil)}$ in oocytes injected with xShroom1 sense and this current was blocked by the addition of 10 μM amiloride (Fig. 3). On the other hand, trypsin had no effect on the currents generated by most of the oocytes coinjected with ENaC and xShroom1 antisense. In only 2 out of 16 oocytes there was an increment in current under these conditions.

High concentration of trypsin induced a greater increase in current in oocytes coinjected with ENaC and xShroom sense and this was amiloride sensitive. We clamped the oocytes to 0 mV and stimulated them with -160 to +40 mV pulses for 500 ms in the absence and presence of 2 μg/ml trypsin for 2.5 minutes. The amplitude of the increase of the $INa_{(amil)}$ was quite variable, ranging in our experiments (-100 mV pulse) from 1.14 to 9.2 fold and a mean of 2.65 (n=7). On the other hand, trypsin had no effect on the currents generated by oocytes coinjected with ENaC and xShroom1 antisense: - 0.13 ± 0.12 μA with a -100 mV pulse, (n= 9, four frogs). Examples of these experiments are in Fig. 4. These data are consistent with the idea that the absence of $INa_{(amil)}$ in oocytes with blocked expression of xShroom1 most probably is due to a lack of functional ENaC channels in the plasma membrane. Low levels of trypsin were without effects on the oocyte endogenous conductances (n= 22) whereas 2 μg/ml of trypsin induced a transitory increment in the endogenous conductance in some oocytes as it was demonstrated by Duriex et al., 1994 [20].

Immunohistochemical localization of α ENaC

For the fluorescence examination of ENaC expression, we injected the three subunits of ENaC with xShroom1 sense or antisense into the *Xenopus* oocytes

and measured the fluorescence signal of the α protein at the plasma membrane using an inverted fluorescence microscope. Oocytes incubated with xShroom1 sense exhibit a fluorescence positive staining of plasma membrane and this was not observed in oocytes co injected with ENaC and xShroom1 antisense oligonucleotides (Fig. 5, n=3).

Discussion

In this work we analyze the regulation of ENaC by xShroom1 protein in oocytes measuring $INa_{(amil)}$ in oocytes coinjected with either xShroom1 sense or antisense oligonucleotides. Although $Ina_{(amil)}$ represents the overall channel activity expressed at the cell membrane it is a small fraction, less than 1 % of the total amount of subunits expressed in these cells [21]. Zuckerman et al. [14] demonstrated the necessity for xShroom1 protein to generate $INa_{(amil)}$ in oocytes. Here we extend these results by studying the effect of blocking xShroom1 in a DEG mutant β mENaC (β-S518K) along with wild type α and γ mENaC subunits, which has an open probability (Po) of nearly 1, and the stimulatory effect of trypsin upon the $INa_{(amil)}$ [18-19].

A number of diverse mechanisms including the regulation of channel synthesis, intracellular channel trafficking, membrane insertion and retrieval, and open probability (Po) could be the main factors in controlling ENaC activity by xShroom1 [21, 22-24]. One possibility is that xShroom1 increases Po. Proteases such as furin and prostasin increase ENaC Po by converting immature channels with low activity into mature highly active channels through proteolytic cleavage of ENaC subunits [21]. The DEG mutant β mENaC (αβ-S518Kγ-ENaC has been shown to increase the Po of ENaC to nearly 1 [15-16]. We found that the coinjection of this mutant with the xShroom1 antisense reduced the $INa_{(amil)}$ to the same extent that with the wild type of channels. Thus, the regulatory effect of xShroom1 upon $INa_{(amil)}$ is most probably not due to an increase in Po but to other factor, i.e an increase in the number of channels inserted in the plasma membrane. The possibility that xShroom1 is unexpectedly affecting the conductance properties of the ENaC channels is unlikely since subunit composition, but not subunit ratio, is a determinant of ENaC properties [25-27]. Single channels studies are necessary to exclude this possibility.

The possibility that xShroom1 promotes in some way the expression of functionally active ENaC channels in the membrane is supported by the results obtained in oocytes treated with trypsin and the results of the immunohistochemical localization of αENaC. Trypsin activates the $INa_{(amil)}$ in oocytes coinjected with ENaC and xShroom1 sense but did not affect the currents in oocytes coinjected with ENaC and xShroom1 antisense. On the other hand in the oocyte expressing ENaC and xShroom1 sense we observed a positive staining of plasma membrane whereas this was not the case in oocytes coinjected with ENaC and xShroom1 antisense. The fluorescence measurements of αENaC expression confirm and extend our electrophysiological data and show that expression and subsequent ENaC protein density is influenced by the treatment with either xShroom1 sense or antisense oligonucleotides. We used the same oligonucleotides published by Zuckerman et al. [14] and Staub et al. [28]. The latter authors found that more than 90 % of xShroom1 mRNA was degraded by endogenous RNase H in the presence of antisense but not sense oligonucleotides. As far as the analysis of whole cell ENaC protein expression is concerned, we had the problem of yolk interference in the immunoblots when preparing protein extracts of *Xenopus oocytes*. Although we tested different protocols in order to reduce this interference we were unable of obtain successful blots.

The published reports about the role played by Shroom proteins as transporters of Na^+ generated conflictive results. xShroom1 was initially identified as a protein participating in amiloride sensitive Na channels [28] and later on as member of a family of proteins that binds the actin cytoskeleton and controls its organization [12-13]. On one hand Prat et al. [28] proposed that xShroom1 (APX) is by itself an actin cytoskeleton-regulated Na^+ channel because its transfection in melanoma cells deprived of actin resulted in an increment in amiloride-sensitive Na^+ currents. In the other hand, works by Staub et al. [28] and Zuckerman et al. [14] indicated that xShroom1 was not a channel but it is required for the expression of $INa_{(amil)}$. Zuckerman et al. [14] proposed that in A6 epithelial cells ENaC occurs in a macromolecular complex with xShroom1 and α-spectrin stabilizing the channels.

Cell migration has an important role in many physiological processes, such as implantation and embryogenesis, immunity and inflammation, tissue regeneration, angiogenesis and metastasis [29-31] and depends on many factors, among them, in the cytoskeleton regulation and ionic activity. Interactions between ion channels and actin or actin-binding/

scaffolding proteins play a role in the regulation of channel activity and in their intracellular trafficking. Na^+ currents mediated by ENaC and other channels are involved in cell migration, well documented in epithelial and vascular cells [31-32] and in the proliferation of tumor cells [33-37]. Perhaps the close association between ENaC and Shroom proteins has an important role in this process [38].

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