The Journal of Membrane Biology (2022) 255:61–69
https://doi.org/10.1007/s00232-021-00212-y

The Effect of Dynasore Upon the Negative Interaction Between ENaC and CFTR Channels in *Xenopus laevis* Oocytes

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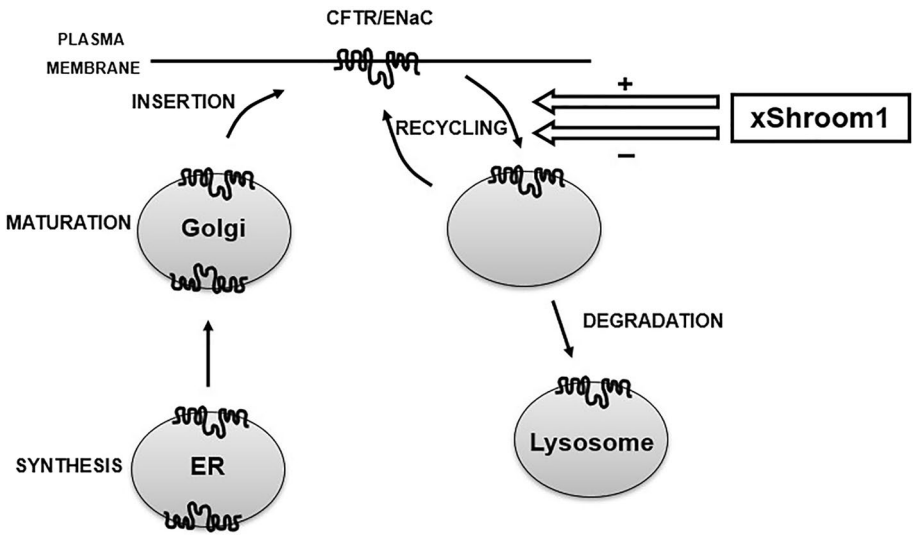
Received: 17 October 2021 / Accepted: 22 December 2021 / Published online: 21 January 2022

Abstract

Shroom is a family of related proteins linked to the actin cytoskeleton, and one of them, xShroom1, is constitutively expressed in *Xenopus laevis* oocytes which is required for the expression of the epithelial sodium channel (ENaC). On the other hand, ENaC and the cystic fibrosis transmembrane regulator (CFTR) are co-expressed in many types of cells with a negative or positive interaction depending on the studied tissues. Here, we measured the amiloride-sensitive ENaC currents (INa_{amil}) and CFTR currents (I_{CFTR}) with voltage clamp techniques in oocytes co-injected with ENaC and/or CFTR and xShroom1 antisense oligonucleotides. The objective was to study the mechanism of regulation of ENaC by CFTR when xShroom1 was suppressed and the endocytic traffic of CFTR was blocked. CFTR activation had a measurable negative effect on ENaC and this activation resulted in a greater inhibition of INa_{amil} than with xShroom1 antisense alone. Our results with Dynasore, a drug that acts as an inhibitor of endocytic pathways, suggest that the changes in INa_{amil} by xShroom1 downregulation were probably due to an increment in channel endocytosis. An opposite effect was observed when I_{CFTR} was measured. Thus, when xShroom1 was downregulated, the I_{CFTR} was larger than in the control experiments and this effect is not observed with Dynasore. A speculative explanation could be that xShroom1 exerts a dual effect on the endocytic traffic of ENaC and CFTR and these actions were canceled with Dynasore. In the presence of Dynasore, no difference in either INa_{amil} or I_{CFTR} was observed when xShroom1 was downregulated.

Graphic Abstract



Keywords Oocytes · ENaC · CFTR · Dynasore

Extended author information available on the last page of the article

Introduction

Two main pieces of information are associated with the objective of this investigation: the regulation of function and the interaction of the epithelial sodium channel (ENaC) with the cystic fibrosis transmembrane regulator (CFTR) channel. ENaC is a member of the ENaC/degenerin ion channel family composed of three homologous subunits (α, β, and γ). It mediates entry of Na^+ from the luminal fluid into the cells in many reabsorbing epithelia, it is blocked by the diuretic amiloride, it is sensitive to many hormones such as aldosterone, cytosolic, and extracellular pH (Collier and Snyder 2009; Kashlan et al. 2015; Reddy et al. 2008), and it is activated by proteases which cleave specific sites in the extracellular loops of the α, γ subunits but not the β subunit (Gentzsch et al. 2010; Kashlan et al. 2011; Zachar et al. 2015). Shroom is a family of four different proteins (Shroom1 to Shroom4) involved in the regulation of cytoskeletal architecture by binding to actin, morphogenesis of embryonic epithelial tissues, and neuronal growth (Hagens et al. 2006; Hildebrand et al. 2021). Of particular interest for us is xShroom1 (APX), a large protein constitutively expressed in *Xenopus laevis* oocytes and initially identified as a molecule required in ENaC activity in *X. laevis* epithelial cells (Staub et al. 1992). It has been well described that Shroom family proteins influence both microtubules and actin cytoskeletons. xShroom1 is associated with α-spectrin, a cytoskeletal protein known to shape the plasma membranes of cells (Zuckerman et al. 1999), and ectopic expression of xShroom1 causes accumulation of γ-tubulin, a microtubule nucleating protein, at the apical surface of epithelial cells (Lee et al. 2007). In addition, Shroom family genes are expressed in many thickened epithelial sheets. xShroom1 and xShroom2 are expressed in the deep layer of neuroepithelium and control apicobasal cell elongation (Lee et al. 2009). It has been shown that xShroom1 has a dual action on the expression and level of activity of ENaC and CFTR. Suppression of xShroom1 resulted in a decrement of ENaC function (Assef et al. 2011; Prat et al. 1996; Zuckerman et al. 1999), whereas the opposite was found on CFTR in *X. laevis* (Palma et al. 2016).

Second, ENaC and CFTR are co-expressed at the apical surface of epithelia and other tissues. CFTR is a cAMP activated, ATP-dependent Cl^- channel, which transports Cl^- and also HCO_3^- ions from the intracellular to luminal space in several tissues. In addition to these functions, both ENaC and CFTR channels are involved in cell migration and proliferation (Liao et al. 2018; del Mónaco et al. 2009; Marino and Kotsias 2014; Schiller et al. 2010; Sun et al. 2011). CFTR is also a regulator of other channels, and the modulation of ENaC function serves as a prime example of the regulatory function of CFTR in tissues. It is also an extracellular chloride sensor (Broadbent et al. 2015). The functional positive or negative interplay between ENaC and CFTR is complex and incompletely understood (see "Discussion").

In a past publication (Palma et al. 2016), we reported an increment in CFTR currents and CFTR cell-surface expression in oocytes co-injected with xShroom1 antisense oligonucleotides, and we suggested a number of factors controlling the expression or activity of CFTR including membrane insertion, degradation, channel synthesis, intracellular channel trafficking, and open probability. In this investigation, we further pursued these experiments using oligonucleotides against xShroom1 and Dynasore to block the dynamin-dependent endocytosis in oocytes expressing the wild-type mouse ENaC and human CFTR. Our results suggest that xShroom1 downregulation decreases CFTR endocytosis, and in this way, CFTR caused a greater inhibition of the amiloride-sensitive Na^+ currents (INa_{amil}) than would be predicted by the downregulation of xShroom1 alone. In addition, our results also confirm that heterologous expression in *X. laevis* oocytes is a suitable system for the study of this interaction.

Material and Methods

Xenopus laevis Oocytes

Adult female *Xenopus laevis* frogs were anesthetized with 0.3% tricaine (MS-222), and the oocytes were surgically removed from the abdominal incision. Oocytes were defolliculated by incubation with 1 mg/ml type IV collagenase for 40 min. The oocytes were placed in ND96 medium containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, and HEPES 5 (pH 7.4) supplemented with 1 μg/ml gentamicin. We synthesized complementary RNAs (cRNAs) for human wild-type CFTR using the T7 mMessagemMachine kit (Ambion, Austin, TX), and for α, β, and γ mouse wild-type ENaC subunits using the T3 mMessage mMachine kit (Ambion, Austin, TX). We used synthetic oligodeoxynucleotides complementary to nucleotides + 455 to + 479 of xShroom1 (Zuckerman et al. 1999) (sense, 5′-GCA TTA AGC AGA ATC GCC CTA ACC AC-3′; antisense, 5′-GTG GTT AGG GCG ATT CTG CTT ATG C-3′, Integrated DNA Technologies, Biodynamics SRL). Oocytes were injected with a Drummond injector (Drummond, Broomall, PA) with 4 ng of CFTR cRNA, 2 ng of α, β, and γ ENaC cRNA and/or 25 ng of xShroom1 sense or antisense oligonucleotides (total volume 50 nl).

Reagents

The reagents used were amiloride 10 µM (Alomone Labs, Jerusalem, Israel), forskolin 10 µM (Alomone Labs, Jerusalem, Israel), IBMX 1 mM (Sigma-Aldrich, St. Louis, USA), and dynasore 80 µM (Sigma-Aldrich, St. Louis, USA).

Electrophysiology

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. Micropipettes had resistances of 1–3 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 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 (0.6 ml/min) with a peristaltic pump (Dynamax RP-1; Rainin Instruments, 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 min before starting the experiment. We ran two sets of records with a delay of 5 min to be sure that the currents were stable. Then we applied amiloride and we recorded the currents at 3 and 5 min, enough time to have a stable blocking effect. For activation of CFTR, we applied 10 µM of forskolin + 1 mM of IBMX, and the currents were recorded at 15 min of incubation, enough time to have a stable channel activation effect. For the current–voltage (I–V) relationships, we applied a series of 500 ms voltage steps from − 140 to + 60 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. CFTR-mediated Cl^- currents were defined as the current difference measured in the absence versus the presence of forskolin + IBMX in the bath solution (Kunzelmann 2011; Qadri et al. 2011).

Statistical Analysis

Data were expressed as mean values ± standard error (SE) (n = number of cells and repetitions). Statistical analysis for differences between experimental groups was performed using Graphpad Prism software, applying unpaired Student's t test. Differences were considered statistically significant when $p < 0.05$.

Results

Basic ENaC Currents in Oocytes

The first experiments were done to determine the expression of ENaC currents in *X. laevis* oocytes as the basis for subsequent experiments. We recorded ENaC currents in oocytes co-injected with human CFTR cRNAs and mouse ENaC cRNAs and the results are shown in Fig. 1 when ENaC was inhibited with amiloride. Under these conditions, only ENaC currents are present because CFTR is inactive (Bachhuber et al. 2005; Drumm et al. 1991, see

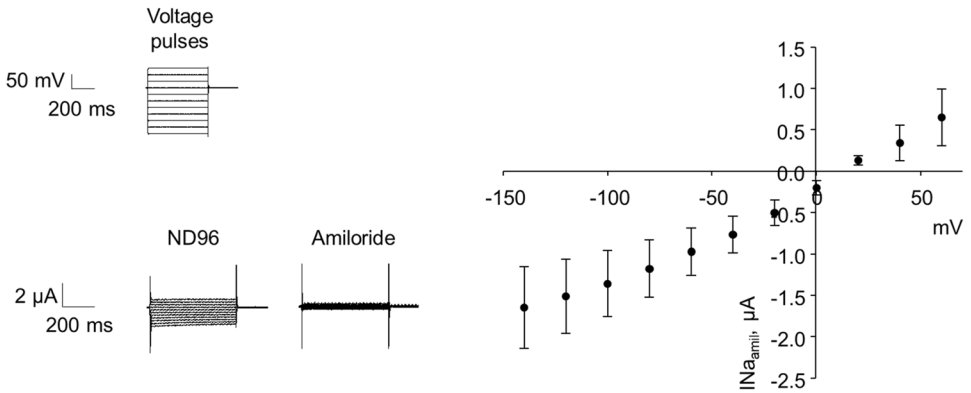


Fig. 1 Left panel. Records of ENaC currents in oocytes co-injected with human CFTR cRNAs and mouse ENaC cRNAs and the inhibition of these currents when 10 µM amiloride was added to the bath. The potential of the cell was held at 0 mV and switched to values of between − 160 and + 40 mV for 500 ms. Under our experimental conditions, only ENaC currents are present because CFTR is inactive. The right panel shows the I-V plot with the average results of INa_{amil} obtained from oocytes after subtracting the current remnant in amiloride from the control values

below). The left panel shows the currents in response to negative or positive pulses in the control solution (ND96) and after the inhibition with amiloride. After the subtraction of the blocked component, we obtained amiloride-sensitive sodium currents (INa_{amil}) and the average results obtained from 8 experiments are shown in the right panel with the I–V curves. With a pulse of − 100 mV amiloride significantly reduced the currents from − 1.96 ± 0.44 to − 0.60 ± 0.23 µA ($p < 0.05$, $n = 8$).

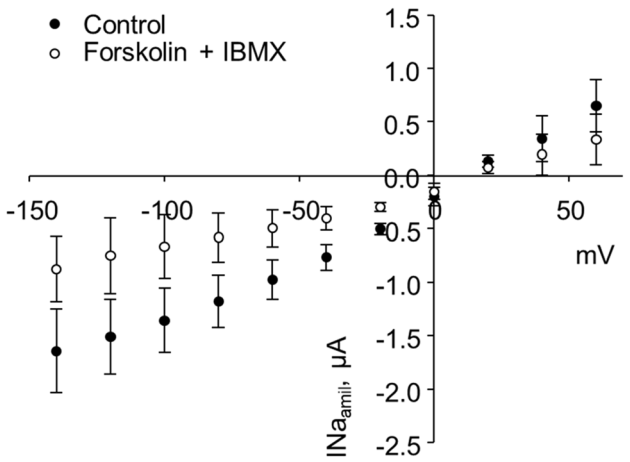


Fig. 2 I-V plot showing the reduction in INa_{amil} when the CFTR channels were activated. In these experiments, the CFTR and ENaC co-injected oocytes were incubated in 10 µM of forskolin and 1 mM of IBMX to stimulate the CFTR channels. The INa_{amil} was obtained with the same protocol as in Fig. 1

Activation of CFTR Inhibits Amiloride-sensitive Sodium Currents

The next experiments were done to evaluate INa_{amil} when the CFTR channels were active. To do so, we incubated CFTR and ENaC co-injected oocytes with 10 µM of forskolin and 1 mM of IBMX, both drugs known to stimulate CFTR (Bachhuber et al. 2005; Drumm et al. 1991), and the results are shown in Fig. 2. As it can be seen, the activation of CFTR clearly diminished the INa_{amil} obtained with the same protocol as in Fig. 1. With a − 100 mV pulse, the INa_{amil} was − 1.38 ± 0.40 µA vs. − 0.67 ± 0.40 µA ($n = 8$, $p < 0.01$).

xShroom1 Downregulation Enhances Amiloride-Sensitive Sodium Current Inhibition by CFTR

To determine if xShroom1 protein is involved in the CFTR and ENaC regulation, we studied oocytes expressing both channels and co-injected with xShroom1 antisense oligonucleotides. In Fig. 3a, the I–V curves show that xShroom1 downregulation reduced the INa_{amil} in every pulse applied. Thus, the INa_{amil} with a − 100 mV pulse was about a quarter (− 0.26 ± 0.11 µA, $n = 5$) with respect to control oocytes co-injected with xShroom1 sense oligonucleotides (− 1.97 ± 0.62 µA, $n = 6$, $p < 0.05$).

Figure 3b shows the I-V curve of INa_{amil} in oocytes injected with xShroom1 antisense and incubated with forskolin and IBMX to induce CFTR activity. The incubation was for 15 min, enough time to stably activate the channel (Palma et al. 2016). Under these conditions, i.e., downregulation of CFTR by xShroom1, we observed an additional reduction in amiloride-sensitive ENaC current beyond that observed with xShroom1 alone (100 mV pulse:

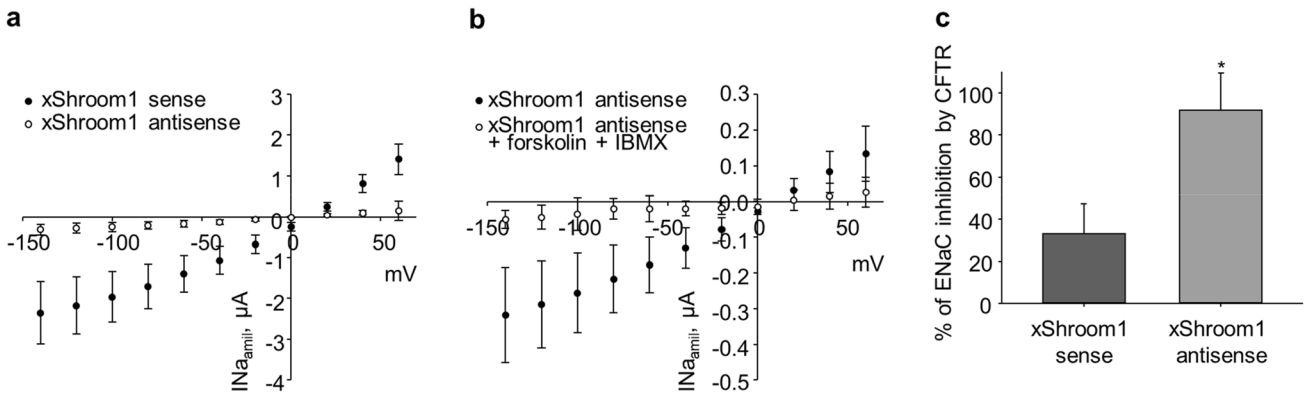


Fig. 3 Panels a and b show the INa_{amil} in oocytes expressing ENaC and CFTR. **a** I-V curve with the average values when xShroom1 was downregulated with antisense oligonucleotides in comparison with the control ones (sense). **b** INa_{amil} when CFTR was activated with Forskolin plus IBMX in oocytes with xShroom1 downregulated. **c** Comparison in the reduction of INa_{amil} (pulse − 100 mV) when CFTR was activated in oocytes injected with sense or antisense oligonucleotides against xShroom1. It is evident that the INa_{amil} inhibition by activation of CFTR was higher when xShroom1 was downregulated

-0.07 ± 0.05 µA, $n = 8$, $p < 0.05$). Figure 3c shows a summary of the inhibition of INa_{amil} (− 100 mV pulse) by activation of CFTR in oocytes injected with sense or antisense oligonucleotides against xShroom1.

xShroom1 Downregulation Effects Upon INa_{amil} and I_{CFTR} in the Presence of Dynasore

The next experiments were done to compare the effect of downregulating xShroom1 on the INa_{amil} and I_{CFTR} and also to see the effect of blocking the endocytic traffic of these channels by means of Dynasore. I_{CFTR} was recorded in oocytes injected with antisense oligonucleotides for xShroom1 and incubated for 24 h in the absence or presence of 80 µM of Dynasore, a concentration enough to inhibit dynamin, a protein necessary for the formation of clathrin-coated vesicles and used in endocytic-trafficking studies of ion channels (Pergel et al. 2021; Wesch et al. 2012; Young et al. 2009). The left panels of Fig. 4a and b show the effect of downregulation of xShroom1 on the I_{CFTR} and INa_{amil} (− 100 mV pulse) in the absence of Dynasore. When xShroom1 was downregulated, the currents through IBMX/forskolin-activated CFTR were increased by a factor of 5 over the control. Regarding the effect of xShroom1 on ENaC, the comparison between Fig. 4a and b (Control) shows a dual action of xShroom1 on the level of activity of ENaC and CFTR. Suppression of xShroom1 resulted in an increment in CFTR function whereas the opposite was found for ENaC, although this negative effect of xShroom1 antisense on ENaC was not observed in the presence of Dynasore. In addition, our results also confirm that heterologous expression in *X. laevis* oocytes is a suitable system for the study of this interaction.

Dynasore Effect of CFTR Activation on the INa_{amil}

Next, we measured the effect of Dynasore upon the reduction in INa_{amil} once CFTR was activated. Figure 5a, b shows that the reduction in INa_{amil} when CFTR was activated was not changed with the xShroom1 antisense. Thus, the negative effect of CFTR activation on the INa_{amil} is not dependent on xShroom1.

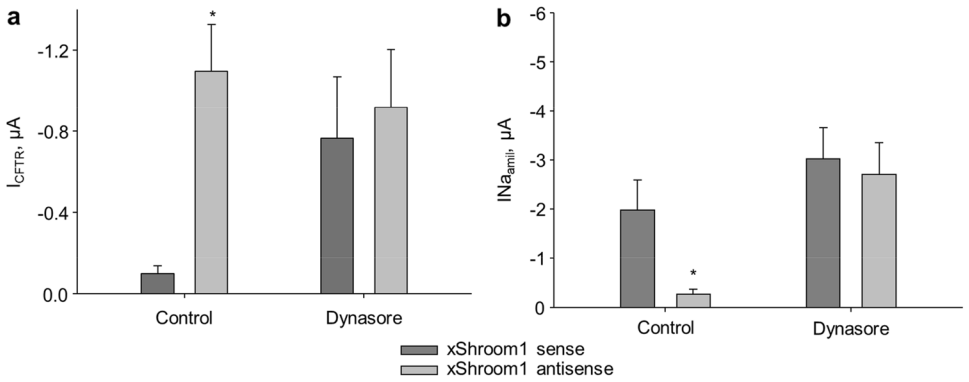


Fig. 4 I_{CFTR} and INa_{amil} in oocytes injected with xShroom1 antisense or sense oligonucleotides in the absence (control) and presence of 80 µM Dynasore for 24 h. When xShroom1 was downregulated, the I_{CFTR} was increased by a factor of 5 over the control and the opposite result was obtained in INa_{amil} (left panels). In the presence of Dynasore, no difference in these currents was recorded in oocytes with xShroom1 downregulated (right panels)

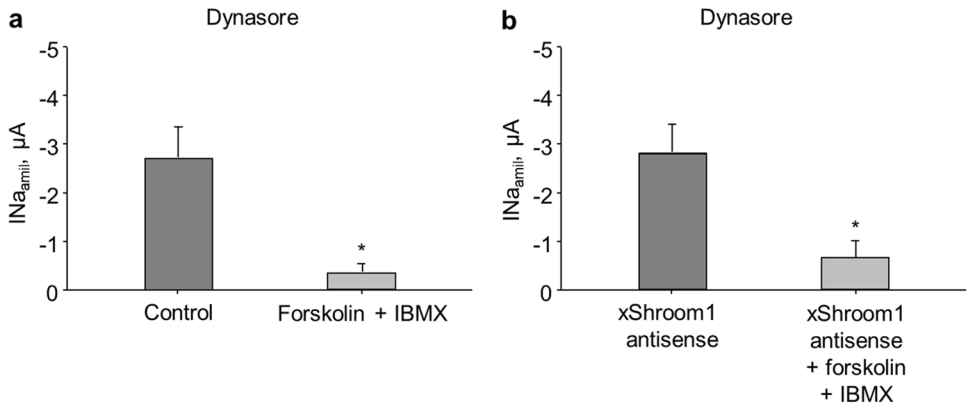


Fig. 5 In these sets of panels, the INa_{amil} (− 100 mV pulse) was recorded when CFTR was activated in the presence of Dynasore in oocytes with xShroom1 expressed (**a**) and when it was downregulated with antisense oligonucleotides (**b**). In both conditions, the activation of CFTR with forskolin plus IBMX reduced the INa_{amil} (see Fig. 3c for comparison)

Discussion

In this work, we analyzed the regulation of the epithelial sodium channel (ENaC) by proteins of the Shroom family involved in the cytoskeletal function (see "Introduction") and the interaction of ENaC with the cystic fibrosis transmembrane regulator (CFTR) channel. First, we will discuss the role of xShroom1 protein on the activity of ENaC and CFTR, second, the interaction between these two proteins, then the role of endocytic trafficking in the mentioned effects and finally the comparison between different species of these channels.

It is evident from our previous results (Assef et al. 2011; Palma et al. 2014, 2016) and the comparison between Fig. 4a and b (Control) that there is a dual action of xShroom1 on the level of activity of ENaC and CFTR in oocytes from *X. laevis*. Much to our surprise, suppression of xShroom1 resulted in an increment in CFTR function whereas the opposite was found for ENaC. Many of the functions of ENaC and CFTR are through interactions with actin, actin-binding proteins, or scaffolding proteins (Karpushev et al. 2010; Santos et al. 2020). Thus, several ENaC-regulatory proteins function within a multiprotein complex which controls the channel expression and activity. This is the case of CNK3, a scaffold protein which has a PDZ domain and serves as a stimulatory factor for ENaC (Soundararajan et al. 2012). On the other hand, Boucherot et al. (2001) found that a CFTR mutant, which lacks the last six amino acids encoding the PDZ-binding domain, resulted in a larger current than wild-type CFTR.

CFTR activation had a measurable negative effect on ENaC as it is shown in Fig. 3, and this activation resulted in a greater inhibition of INa_{amil} than the one obtained with the xShroom1 antisense alone. The interplay between CFTR and ENaC is complex and incompletely understood. Tissues and species differences may account for the discrepant findings reported in the literature. For example, activation of ENaC requires CFTR function in sweat ducts (Reddy et al. 1999; Reddy and Quinton 2005) and in human alveolar type II cell (Bove et al. 2010), whereas Na^+ absorption is elevated in defective airways in cystic fibrosis (see Collawn et al. 2012; Strandvik 2021, for references). There are reports showing that CFTR activation by cAMP caused an inhibition of INa_{amil} in oocytes co-expressing rat α, β, and γ ENaC and CFTR (Bachhuber et al. 2005; Briel et al. 1998; Chabot et al. 1999), and similar results were obtained with mouse α,β,γ ENaC co-expressed with CFTR (Yan et al. 2004) but not in human α,β,γ ENaC when co-expressed with CFTR (Nagel et al. 2005; Yan et al. 2004; see references in Rauh et al. 2017). In addition, we showed that the inhibition of ENaC by the activation of CFTR is greater when xShroom1 is blocked with the antisense oligonucleotides, and this is also in agreement with the results of Boucherot et al. (2001), showing that inhibition of ENaC was linked to Cl^- currents generated by CFTR and was observed in the presence of Cl^-, I^-, or Br^- but not gluconate, although Suaud et al. (2007) found that chloride transport is not necessary for inhibition of ENaC.

Third, we will discuss the effects of Dynasore upon the interplay between ENaC and CFTR. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin, essential for clathrin-dependent coated vesicle formation, by rapidly blocking coated vesicle formation (Macia et al. 2006). It has been previously used (at the same concentration as in our experiments) to probe the role of dynamin in the endocytic trafficking of CFTR and ENaC by Young et al. and Wesch et al. with similar results. Our experiments support the idea that CFTR and ENaC undergo endocytosis, at least in part, through the classically described dynamin-dependent, clathrin-mediated endocytosis. The results presented in Figs. 4 and 5 with Dynasore suggest that the changes in INa_{amil} by xShroom1 downregulation were probably due to an increment in the endocytosis of the channels. In other words, xShroom1 impairs in some manner the endocytic traffic of ENaC, and this effect is antagonized with Dynasore. Unexpectedly, an opposite effect was observed when I_{CFTR} was measured. Thus, when xShroom1 was downregulated, the I_{CFTR} was larger than in the control experiments and this effect is not observed with Dynasore (Fig. 4a).

Finally, the use in the present study of two channels from different species to analyze the interaction between them will be discussed.

Although there is a possibility that the relationship between CFTR and ENaC channels from same species may be different to the found in our investigation, it has been well described that αβγ-ENaC subunits are highly conserved between different species, including rat and human ENaC (Hanukoglu and Hanukoglu 2017; Voilley et al. 1994). Additionally, β and γ rat ENaC share sequence identity to human ENaC in the proline-rich P2 regions (Staub et al. 1996), which have been shown to bind the E3 ubiquitin ligases Nedd4 suppressing ENaC activity by decreasing its cell-surface stability (Lu et al. 2007).

Moreover, the literature shows that mouse ENaC has several characteristics in common with human ENaC that could suggest that the relationship between human CFTR and mouse ENaC could be the same as the relationship between human CFTR and human ENaC.

In previous studies, it was reported that the relationship between CFTR and ENaC is inhibitory in human cells, as the results presented here. In human primary culture from airways, it was shown that CFTR impedes the proteolytic processing of ENaC, regulating the channel negatively

(Gentzsch et al. 2010). Besides, Mall et al. (1999) found that the amiloride-sensitive sodium currents are inhibited by CFTR activation in normal human colon biopsies but not in tissue biopsies from cystic fibrosis patients.

In addition, both human and mouse ENaC are endocyted from the plasma membrane through clathrin-mediated endocytosis; thus, we would expect the same results from human and mouse ENaC. Evidence from oocytes studies demonstrate that the process is dynamin dependent, consistent with a role for clathrin-mediated endocytosis. With the use of two different tools, Pitstop-2, an inhibitor of the clathrin-mediated endocytosis or mutating clathrin adaptor protein 2 (AP-2) recognition motifs in the C-termini of β- and/or γ-ENaC, it was shown that ENaC endocytosis is clathrin mediated in *X. laevis* oocytes injected with human ENaC cRNA (Ilyaskin et al. 2021). Moreover, Shimkets et al. (1997) found that rat ENaC channels are also removed from the plasma membrane through clathrin-mediated endocytosis in *X. laevis* oocytes. Wang et al. (2006) demonstrated that ENaC is present in clathrin-coated vesicles in mouse mpkCCDc14 cells and is efficiently endocytosed. They also showed in *X. laevis* oocytes that the co-expression of mouse ENaC and epsin, a clathrin adaptor protein, resulted in the downregulation of the channel activity.

Furthermore, several studies demonstrated that the endocytosis inhibitor used in the present study, Dynasore, blocked both human and mouse ENaC endocytosis, as would be expected for a channel whose surface expression is regulated by clathrin-mediated endocytosis. ENaC endocytosis was inhibited with dynasore in *X. laevis* oocytes injected with rat ENaC cRNAs and in mouse M1 cortical-collecting duct cells (Almaça et al. 2009). In addition, Wesch et al. (2012) showed a significant increase of amiloride-sensitive sodium current due to blocked endocytosis of the channel in the presence of dynasore, in *X. laevis* oocytes injected with human ENaC cRNAs.

The number of channels at the cell surface is determined by the balance between insertion of new channels into the plasma membrane and the endocytosis and degradation of channels from the membrane, whereas other additional factors influence the amount, stability, half-life, and activity of CFTR and ENaC at the surface membrane (see Butterworth 2010; Farinha et al. 2013 for references). In this context, a speculative explanation could be that xShroom1 exerts a dual effect on the endocytic traffic of ENaC and CFTR, a negative action upon ENaC and a positive one with CFTR, and both of these actions were canceled with Dynasore. In its presence no difference in either INa_{amil} or I_{CFTR} was observed when xShroom1 was downregulated.

For the maintenance of cellular homeostasis a coordinated interaction between ENaC and CFTR is necessary. The importance of a correct balance of these channels' activity is demonstrated in several pathologies. Therefore, it is essential to understand the mechanism of regulation of ENaC by CFTR. Our data show an interaction between CFTR and ENaC and suggest that xShroom1 regulates both channels, indicating that xShroom1 could have a role in the channels' deregulation in several pathologies.

Acknowledgements Many thanks to Dr CM Fuller from the University of Alabama at Birmingham, AL and GI Marino and L Galizia from the University of Buenos Aires for their help.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by AGP. The first draft of the manuscript was written by BAK, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PRESTAMO BID PICT 2010-1861).

Declarations

Conflicts of interest Authors declare no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Availability of Data and Material Not applicable.

Code Availability Not applicable.

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