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The F508del-CFTR trafficking correctors elexacaftor and tezacaftor are CFTR-independent Ca^{2+} -mobilizing agonists normalizing abnormal Ca^{2+} levels in human airway epithelial cells

Manuella Lévêque¹, Sandra Mirval¹, Christine Barrault¹, Isabelle Fixe¹, Christelle Coraux², Edouard Sage^{3,4}, Frédéric Becq¹ and Clarisse Vandebrouck^{1*}

Abstract

Background Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) channel. For people with CF (pwCF) affected by the most common pathogenic variant F508del, a tritherapy, named Trikafta/Kaftrio (ETI: elexacaftor (VX-445) /tezacaftor (VX-661) / ivacaftor (VX-770)) was successfully developed. However, in CF airway epithelial cells the calcium homeostasis is also disturbed; it is observed an increased calcium mobilization in CF cells compared to non-CF cells. Here, we studied the effects of ETI on intracellular calcium levels in F508del-CFTR airway epithelial cells to determine whether these compounds, individually or collectively, could normalize intracellular calcium levels.

Methods We measured intracellular calcium variations using human airway epithelial cells (hAEC) from pwCF, human bronchial epithelial CFBE41o- F508del-CFTR cells and Chinese Hamster Ovary (CHO) cells using the fluorescent probe Fluo4-AM, in the presence or absence of extracellular calcium. The rescue to the plasma membrane of F508del-CFTR protein by ETI was determined by western blot. The SarcoEndoplasmic Reticulum Calcium ATPase (SERCA), was also analysed by western blotting and by interference assay.

Results We show that ETI normalizes calcium homeostasis in our cellular models. However, we also found that (1) each ETI-corrector compound is capable of mobilizing calcium acutely in the absence of CFTR, and (2) tezacaftor mobilizes calcium from the endoplasmic reticulum (ER) probably via inhibition of the SERCA pump.

Conclusions We show that ETI not only corrects the abnormal trafficking and function of F508del-CFTR but also normalizes calcium homeostasis in our cellular models. Finally, we identified SERCA as a potential intracellular target for tezacaftor.

Keywords Cystic fibrosis, ETI, Calcium, SERCA, CFTR, Endoplasmic reticulum

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Introduction

Cystic fibrosis (CF), one of the most common, lethal and autosomal recessive diseases, is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) channel. CFTR is a plasma membrane protein that ensures the transport of chloride and bicarbonate across epithelia, thereby playing a pivotal role in transepithelial fluid secretion. Epithelial cells expressing CFTR pathological variants are characterized by abnormal ion transport across their apical plasma membrane leading to severe pulmonary and digestive impairments [1]. If more than 2000 diverse variants are described regarding CFTR [2], the most common pathogenic variant is a deletion of phenylalanine at position 508 (F508del), a class-2 variant presents in more than 80% of people with CF (pwCF) [3, 4]. Mainly, F508del-CFTR is inefficiently matured, and its plasma membrane expression is reduced [5–7] but F508del-CFTR also exhibits a gating defect, reduced stability at the plasma membrane and thermal instability at physiological temperature [8–12].

Recently, a tritherapy, named Trikafta® (Kaftrio® in EU) was made available for pwCF carrying the F508del-CFTR mutation. The triple combination therapy is composed of two folding correctors elexacaftor/tezacaftor (VX-445/VX-661) and the gating potentiator ivacaftor (VX-770) (hereafter noted ETI). Briefly, the two correctors act on protein maturation/folding inducing the rescue of the protein to the plasma membrane where it is functional and the potentiator acts to increase channel function [13, 14]. This therapy is currently the leading therapeutic for CF and has demonstrated a real clinical benefit for pwCF and is also effective on rare misfolded CFTR mutants [15–17]. Nevertheless, some pwCF present side effects affecting their life quality and leading in some cases to treatment discontinuation [18].

Previously, a non-intuitive consequence of CFTR mutation on the deregulation of Ca^{2+} homeostasis has been described in several epithelial cell lines [19, 20] and human CF airway epithelial cells [21]. This deregulation can be due to either the persistent infection in CF cells [21–23], or to the mutated CFTR itself [24]. These deregulations, related to the mutated CFTR localization, implicate other proteins as ion channels and calcium transporters at the plasma membrane (TRPC, Orai1, PMCA [20, 25–29]) or the ER membrane (IP3R, SERCA [20, 24, 27, 30–32]). In addition, mitochondria Ca^{2+} buffering activities were also shown to be deregulated in CF epithelial cell lines [19, 33, 34]. Finally, modulation of the intracellular Ca^{2+} concentration was also shown to down-regulate the CFTR mRNA level [35], to involve changes in immune, respiratory or antimicrobial responses [36, 37] or to be involved in CFTR internalization [38].

Finely tuned Ca^{2+} signalling is recognized as a key regulator of normal cell functions, and deregulation of this pathway leads to several pathological processes [30, 31]. Such abnormal Ca^{2+} profile has been shown to impact cell function, viability, and susceptibility to pathogens, contributing to proinflammatory overstimulation, organelle dysfunction, oxidative stress, and excessive cytokines release in CF lung [29, 39]. In CF, rescued CFTR normalizes the deregulated calcium homeostasis [29]. Here, we studied the effects of ETI on intracellular calcium levels in F508del-CFTR airway epithelial cells to determine whether these compounds, individually or collectively, could normalize intracellular calcium levels.

Materials and methods

Cell culture

Human airway epithelial cells (hAEC) were obtained from the department of thoracic surgery and lung transplantation of the Foch hospital (Suresnes, France) from explanted CF lungs at the time of the patient's transplantation [40]. Human tissues from four F508del/F508del donors were collected and used according to French law, with the informed consent of pwCF and through the authorization of Biological Collection noDC-2012–1583 obtained from the French Ministry of Higher Education and Research, and with the approval no21–775 of institutional review board 00003888. Airways were dissected, and epithelial cells were obtained after overnight enzymatic dissociation using $0.5 \text{ mg}\cdot\text{mL}^{-1}$ pronase E (Sigma Aldrich, USA). Primary HAE cells were seeded on type IV collagen-coated dishes and cultured in Pneumacult™-Ex basal medium (StemCell Technologies, France) supplemented with Pneumacult™-Ex 50X Supplement (StemCell Technologies, France), hydrocortisone ($0.48 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$; StemCell Technologies, France), penicillin ($100 \text{ units}\cdot\text{mL}^{-1}$) and streptomycin ($100 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$) (Sigma Aldrich, France). For polarized culture, primary HAE were seeded on type IV collagen-coated Corning® 12 mm Snapwell Insert at $0.175 \times 10^6 \text{ cell}/\text{cm}^2$ in PneumaCult™-Ex Plus Complete Medium. Upon reaching confluency (2 days later) the apical media was removed, and an air-liquid interface (airlift) was set using StemCell™ PneumaCult™-ALI maintenance medium (StemCell Technologies, France), to the basal chamber. Basal culture medium was renewed every two days [41].

The human bronchial epithelial cell lines CFBE41o-wild-type CFTR cells and CFBE41o-F508del-CFTR cells [42] were grown at $37 \text{ }^\circ\text{C}$ in 5% carbon dioxide (CO_2)–95% air and media were replaced every 2 days. These cell lines were grown in Eagle's minimum essential medium (EMEM) containing nonessential amino acids (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Eurobio, France), 2 mM L-glutamine and were selected using $5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ puromycin (Gibco).

F508del-CFTR proteins were restored to the plasma membrane by incubating cells with various combinations of VX-445, VX-661, VX-809, and VX-770 over 24 h, before experiments.

CHO cells stably transfected with pNut vector (CHO-K1) were kindly provided by J. R. Riordan [43]. Cells cultured at 37 °C in 5% CO₂-95% air were maintained in α -minimal essential medium containing 7% fetal bovine serum, antibiotics (50 IU/ml of penicillin and 50 μ g/ml of streptomycin), and 20 μ M methotrexate (all from Sigma Aldrich, France).

Treatments

F508del-CFTR expressing cells or CHO cells used in the present study were treated by modulators in acute or 24 h before the experiments. The following modulators (see Additional file 1) were from Selleckchem (Selleck Chemicals, USA) (IUPAC name): VX445 (elixacaftor), (*N*-(1,3-dimethylpyrazol-4-yl) sulfonyl-6-[3-(3,3,3-trifluoro-2,2-dimethylpropoxy) pyrazol-1-yl]-2-[(4*S*)-2,2,4-trimethylpyrrolidin-1-yl]pyridine-3-carboxamide); VX-661 (tezacaftor), (1-(2,2-difluoro-1,3-benzodioxol-5-yl)-*N*-[1-[(2*R*)-2,3-dihydroxypropyl]-6-fluoro-2-(1-hydroxy-2-methylpropan-2-yl) indol-5-yl]cyclopropane-1-carboxamide); VX-770 (ivacaftor), (*N*-(2,4-ditert-butyl-5-hydroxyphenyl)-4-oxo-1*H*-quinoline-3-carboxamide); VX-809 (lumacaftor), (3-[6-[[1-(2,2-difluoro-1,3-benzodioxol-5-yl)cyclopropanecarbonyl]amino]-3-methylpyridin-2-yl]benzoic acid). In our study, we selected the concentrations used by Keating et al. [44] describing the in vitro effect of these compounds on CF airway epithelial cells; VX-661 at the concentration of 18 μ M, VX-445 at 3 μ M and VX-770 at 1 μ M.

To study the origin of the calcium release, ATP (Adenosine 5'-triphosphate disodium salt hydrate; 100 μ M), thapsigargin (1 μ M), 2-APB (2-Aminoethyl-diphenylborinate; 100 μ M) or CPA (Cyclopiazonic acid from Penicillium cyclopium; 10 μ M) were used in acute treatment. All these molecules are from Sigma (Sigma Aldrich, France).

Calcium measurements

Non-polarized cells at 80% of confluence and polarized HAE cells were loaded with 3 μ M Fluo-4-acetoxymethyl ester (Invitrogen, ThermoFisher Scientific, France) for 20–45 min respectively at room temperature in Krebs buffer solution containing: (in mM) 130 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.8 MgCl₂, 5.6 glucose, 10 HEPES, pH 7.4 (adjusted with Tris base), rinsed and allowed to equilibrate for 5–10 min. Ca²⁺ activity was recorded using a Zeiss Axio observer Z1 inverted microscope, 250 ms duration of laser stimulation was applied to the region of interest. For polarized HAE cells, images were collected using a long working distance objective (Zeiss LD-Plan

NEOFLUAR). The recording was performed under Carl Zeiss AxioVision Release 4.8.2 software, with a physiology acquisition module. The basal fluorescent level is recorded for 60 s, afterwards the coverslips are available for treatments. Intensity profiles are normalized by dividing the fluorescence intensity of each pixel (F) by the average resting value before stimulation (F₀) to generate an (F/F₀) image. The background is subtracted from the Ca²⁺ signal recording. With this intensity profile, we compared the different Ca²⁺ responses by measuring the area under the curve (AUC) using GraphPad Prism version 8.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Average AUC from multiple independent coverslips was used to calculate the sample mean \pm SEM. All experiments were performed on a minimum of two different cell passages (2 < N < 6) and cells selected in each field were noted n in each histogram.

Gene silencing by siRNA

siRNA against SERCA2 (Santa Cruz Biotechnology, USA) was transfected using the Lipofectamine RNAiMAX Reagent (Invitrogen, Thermo Fisher Scientific, France). The transfection reagent and siRNAs were mixed, and complex formation was allowed to proceed for 5 min at room temperature before the complex was added to the cells. After 72 h, the cells were available for calcium measurement or western blotting analysis.

Western blotting

Total proteins were extracted from CFBE41o- F508del-CFTR by lysing cells with RIPA lysis buffer (Thermo Scientific, Thermo Fisher Scientific, France) supplemented with 1 mM Pefabloc[®] SC (Sigma Aldrich, France) and the complete[™] Protease Inhibitor Cocktail (Roche, Sigma Aldrich, France). Next, protein extracts were quantified using a BCA protein assay kit (Thermo Scientific, Thermo Fisher Scientific, France) and 30 μ g of protein samples were separated on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then subjected to Western blotting using a mouse anti-CFTR antibody (MAB3480, aa 1370–1380, clone M3A7, 1:1000; Merck Millipore, Sigma Aldrich, France), a mouse anti-SERCA2-HRP (1:1000; SantaCruz biotechnology, USA) and a mouse anti-GAPDH (1:2000; SantaCruz biotechnology, USA). Horseradish peroxidase-conjugated sheep anti-mouse antibody (1:5000; Amersham, GE Healthcare, UK) was used as the secondary antibody, and proteins were detected using enhanced chemiluminescence (Cytiva, Dutscher, France). Images were obtained using the GeneGnome Imager (SynGene Ozyme, France) and analyzed for densitometry with the Genetools software (SynGene Ozyme, France). The intensity of the bands was normalized to the loading control, and the GAPDH protein and CFTR maturation status were estimated by

the band C/ (band B+band C) ratio. The PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Thermo Fisher Scientific, France) was used for the identification of proteins on SDS-polyacrylamide gels.

Statistical analysis

The number of experiments (N) and cells (n) used in each group is stated in the respective figures. Statistical significance was evaluated by using the GraphPad Prism software v.8.0 (GraphPad Software Inc., San Diego, CA, US). Data are presented as mean \pm SEM of *n* observations. An ordinary one-way ANOVA or unpaired parametric Student's t-test were used to assess the statistical significance between treatments after calcium measurement. A paired Student's t-test was used to assess the statistical significance of densitometric analysis between treatments. Differences were considered statistically significant at $P < 0.05$. ns, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$.

Results

Rescue of the calcium mobilization by F508del-CFTR correctors in CFBE41o- F508del-CFTR cells

Given the increase of calcium mobilization in CF cells compared to WT cells and its rescue in response to temperature correction [20], we first measure the intracellular calcium mobilization in CFBE41o- F508del-CFTR cells using ATP in response to ETI. After 24 h treatment with ETI or control, cells expressing F508del-CFTR or WT-CFTR were loaded with Fluo-4 and subjected to exposure with ATP (100 μ M). Following the addition of ATP to the bath perfusing cells, we recorded a biphasic calcium response: first, an initial calcium peak then a sustained rise of intracellular calcium that persisted during the five-minute stimulation. The measure of the height of the peak and the area under the curve (AUC) were used to quantify the intracellular calcium mobilization. Firstly, we confirmed the significant increase of calcium mobilization in CFBE41o- F508del-CFTR compared to CFBE41o- WT-CFTR and the corrector effect of the temperature (27 °C) (Fig. 1a-b). Secondly, we showed that ETI induced a significant decrease of the intracellular calcium mobilization in response to ATP, similar to the treatment of CFBE41o- F508del-CFTR at 27 °C or CFBE41o- WT-CFTR (Fig. 1a-b). As ETI contains two correctors we measured the effect of each on intracellular calcium mobilization. We experimented using correctors alone compared to the effect of VX-809 (24 h treatment). VX-809, VX-661 and VX-445 induced a significant decrease in intracellular calcium mobilization compared to untreated cells and similar to ETI (Fig. 1c-d).

Mobilization of calcium by acute addition of VX-661 and VX-445 is dependent on intracellular calcium store

We investigated the effect of these correctors in acute addition on the intracellular calcium mobilization in CFBE41o- CFBE-F508del. Surprisingly, the addition of VX-661 or VX-809 induced a significant mobilization of intracellular calcium, in terms of peak height and sustained signal, particularly with VX-661 (Fig. 2a-b). Regarding VX-445, we recorded a small intracellular calcium mobilization in response to treatment, which was significantly reduced compared to VX-661 (Fig. 2b). We determined the dose-response relationship for the three compounds (Fig. 2c). The order of efficacy of the three correctors to stimulate a Ca^{2+} response in CFBE41o- CFBE-F508del cells is VX-661 > VX-809 > VX-445.

The intracellular calcium mobilization observed in response to treatment can have two origins. In the first one, the Ca^{2+} is released in the cytoplasm from the extracellular medium due to the activation of calcium channels (e.g. TRP channels) present at the plasma membrane [28]. In the second one, the Ca^{2+} is released from the Ca^{2+} -stores, for example, endoplasmic reticulum (ER) or mitochondria [33]. In this case, specific calcium channels implicated in the release or recapture of calcium are involved [29]. To test these hypotheses, we compared the effect of correctors in the absence of calcium in the extracellular medium (Fig. 2d and f). Using VX-661, we observed a 60% decrease in the level of calcium mobilization in presence of a Ca^{2+} -free extracellular medium, suggesting that VX-661 induces calcium mobilization from the Ca^{2+} -store on the one hand (60%) and the extracellular medium on the other hand (40%). In contrast, VX-445 induces comparable Ca^{2+} mobilization in the presence or absence of extracellular calcium, indicating that VX-445 induces an increase in calcium that does not originate from external sources. So, both correctors have a direct effect on the release of the calcium store.

To confirm our observations, HAE cells were treated with VX-661 in acute in the presence or absence of calcium in the extracellular medium (Fig. 2g-h). We obtained a similar result compared to CFBE41o- F508del-CFTR cells. The Ca^{2+} signal is even more important in the presence of calcium and sustained for a longer time compared to CFBE41o- F508del-CFTR cells. In the absence of calcium in the extracellular medium, we observed an 80% decrease in Ca^{2+} signal compared to the Ca^{2+} mobilization in the presence of calcium. This result confirms that VX-661 triggers an important calcium mobilization during acute treatment of both CFBE41o- F508del-CFTR and HAE cells. The acute effect of VX-661 (folding corrector) was also compared with the effect of acute addition of VX-770 (gating potentiator) on HAE cells from 2 pwCF. We show that, in contrast to VX-661, VX-770 did

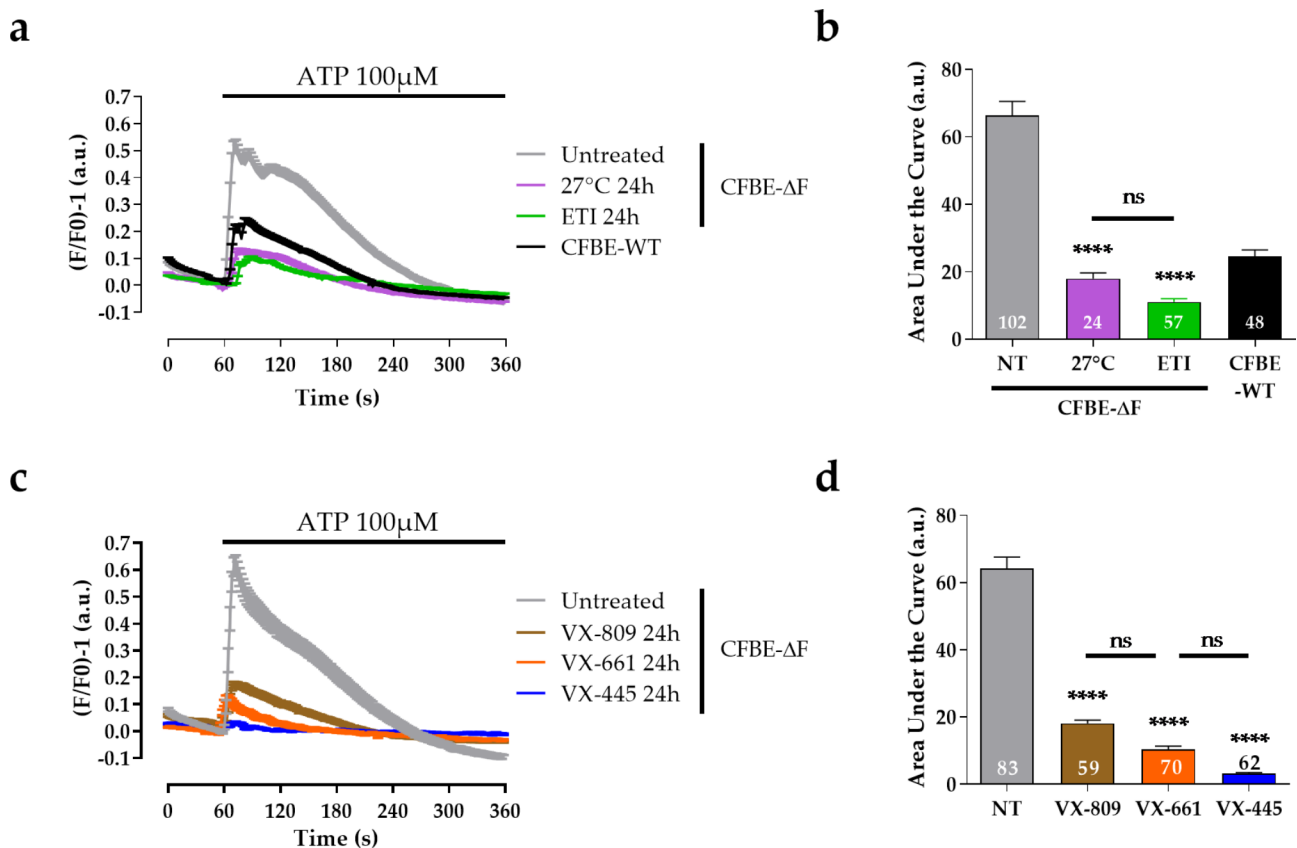


Fig. 1 Effects of F508del-CFTR trafficking correctors on the Ca^{2+} mobilization in CFBE41o- F508del-CFTR cells. **(a)** Typical traces of Ca^{2+} mobilization in corrected or uncorrected CFBE41o- F508del-CFTR cells during 5 min stimulation (black line on the top of traces) by 100 μM ATP. The CFBE41o- F508del-CFTR cells were incubated for 24 h at 27 $^{\circ}\text{C}$ or 24 h at 37 $^{\circ}\text{C}$ with ETI. **(b)** Histogram of the normalized area under the curve (AUC) corresponding to Ca^{2+} mobilization induced by 100 μM ATP (N: 2–5; n, number of cells recorded). **(c)** Typical traces of Ca^{2+} mobilization in corrected or uncorrected CFBE41o- CFBE-F508del cells during 5 min stimulation (black line on the top of traces) by 100 μM ATP. The CFBE41o- CFBE-F508del cells were incubated for 24 h at 37 $^{\circ}\text{C}$ with 3 μM VX-809, 18 μM VX-661 or 3 μM VX-445. **(d)** Histogram of the normalized area under the curve (AUC) corresponding to Ca^{2+} mobilization induced by 100 μM ATP (N: 3–4; n, number of cells recorded). Results are presented as mean \pm SEM. **** $p < 0.0001$; ns, not significant

not induce an increase in intracellular calcium (see Additional file 2).

Finally, we tested VX-661 in acute on polarized cultures of 3 pwCF (Fig. 2i–j). We observed a calcium mobilization induced by the addition of VX-661 on these pseudostratified epithelia similar to that observed on HAE cells.

Mobilization of calcium by acute addition of VX-661 is independent of CFTR

To determine whether these effects on calcium mobilization are due to CFTR, we tested the acute addition of VX-661 or VX-445 on CHO-K1 cells without CFTR expression (Fig. 3a and c). As shown in Fig. 3a–b, we still recorded a calcium response in CHO-K1 cells after adding VX-661 and VX-445. Thus, both correctors induce a calcium mobilization independent of CFTR expression. However, as CHO-K1 is a non-human cell model, care must be taken as calcium regulation may differ from that in human cells.

The calcium mobilization induced by VX-661 is dependent on ER

In the next experiments, we pretreated CFBE41o- F508del-CFTR cells with 2-APB, an IP3R and a SERCA inhibitor, (100 μM ; Fig. 4a and c) [45, 46] and measured the Ca^{2+} response induced by VX-661. In the presence of 2-APB, the VX-661-dependent Ca^{2+} response was significantly decreased compared to the control, suggesting that at least part of the Ca^{2+} response is due to an ER release of calcium. On the contrary, VX-445 does not show a significant difference between the presence or absence of 2-APB, suggesting that the acute addition of VX-445 allows a calcium mobilization that is not dependent on an ER release of calcium. However, the Ca^{2+} response in the presence of 2-APB could alternatively be due to a calcium influx from the extracellular medium or other intracellular stocks. To study this, we performed the experiment in the presence of 2-APB and in the absence of extracellular calcium (Fig. 4d–e). In this case, we observed that after

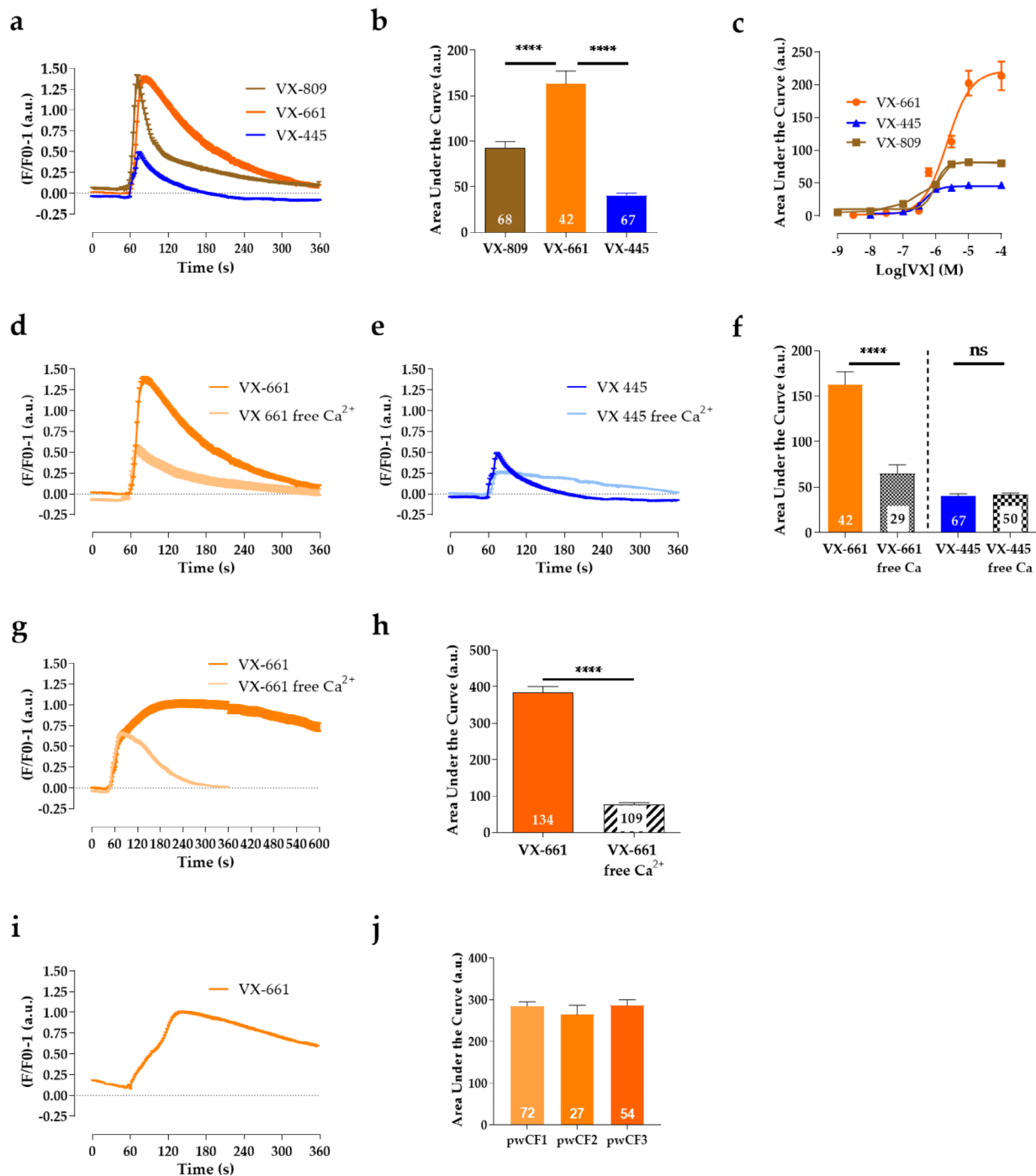


Fig. 2 Effect of acute addition of correctors on calcium homeostasis of CFBE41o- F508del-CFTR cells and HAE cells. **(a)** Typical traces of Ca^{2+} response induced by the addition of $3 \mu\text{M}$ VX-809, $18 \mu\text{M}$ VX-661, or $3 \mu\text{M}$ VX-445 to the bath of CFBE41o- F508del-CFTR cells during 5 min stimulation. **(b)** Histogram of the normalized area under the curve (AUC) corresponding to Ca^{2+} mobilization in CFBE41o- F508del-CFTR cells induced by $3 \mu\text{M}$ VX-809, $18 \mu\text{M}$ VX-661, or $3 \mu\text{M}$ VX-445 (N: 2–4; n, number of cells recorded). **(c)** Dose-response curves for correctors on the calcium response on CFBE41o- F508del-CFTR cells. **(d)** Paired calcium responses of CFBE41o- F508del-CFTR cells to VX-445 ($3 \mu\text{M}$) in the presence and absence of extracellular calcium. **(e)** Paired calcium responses of CFBE41o- F508del-CFTR cells to VX-445 ($3 \mu\text{M}$) in the presence and absence of extracellular calcium. **(f)** Area under the curve value with or without extracellular Ca^{2+} in response to correctors in CFBE41o- F508del-CFTR cells (N: 2–5; n, number of cells recorded). **(g)** Typical traces of Ca^{2+} response induced by the addition of $18 \mu\text{M}$ VX-661 in human airway epithelial (HAE; pwCF) cells during 5 min stimulation. **(h)** Histogram of the area under the curve (AUC) corresponding to Ca^{2+} mobilization induced by $18 \mu\text{M}$ VX-661 in pwCF cells (N: 4; n, number of cells recorded). **(i)** Typical traces of Ca^{2+} response induced by the addition of $18 \mu\text{M}$ VX-661 to the bath of polarized pwCF cells during 5 min stimulation. **(j)** Histogram of the normalized area under the curve (AUC) corresponding to Ca^{2+} mobilization in polarized pwCF cells induced by $18 \mu\text{M}$ VX-661 (N: 3; n, number of cells recorded). Results are presented as mean \pm SEM. **** $p < 0.0001$

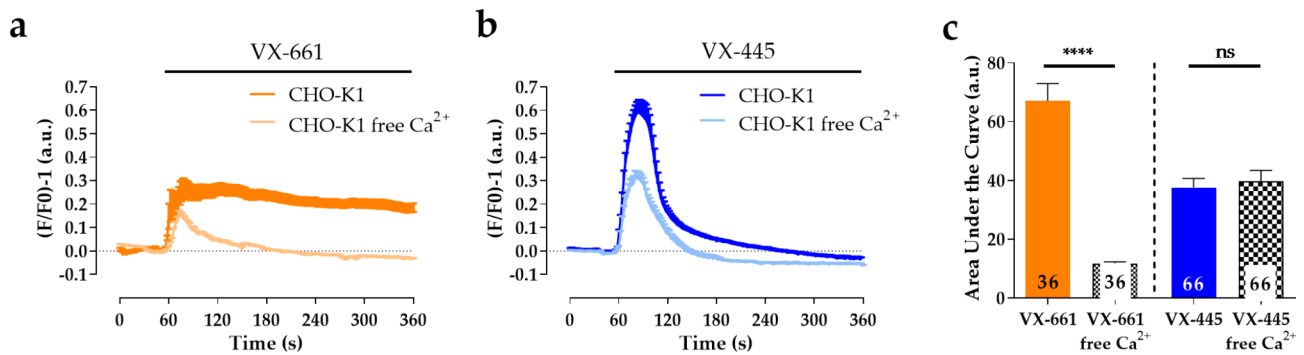


Fig. 3 Effect of acute addition of correctors on calcium homeostasis in CHO-K1 cells (a) Typical traces of Ca^{2+} response on CHO-K1 cells by adding 18 μM VX-661 (a) or 3 μM VX-445 (b). In c, a summary of the area under the curve (AUC) values corresponding to Ca^{2+} mobilization induced by 18 μM VX-661 and 3 μM VX-445 (N: 2–4; n, number of cells recorded). Results are presented as mean \pm SEM. **** $p < 0.0001$; ns, not significant

treatment with VX-661, the response was significantly reduced and almost totally abolished (Fig. 4e).

To further understand our results suggesting that the bulk of the calcium mobilization from Ca^{2+} stores is ER-related and targets the SERCA protein, a new experiment was performed using the CPA compound, a reversible SERCA inhibitor (Fig. 4f). We tested two strategies. In the first one, we first added CPA and then VX-661. In this case, we expected not to get a response after adding VX-661 if its target is the SERCA pathways. In the second one, we first added VX-661 and then CPA. In this case, if SERCA is a direct target of VX-661, the inhibition of SERCA by CPA should suppress the signal due to VX-661. Our results show that CPA induces a signal due to the release of ER Ca^{2+} stores and that the addition of VX-661 in a second step does not induce a new signal. Moreover, VX-661 induced a signal as previously described and the addition of CPA in a second step was no longer able to mobilize ER calcium by inhibiting SERCA, suggesting that VX-661 directly targets the SERCA protein.

Mobilization of calcium by acute addition of VX-661 is dependent on SERCA

To confirm the implication of the SERCA protein in the mobilization of calcium by VX-661, we used RNA interference. The efficiency of transfection of SERCA siRNA was assessed via Western blotting of the total protein fraction from CFBE41o- F508del-CFTR cells. We observed a decrease of 80% in the expression of the SERCA protein (Fig. 5a). Regarding functionality, we confirmed the low expression of SERCA protein at the ER surface using thapsigargin, an irreversible SERCA inhibitor. Thapsigargin induces a cellular Ca^{2+} response due to ER Ca^{2+} release (Fig. 5b). As expected, this release from ER was decreased in the CFBE41o- F508del-CFTR cells treated with SERCA siRNA (Fig. 5b). Interestingly, the extinction of the SERCA protein abolished the signal induced by VX-661 compared to cells treated with

control siRNA or untreated (Fig. 5c-d). We also tested the effect of VX-445 on CFBE41o- F508del-CFTR cells treated with SERCA siRNA and observed that the corrector induces a similar calcium response in the presence or absence of SERCA protein (Fig. 5e-f), suggesting that ER is not implicated in the calcium mobilization mediated by the acute addition of VX-445.

Mobilization of calcium by acute addition of VX-661 did not rescue CFTR maturation

Finally, as VX-661 has been identified for its role in the rescue of CFTR to the plasma membrane, we studied whether the calcium mobilization mediated by VX-661 on the SERCA protein is linked to the plasma membrane rescue of F508del-CFTR. To this end, we treated CFBE41o- F508del-CFTR cells with SERCA siRNA followed by VX-661 for 24 h and quantified the F508del-CFTR protein B and C bands by western blotting (Fig. 6a-b). We observed no significant variation in the ratio C/(B+C) in VX-661-treated cells in the presence or absence of SERCA siRNA, suggesting that calcium mobilization mediated by VX-661 neither promotes nor alters the rescue of F508del-CFTR protein at the plasma membrane.

Discussion

Our study shows several salient results. First, the use of the F508del-CFTR trafficking correction strategy, including ETI treatment and temperature modulation, leads to the normalization of calcium homeostasis in human CF airway epithelial cells. Second, individually, VX-661 (Tezacaftor) and VX-445 (Elexacaftor) can mobilize calcium acutely, like VX-809 (Lumacaftor). VX-809 and VX-661 belong to the same cluster called type I correctors, whereas VX-445 belong to the type III correctors [47]. VX-661 therefore interacts with the F508del-CFTR protein at a different binding site to VX-445, facilitating the folding and presentation of mature CFTR protein at the cell surface, thereby enhancing F508del-CFTR

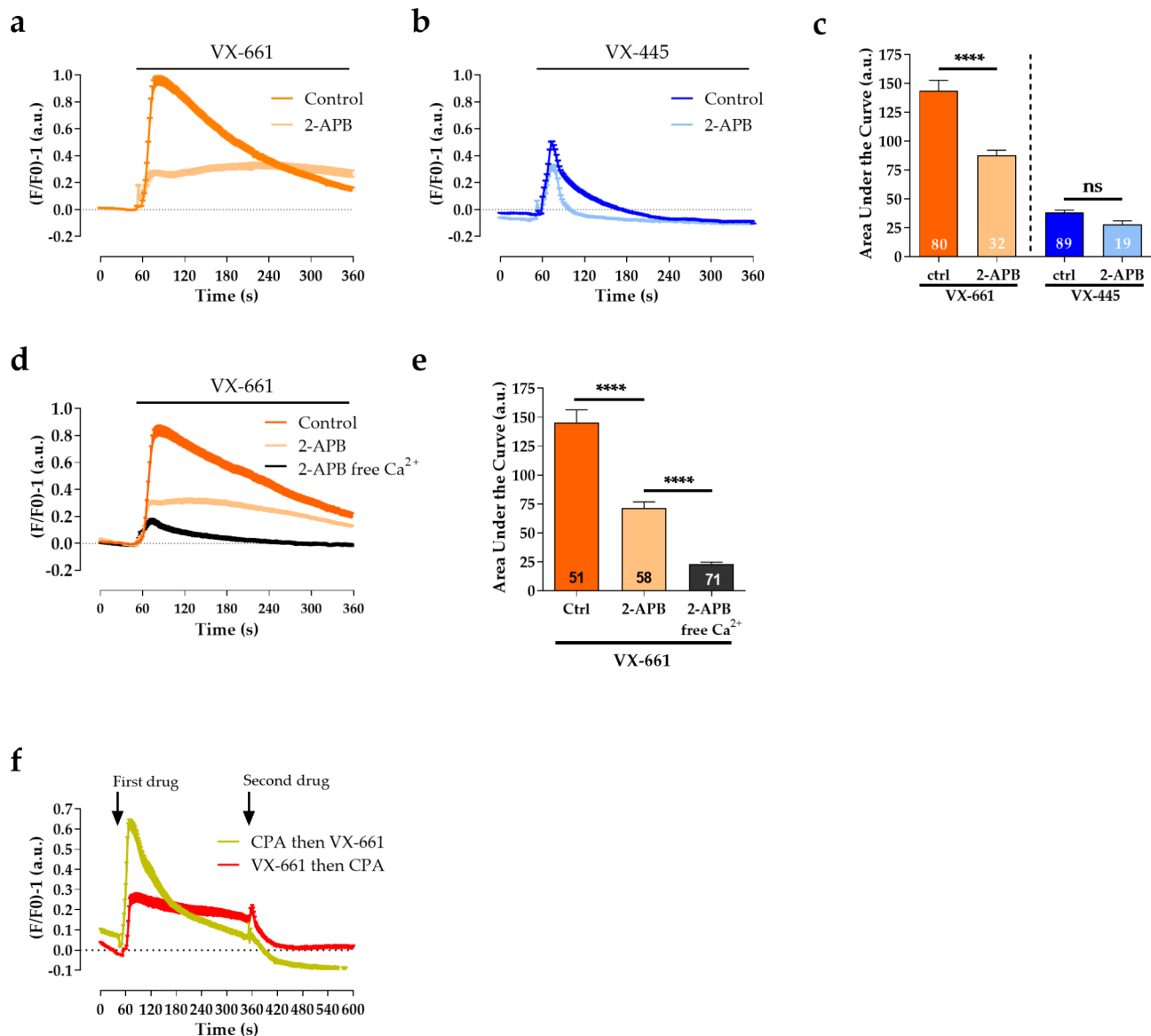


Fig. 4 Role of ER calcium stores in the global Ca^{2+} mobilization induced by correctors in CFBE41o- F508del-CFTR cells. (a) Typical traces of Ca^{2+} mobilization in pretreated CFBE41o- F508del-CFTR cells with 100 μM 2-APB (preincubation: 15 min) maintained at 37 $^{\circ}\text{C}$ during 5 min stimulation (black line on the top of traces) by 18 μM VX-661 (a) and 3 μM VX-445 (b). (c) Histogram of the area under the curve corresponding to the cytoplasmic mobilization induced by correctors stimulations with or without 2-APB treatment (N: 2–4; n, number of cells recorded). (d) Typical traces of Ca^{2+} mobilization in pretreated CFBE41o- F508del-CFTR cells with 100 μM 2-APB (preincubation: 15 min) maintained at 37 $^{\circ}\text{C}$ during 5 min stimulation by 18 μM VX-661 in the presence or absence of extracellular calcium. (e) Histogram of the area under the curve corresponding to the cytoplasmic mobilization induced by 18 μM VX-661 stimulation with or without 100 μM 2-APB treatment and in the presence or not of extracellular calcium (f) Typical traces of Ca^{2+} response on CFBE41o- F508del-CFTR cells induced following the addition on acute successively of 18 μM VX-661 and 10 μM CPA and vice versa. (N: 4–6; n, number of cells recorded). Results are presented as mean \pm SEM. **** $p < 0.0001$

function. Specifically, for VX-661, our results indicate that the Ca^{2+} mobilization is comparable between non-polarized CFBE or pwCF and polarized pwCF cells. Third, the present study has shown that the calcium mobilization by VX-661 involves inhibition of SERCA proteins, an effect that appears to be independent of its activity in rescuing CFTR to the plasma membrane. Therefore, our work suggests that SERCA is another target of VX-661 in human airway epithelial cells. SERCA

pump blocked by VX-661 could cause Ca^{2+} leakage via IP_3 receptors, leading to an increase in intracellular calcium concentration.

As Ca^{2+} is a second messenger, fulfilling a plethora of intracellular functions [48], the ER calcium depletion and increased intracellular calcium concentration in the cytoplasm of airway epithelial cells induced by VX-661 is not without consequences in the pathophysiological context of CE, particularly concerning inflammation

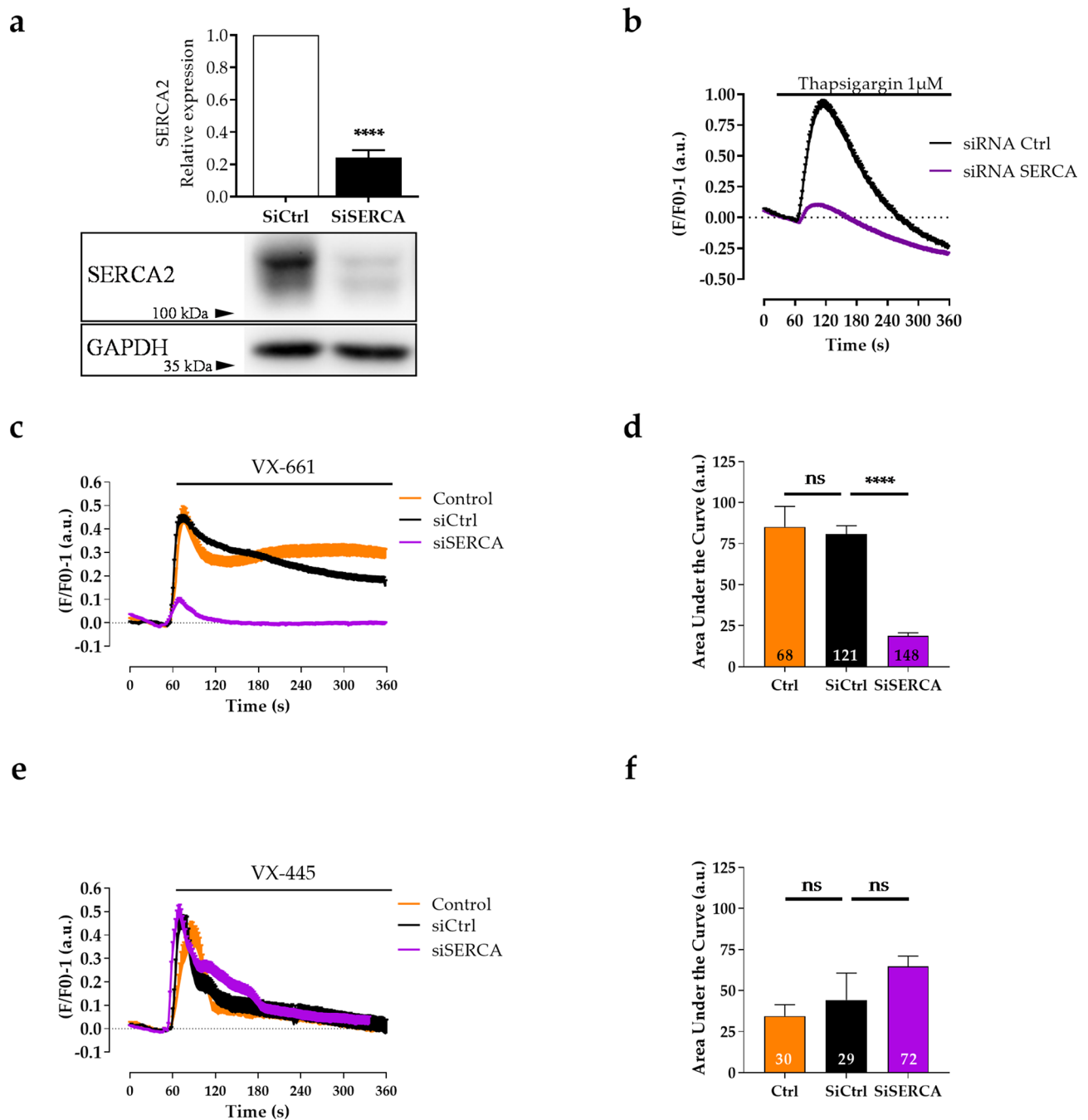


Fig. 5 ER calcium stock mobilization by acute addition of correctors in CFBE41o- F508del-CFTR cells. **(a)** SERCA2 expression in the total protein fraction of CFBE41o- F508del-CFTR cells treated with siSERCA or siCtrl (100 nM; 72 h pretreatment). Equal protein loading was controlled using GAPDH detection. Immunoblots are representative of eight independent experiments. The histogram represents densitometric analysis vs. the relative intensity observed in control siRNA-treated CFBE41o- F508del-CFTR cells. **(b)** Typical traces of Ca²⁺ mobilization in CFBE41o- F508del-CFTR cells pretreated with siSERCA or siCtrl (100 nM; 72 h) maintained at 37 °C during 5 min stimulation by 1 μ M thapsigargin. Traces are presented as mean \pm SEM. (N=2; n=81–83 cells). **(c)** Typical traces of Ca²⁺ mobilization in CFBE41o- F508del-CFTR cells untreated or pretreated with siSERCA or siCtrl (100 nM; 72 h) maintained at 37 °C during 5 min stimulation by 18 μ M VX-661. **(d)** Histogram of the area under the curve (AUC) corresponding to Ca²⁺ mobilization induced by 18 μ M VX-661 (N: 6; n, number of cells recorded). **(e)** Typical traces of Ca²⁺ mobilization in CFBE41o- F508del-CFTR cells untreated or pretreated with siSERCA or siCtrl (100 nM; 72 h) maintained at 37 °C during 5 min stimulation by 3 μ M VX-445. **(f)** The histogram shows the area under the curve (AUC) corresponding to Ca²⁺ mobilization induced by 3 μ M VX-445 (N: 3; n, number of cells recorded). Results are presented as mean \pm SEM. **** $p < 0.0001$; ns, not significant

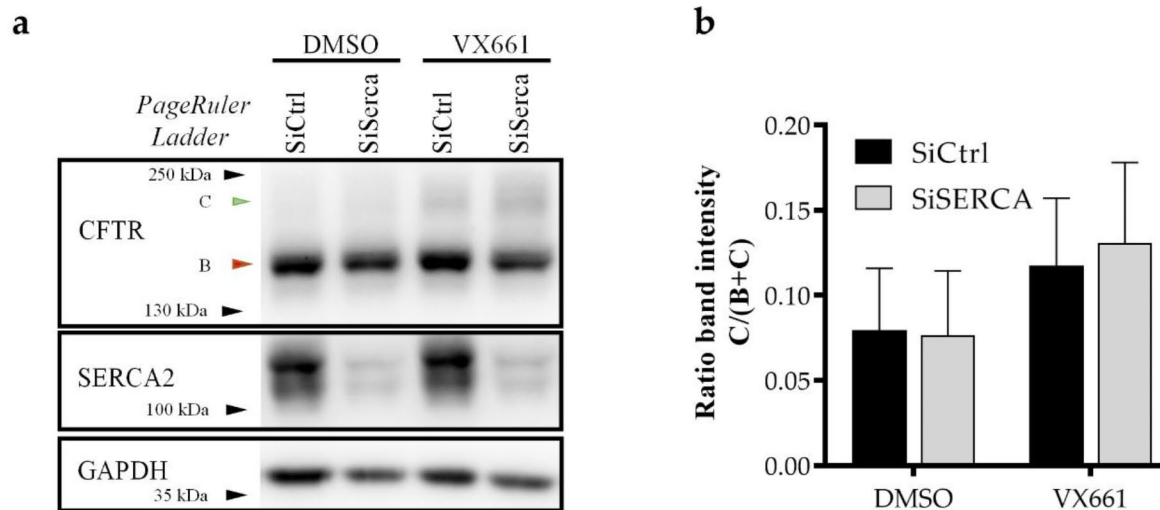


Fig. 6 Effect of SERCA silencing on the expression of VX-661-rescued F508del-CFTR proteins. **(a)** CFTR and SERCA2 expression in the total protein fraction of CFBE41o- F508del-CFTR cells treated by siSERCA or siCtrl (100 nM, 72 h pretreatment) and VX-661 (18 μ M, 24 h). Equal protein loading was controlled using GAPDH detection. Immunoblots are representative of four independent experiments. **(b)** Results of densitometric analysis of F508del-CFTR expressed as ratio of mature form C to the sum of forms B + C from CFBE F508del cells (N:4). Results are presented as mean \pm SEM

[49]. Indeed, in addition to its role in energy metabolism and cell death, the level of intracellular calcium Ca^{2+} in the cytoplasm is intimately involved in various cellular processes, such as autophagy and inflammation [50, 51]. It is therefore not surprising that altered Ca^{2+} signalling represents a key factor in several inflammatory diseases, including CF. For this reason, airway epithelial cells are critically dependent on the function and integrity of the Ca^{2+} signalling. Disturbances in Ca^{2+} signalling have been observed in CF [29, 39]. Some are due to intrinsic defects associated with CFTR deficiency, and others to environmental stress related to recurrent bacterial infections [29]. The result is an exacerbated inflammatory response that favors lung injury [39, 52]. For example, SOCE has already been demonstrated to be altered in CF cells and the mobilization of calcium from intracellular stores by VX-661 has the potential to trigger store-operated calcium entry (SOCE). This increase in calcium influx is mediated by Orai-1, and Orai-1-dependent calcium influx induces an increase in interleukin-8 levels in these cells [26]. This suggests that an increase in intracellular calcium may also affect the inflammatory status of the airways in CF patients. Then, the fact that VX-661 induces an increase in the Ca^{2+} level in the cytosol could, despite its role as a corrector of the F508del-CFTR protein, accentuate the effects on hyperinflammation and infection [29], abdominal pain [53] and vomiting [54]. This may be related to the common not-serious adverse effects (diarrhea, abdominal pain, vomiting, fatigue, cough, headache) reported in the clinical trial of Trikafta/

Kaftrio therapy in CF subjects homozygous for the F508del mutation [55].

In addition, an increasing number of studies suggest that both individual components or their combinations may have potential off-target effects. VX-661 has been demonstrated to inhibit sphingolipid delta-4 desaturase [56], VX-770 and its metabolites to possess a notable affinity for serotonin and β 3-adrenergic and δ -opioid receptors [57], VX-445 to induces K^{+} secretion via a potentiation of BKCa channels [58] or ETI to exhibit antimicrobial activity against *Staphylococcus aureus* [59]. Our results show that the acute addition of folding correctors (VX-809, VX-661 or VX-445), in contrast to the gating potentiator (VX-770) affect calcium mobilization. We then focused on demonstrating the effect of acute VX-661 in calcium mobilization. Since the calcium mobilization induced by the addition of VX-661 on non-polarized cells is similar to that observed on pseudostratified epithelia, we can assume that VX-661 may also induce calcium variations with each dose taken by CF patients. It would also be interesting to monitor the long-term impact of this daily calcium mobilization. Indeed, authors have recently shown the long-term effects of individual modulators and their combinations in reactive oxygen species (ROS) production and autophagic flux [60]. These data suggest that the off-target effects of CFTR modulators may hurt cell physiology, particularly in cells where their mutant target is absent [60].

The ER calcium depletion has been demonstrated to be a possible way of correcting F508del-CFTR by bypassing

the quality control system of the mutated protein using short-term calcium-pump inhibitor treatments [30–32]. This effect could therefore contribute to the correction of the F508del-CFTR traffic defect. If our results did not confirm the rescue of F508del-CFTR to the plasma membrane in response to ER calcium mobilization by SERCA inhibition under 24 h VX-661 treatment, SERCA inhibitor (curcumin) were investigated in the traffic correction of F508del-CFTR to the plasma membrane [31]. One clinical study was undertaken by Ramsey in 2005 to consider the use of curcumin in adult pwCF but the results have not been published (NCT00219882). More recently, two other clinical studies assessed the effect of curcumin supplementation in children with cystic fibrosis [61, 62]. Rafeey et al. showed that the percentage of pwCF with weight gain was significantly higher in the group of CF children treated with curcumin with an improved quality of life [61]. While Tabeli et al. reported that curcumin has a shorter hepatic metabolism and is more readily absorbed in the gastrointestinal tract [62]. These results indicate that the use of curcumin improves the status of children with cystic fibrosis. Taken together, these results suggest that ER calcium level need to be maintained at a low level over time to maintain the CFTR rescue at the plasma membrane or at least a beneficial effect on pwCF's quality of life.

In conclusion, VX-661 and VX-445, two correctors of the F508del-CFTR abnormal biogenesis are also Ca^{2+} -mobilizing agonists. However, only VX-661 appears to intracellularly modulate the SERCA activity in addition to target the F508del-CFTR protein. The action of VX-661 *via* SERCA protein may lead to a dual effect of this molecule: i) a short-term negative effect which, in the context of long-term treatment, could become a side-effect by contributing to the alteration of calcium homeostasis already described in pwCF; and 2) a positive effect by reducing the ER calcium level over time and promoting the F508del-CFTR protein trafficking. As part of the development of new correctors, their effect on SERCA should be studied to better assess their impact on calcium mobilization and therefore on their *in vivo* biological effects generated.

Abbreviations

AUC	Area under curve
APB	2- Aminoethylidiphenylborinate
Ca^{2+}	Calcium
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CPA	Cyclopiazonic acid
HAE	Human airway epithelial
SERCA	SarcoEndoplasmic reticulum calcium ATPase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12931-024-03059-8>.

Additional file 1

Additional file 2

Additional file 3

Author contributions

ML and CV designed, experimented and analysed the data. ML, FB, ML and CV wrote the manuscript. SM, CB, IF performed cell cultures; CC and ES collected and provided HAE cells. FB and CV provided resources. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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