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SERCA and PMCA pumps contribute to the deregulation of Ca^{2+} homeostasis in human CF epithelial cells ☆,☆☆

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ABSTRACT

Cystic Fibrosis (CF) disease is caused by mutations in the *CFTR* gene (CF transmembrane conductance regulator). F508 deletion is the most represented mutation, and F508del-CFTR is absent of plasma membrane and accumulates into the endoplasmic reticulum (ER) compartment. Using specific Ca^{2+} genetics cameleon probes, we showed in the human bronchial CF epithelial cell line CFBE that ER Ca^{2+} concentration was strongly increased compared to non-CF (16HBE) cells, and normalized by the F508del-CFTR corrector agent, VX-809. We also showed that ER F508del-CFTR retention increases SERCA (Sarcoplasmic/Reticulum Ca^{2+} ATPase) pump activity whereas PMCA (Plasma Membrane Ca^{2+} ATPase) activities were reduced in these CF cells compared to corrected CF cells (VX-809) and non-CF cells. We are showing for the first time CFTR/SERCA and CFTR/PMCA interactions that are modulated in CF cells and could explain part of Ca^{2+} homeostasis deregulation due to mislocalization of F508del-CFTR. Using ER or mitochondria genetics Ca^{2+} probes, we are showing that ER Ca^{2+} content, mitochondrial Ca^{2+} uptake, SERCA and PMCA pump, activities are strongly affected by the localization of F508del-CFTR protein.

1. Introduction

Cystic Fibrosis (CF) disease is characterized by abnormal gastrointestinal and pulmonary epithelial ion transport and viscous mucus. CF is an autosomal recessive disease caused by mutations in the *CFTR* gene. Normally, CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein is localized at the apical plasma membrane from epithelial cells, acting as a cAMP-regulated anion channel [1–3]. Currently, more than 1900 mutations in the *CFTR* gene are identified and classified regarding their consequences on CFTR activity, expression or localization. The most common mutation is a deletion of a phenylalanine at position 508 (F508del-CFTR), which confers an abnormal conformation to F508del-CFTR and its recognition by several endoplasmic reticulum (ER) chaperone proteins, like calnexin [4,5], calreticulin [6], HSP 70 [7,8] and HSP90 [9]. These chaperones trapped F508del-CFTR into the ER lumen in order to restore its standard conformation, and to process the misfolded protein towards degradation, leading to the absence of the CFTR protein at the cell surface. The absence of F508del-CFTR protein at the plasma membrane and its ER retention will have several significant consequences on a number of directly CFTR associated functions and on cellular mechanisms regulated by protein complexes including CFTR [10–12].

Since the last decade, several groups described a non-intuitive consequence of CFTR mutation, which is an important deregulation Ca^{2+} homeostasis in CF cells. Ca^{2+} signaling deregulations were observed in several epithelial cell lines and also in primary epithelial cells [13,14]. Interestingly, two hypotheses coexist in the literature to explain the CF epithelial cells Ca^{2+} phenotype. The work done by Ribeiro and colleagues Boucher's group showed that Ca^{2+} deregulation in CF epithelial cells results from persistent infection in CF cells and not directly from the presence of a mutated CFTR [15,16]. Others studies realized in the absence of infectious factors showed in CF epithelial cell lines and CF epithelial primary cells several deregulations of Ca^{2+} signaling directly related to the mutated CFTR localization. OAG-mediated TRPC6 Ca^{2+} entry was demonstrated to be abnormally increased in CF epithelial cells and this deregulation was reversed by pharmacological restoration

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of F508del-CFTR trafficking to cell surface [17,18]. In primary epithelial cells, 2D epithelium and cultured epithelial cell lines, Store Operated Ca^{2+} Entry (SOCE) appears significantly increased in CF cells, due to an increased in Orai1 channel plasma membrane insertion [19]. IP3R mediated ER Ca^{2+} release was also abnormally increased in CF epithelial cell lines [20,21]. Mitochondria Ca^{2+} buffering activities were also showed to be deregulated in CF epithelial cell lines [22–24]. This CF abnormal Ca^{2+} phenotype takes a great importance in the CF physiopathology. Calcium is a universal second messenger controlling numerous cellular protein activities such as ion channels and enzymes involved in diverse cellular processes such as cell proliferation [25,26], ionic secretion [27,28], ciliary beat frequency [29,30], inflammation [23], protein maturation [31] or trafficking [32,33]. Importantly, an uncontrolled and persistent deregulation of intracellular Ca^{2+} concentration will lead to pathological processes like apoptosis or to deregulation of Ca^{2+} dependent pathways. In every cell, intracellular calcium concentration ($[Ca^{2+}]$intr) and calcium fluxes are tightly controlled by an organelle compartmentalization of Ca^{2+} ions (ER, Mitochondria) and via the fine tuning of Ca^{2+} permeable ion channels and Ca^{2+} transporter activities. Among them SERCA (Sarcoplasmic/Reticulum Ca^{2+} ATPase) and PMCA (Plasma Membrane Ca^{2+} ATPase) Calcium ATPase localized respectively at the ER membrane and plasma membrane, and Mitochondrial Ca^{2+} Uniporter (MCU) [34–36] localized at mitochondrial membrane, are essential in the control of the $[Ca^{2+}]$intr.

However, all the previous published work related to deregulations in Ca^{2+} signaling in CF cells had never explored the impact of F508del-CFTR mutation expression on organelles Ca^{2+} homeostasis with a direct approach. In the current study, we deciphered the consequence of F508del CFTR mutation on both cytoplasmic, ER and mitochondrial Ca^2 homeostasis using cameleon ER or mitochondrial targeted Ca^{2+} probes in well characterized CF (CFBE) and non-CF (16HBE) bronchial epithelial cells lines [37]. We are showing that ER Ca^{2+} content is strongly increased in CF cells and SERCA and PMCA Ca^{2+} pumps activities are greatly deregulated in CF cells probably as a consequence of their interaction with the CFTR channel.

2. Materials and methods

2.1. Materials

Thapsigargin, ATP and Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were obtained from Sigma. Acetoxymethyl ester form of Fura-2 (Fura-2/AM) was purchased from Molecular probes Europe (Leiden, the Netherlands). The ER-targeted and mitochondrial-targeted cameleon probes $D1_{ER}$ and 4mitD3cpv were kindly provided by Drs. Amy Palmer and Roger Tsien. Forskolin and genistein are from LC Laboratories.

2.2. Cell culture

CFBE (CFBE41o-) and 16HBE (16HBE14o-) cell lines originally developed by Dr. Gruenert, were generously provided by Pr Karl Kunzelmann from the University of Regensburg (Deutschland). BHK cell lines stably expressing F508del-CFTR and Wt-CFTR were obtained from Dr. John Hanrahan from McGill University (Canada). Each cell line was grown in Dulbecco's modified Eagle's medium completed with 10% Fetal Bovine Serum and 1% penicillin/streptomycin, and incubated at 37 °C and 5% CO_2.

2.3. Iodide efflux

CFTR channels activity was assayed on epithelial cell populations by the iodide (^{125}I) efflux technique as described [38,5].

2.4. Cytosolic Ca^{2+} imaging

For Ca^{2+} imaging, cells were plated on 18 mm glass cover slips. Changes in cytosolic Ca^{2+} concentration were measured with Fura-2. Cells were loaded with 4 μM Fura-2/AM plus 2 μM pluronic acid for 45 min in the dark at room temperature in a medium containing (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 Hepes, 10 glucose, and pH adjusted at 7.45 with NaOH. Cells were washed twice and equilibrated for 10–15 min in the same buffer to allow de-esterification of the dye. Ratiometric images of Ca^{2+} signals were obtained using a microscope (IX71, Olympus) equipped with a monochromator illumination system (Polychrome V, TILL Photonics). Emission was collected through a 415DCLP dichroic mirror, by a 14-bit CCD camera (EXiBlue, Qimaging). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging, West Chester, PA, USA). Experiments were performed at room temperature in Hepes-buffered solution containing (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 Hepes, 10 glucose, and pH adjusted at 7.45 with NaOH. The Ca^{2+}-free solution contained 1 mM EGTA instead of 1.8 mM $CaCl_2$.

The amplitude of SOC influx and TG-mediated Ca^{2+} release was measured by calculating the difference between the basal and maximal ratio. The rate of TG induced Ca^{2+} release or SOCE is approximated by the maximal speed of Ca^{2+} concentration increase (first derivation of the signal). ATP induced signals were measured with the area under curve (a.u.c.) of the signal (arbitrary unit, a.u.). PMCA activity was estimated by the time constant τ when a single exponential fits the decrease in the signal.

2.5. ER and mitochondrial Ca^{2+} measurements

CFBE and 16HBE cells were transiently transfected using LipoD293 (Tebu-bio) with 2 μg of cDNA encoding the $D1_{ER}$ or 4mitD3cpv construct 48 h before the experiments. Ratiometric images of Ca^{2+} signals were obtained using a microscope (Axio Observer, Zeiss) equipped with a Lambda DG4 illumination system (Sutter Instrument Company, Novato, CA, USA). Cells were illuminated at 440 nm (440AF21; Omega Optical), and emission was collected through a 455DRLP dichroic mirror, by a cooled, 12-bit CCD camera (CoolSnap HQ, Ropper Scientific, Trenton, NJ, USA) alternatively at 480 nm (480AF30; Omega Optical) and 535 nm (535AF26; Omega Optical). $[Ca^{2+}]_{ER}$ was calculated as previously described [39] from $D1_{ER}$ ratios using the equation.

$R = Rmin + \left([Rmax - Rmin]/1 + 10^{(LogK/d - Log[Ca^{2+}]ER)h}\right)$. Where R_{min} and R_{max} are the minimal and maximal ratio obtained using appropriate solutions, K'_d is the apparent dissociation constant, and h is the Hill coefficient derived from the in situ Ca^{2+} titration of the $D1_{ER}$ probe in semi-permeabilized cells.

To quantify ER Ca^{2+} refilling and mitochondrial Ca^{2+} uptake, the slope of the increase of FRET signal was determined by a linear fit. The amplitude of ER Ca^{2+} depletion and mitochondrial Ca^{2+} uptake was also measured by the difference between basal and TG response and between peak of Ca^{2+} uptake and the end of TG response in Ca^{2+} free, respectively. To measure Ca^{2+} leak rates of the ER, passive ER depletion was induced by TG and the $D1_{ER}$ responses were fitted with a one-phase exponential decay function to extract the half-time ($\tau 1/2$).

2.6. Western blot

Protein extraction was performed with a lysis buffer (in mM): 20 Tris HCl pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton X100, 2.5 Na^+ Pyrophosphate (tetrasodium), 1 glycerophosphate, 1 Na^+ orthovanadate, 1 μg/ml leupeptin, and + complete protease inhibitor tablet (Roche). After 30 min of incubation on ice, protein extracts were centrifugated 12 min at 16,000*g*. 50 μg of protein (or 150 μg for CFTR) lysates were separated on 4–12% Bis/Tris pre-casted poly-acrylamide gels (Invitrogen) or 7.5% poly-acrylamide gels (for CFTR), and transferred onto PVDF

membrane. Blots were incubated with primary antibodies diluted in T-TBS and nonfat milk as follows: mouse monoclonal anti-PMCA (clone 5F10, Pierce) (1/1000) (against all isoform of PMCA), mouse monoclonal anti-SERCA2b ATPase (clone IID8, Santa Cruz) (1/250), mouse monoclonal anti-CFTR M3A7 antibody (1/500) or mouse monoclonal anti-GAPDH antibody (1/30 000). After 3 washes, blots were incubated with horseradish peroxydase (HRP)-conjugated donkey anti-mouse diluted 1:10,000 (Abcam), respectively, and revealed with Luminata Forte reagent (Millipore). Acquisition was performed on Chemi-Smart 5100 acquisition system (Vilber-Lourmat) with the Chemi-Capt 5000 software, and analyses were done on ImageJ.

2.7. Immunoprecipitation

Protein G Dynabeads (Life Technology) were incubated for 1 h at 4 °C in PBS 0.02% Tween-20 with 2 μg of anti-CFTR (clone 24–1, R&D Systems), anti-SERCA (ab3625, Abcam) or anti-PMCA antibodies (clone 5 F10, Pierce). Then 1000 μg of proteins was incubated overnight with antibodies-coupled beads. After several washes, protein–antibody–beads complexes were precipitated in 25 μl of laemmli buffer (2×) and separated on SDS–PAGE electrophoresis before revelation by Western blot.

2.8. Duolink assay

To study PMCA–CFTR and SERCA–CFTR interactions, Proximity Ligation Assay was performed using the Duolink in vivo IP approach (Olink Bioscience, Uppsala, Sweden). Briefly, cells were fixed in 4% PFA solution and permeabilized in 0.25% Triton X-100 solution. Saturation was performed in blocking solution containing 2% goat serum and 0.2% Tween. Cells were then incubated with primary antibodies in a blocking solution at the following dilutions: 1/500 anti-PMCA (clone 5F10, Pierce) and 1/100 anti-CFTR (ab131553, Abcam) for PMCA–CFTR interaction, and 1/500 anti-SERCA (ab3625, Abcam) and 1/100 anti-CFTR (clone 24–1, R&D Systems) for SERCA–CFTR interaction. Secondary antibodies incubation (PLA probe anti-rabbit PLUS Cat. No. 92002, PLA probe anti-mouse minus Cat. No. 92004), probes ligation and amplification were performed following manufacturer's instructions (Detection Kit, Cat. No. 92014). Green fluorescent spots were observed on a confocal microscope (LSM 780, Carl Zeiss). Number of spots by image was counted using the ImageJ software.

2.9. Statistics

Ca^{2+} measurement, Western blot and Duolink quantification data are expressed as mean ± s.e.m. of n observations and were compared with ANOVA analysis with post-hoc analysis with Dunnett. Differences were considered statistically significant when $P < 0.05$. ns: non-significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. All statistical tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software).

3. Results

3.1. ER Ca^{2+} release and SOCE are abnormally increased in CF cells, and normalized by CFTR correction with VX-809 treatment

As described previously, ER Ca^{2+} release and SOCE are increased in cells expressing F508del-CFTR [17,19]. To evaluate ER Ca^{2+} content and SOCE in our cellular models, we performed classical SOCE activation protocol (Fig. 1A). Internal Ca^{2+} stores were depleted by 2 μM of TG in Ca^{2+} free medium and then SOCE was measured after perfusion with 1.8 mM Ca^{2+} extracellular medium (Fig. 1A).

We first confirmed previous observations of a significant increase in SOCE amplitude (Fig. 1B and C) and rate (Supplemental Fig. 1A) in CF (CFBE) cells compared to non-CF cells (16HBE). To investigate if these differences are due to the abnormal F508del-CFTR accumulation in the ER, we have chosen to treat CF cells for 24 h with the pharmacological CFTR corrector, VX-809 (Lumacaftor, Selleck Chemicals) at 10 μM [40,41]. In order to confirm VX-809 potential to rescue mislocalization of F508del-CFTR channels, we performed iodide efflux experiments to evaluate CFTR activity (Fig. 1D and E). CFBE and 16HBE cells were incubated 24 h with 10 μM of VX-809, and then F508del-CFTR (CFBE) or WT CFTR (16HBE) channel activities were stimulated by a cocktail of forskolin (10 μM) and genistein (30 μM) [5]. As expected stimulation of F508del-CFTR activity in uncorrected CFBE cells, did not induced an iodide efflux (Fig. 1D and E).

However, VX-809 treatment rescued functional F508del-CFTR to plasma membrane, as indicated by the significant increase of iodide efflux observed in CF treated cells compared to untreated CFBE cells ($0.0275 \pm 0.0047\ min^{-1}$, $n = 4$ for untreated CFBE cells and $0.1420 \pm 0.0155\ min^{-1}$, $n = 4$ for VX-809 treated CFBE cells; $P = 0{,}0004$). A similar VX-809 treatment had no impact on WT CFTR activity ($0.1950 \pm 0.0202\ min^{-1}$, $n = 4$ in untreated 16HBE cells and $0.2270 \pm 0.01472\ min^{-1}$. $n = 4$ in VX-809 treated 16HBE cells) (Fig. 1D and E).

Based on these findings, we next investigated Ca^{2+} signaling in CFBE (CF cells) treated with VX-809 to determine if this treatment normalized SOCE parameters to values observed in 16HBE cells (non-CF cells). As expected VX-809 treatment had no impact on SOCE amplitude in non-CF cells (Fig. 1C). For an unknown reason, the rate of SOCE was decreased in VX-809 16HBE treated cells (Supplemental Fig. 1A). Interestingly, rescuing F508del-CFTR activity induced a significant decreased in SOCE amplitude to the level measured in non-CF cells (16HBE; 2.066 ± 0.09925 ratio/sec, n = 84 and VX-809 treated 16HBE; 1.826 ± 0.1136 ratio/sec, n = 64 in cells). The absence of significant difference in SOCE amplitude between 16HBE and VX-809 CFBE treated cells reflects the potency of this CFTR corrector to eliminate Ca^{2+} influx defects. In this condition a strong and significant decrease of calcium entry rate was surprisingly observed ending up to a rate value lower than what observed in 16HBE cells (16HBE; 0.08911 ± 0.006924 ratio/sec, n = 87 and VX-809 treated 16HBE; 0.03307 ± 0.002053 ratio/sec, n = 54).

ER Ca^{2+} release was also evaluated by measuring the amplitude (Fig. 1F and G) and speed (Supplemental Fig. 1B) of the TG response in Ca^{2+} free condition. Both parameters were significantly increased by approximately 30% in CF (CFBE) cells compared to non-CF (16HBE) cells (Fig. 1G and Supplemental Fig. 1B). As shown in Fig. 1G and Supplemental Fig. 1B, TG induced Ca^{2+} response was significantly normalized in CF-corrected cells (VX-809 treated CF cells) to the values measured in non-CF cells. Moreover, as observed for SOCE amplitude, VX-809 treatment had no impact on TG-Ca^{2+} response in non-CF treated cells (Fig. 1G).

Measuring TG induced Ca^{2+} response in a free extracellular Ca^{2+} medium with Fura-2 allows us to have an indirect estimation of the ER Ca^{2+} content. Even if we abrogated Ca^{2+} influx in this condition, the Plasma Membrane Ca^{2+} ATPase (PMCA) is always functional allowing cytosolic Ca^{2+} extrusion to the extracellular space. Moreover part of Ca^{2+} released from the ER is also captured by mitochondria. Therefore, to quantify accurately the ER Ca^{2+} concentration and ER Ca^{2+} fluxes, we measured Ca^{2+} variations directly inside the ER compartment with the Ca^{2+} sensitive ER-targeted cameleon probe $D1_{ER}$ [42] (Fig. 2). To measure the ER Ca^{2+} concentration ($[Ca^{2+}]_{ER}$), we performed a $D1_{ER}$ titration in semi-permeabilized cells (data not shown) as described by Shen et al. [43]. $[Ca^{2+}]_{ER}$ in CF cells is significantly increased by 45% compared to non-CF cells (16HBE: 358 ± 26 μM, n = 41; CFBE 525 ± 31 μM, n = 58) (Fig. 2A). In CF cells treated with VX-809, the ER Ca^{2+} concentration was significantly decreased by 20% (treated CFBE: 432.5 ± 31 μM, n = 27) compared to uncorrected CF cells and normalized to the value measured in non-CF cells. No significant difference of ER Ca^{2+} concentration was observed between 16HBE and VX-809 CFBE treated cells. In contrast, the VX-809 treatment did

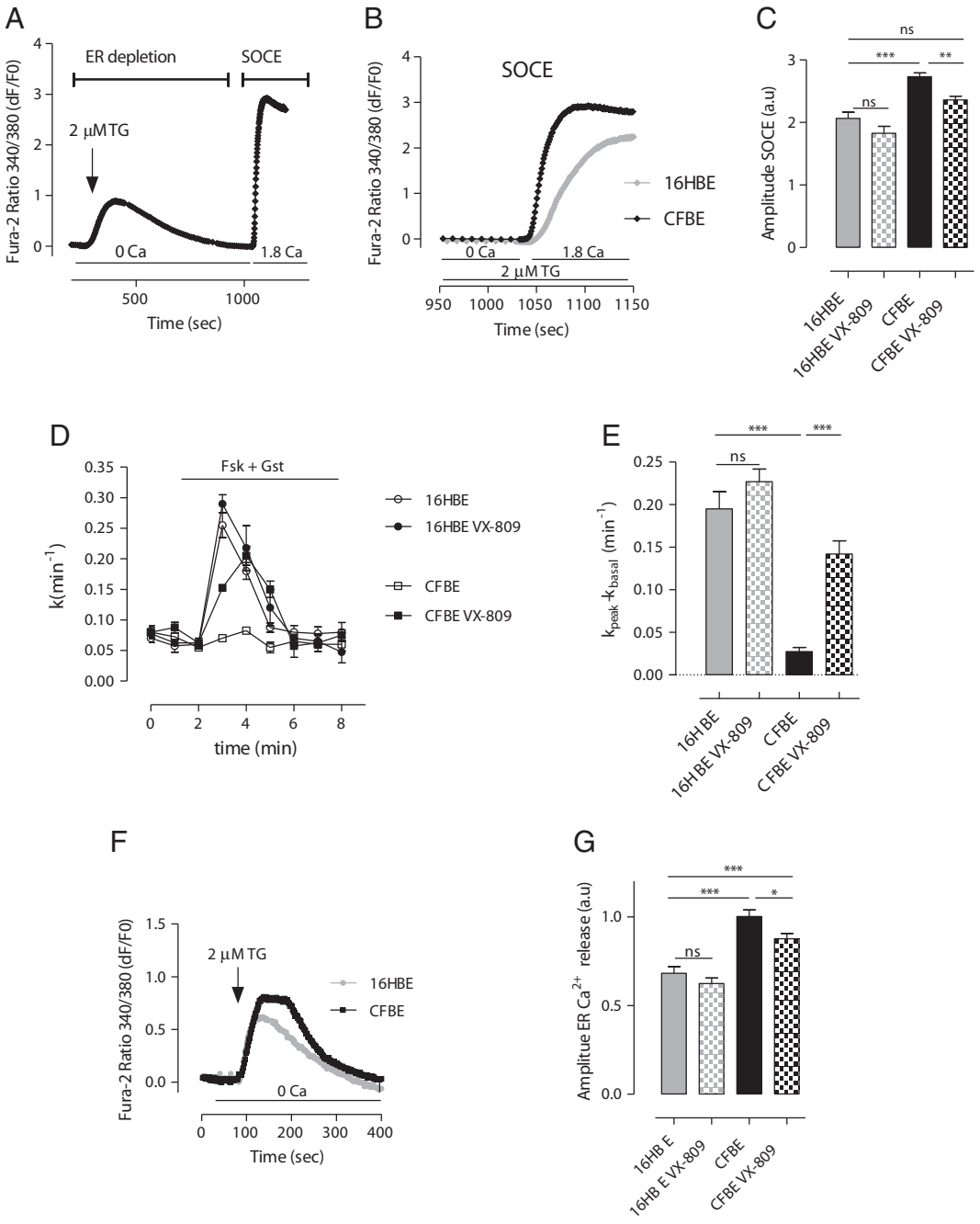

Fig. 1. Impact of VX-809 mediated F508del-CFTR correction on SOCE and ER Ca^{2+} content. A, Typical recording of intracellular Ca^{2+} concentration variations measured using Fura-2 in 16HBE and CFBE cells when SOCE activation protocol is implemented. ER Ca^{2+} content is first totally depleted in Ca^{2+} free medium by application of 2 μM thapsigargin (TG) and SOCE is next measured by addition of 1.8 mM Ca^{2+} in the extracellular medium. B, Representative traces of SOCE measurements in CFBE and 16HBE cells. Each trace represents the mean trace of multiple recordings from a representative coverslip. C, Average values of the amplitude of SOCE in CFBE (n = 77), 16HBE (n = 84) and VX-809 (n = 91 for CFBE and n = 64 for 16HBE) treated cells. D, Average iodide efflux curves after CFTR stimulation by forskolin and genistein in untreated and VX-809 treated CFBE and 16HBE cells. Cells were treated 24 h with 10 μM VX-809. E, Corresponding average values of iodide effluxes in each experimental condition (n = 4 for each condition). F, Representative traces of ER Ca^{2+} release mediated by 1 μM of TG in CFBE and 16HBE cells. Each trace represents the mean trace of multiple recordings from a representative coverslip. G, Average values of the amplitude of TG-induced ER Ca^{2+} release in CFBE (n = 61), 16HBE (n = 75) and VX-809 treated cells (n = 102 for CFBE and n = 61 for 16HBE) in 5 to 8 different experiments.

not modified $[Ca^{2+}]_{ER}$ in non-CF cells (treated 16HBE: 345.7 ± 43 μM, n = 12) (Fig. 2A).

As expected, TG stimulation induced a significantly more important ER-Ca^{2+} depletion in CF cells than in non-CF cells (Fig. 2C). Interestingly, the rate of $[Ca^{2+}]_{ER}$ decrease evoked by TG stimulation was also significantly increased in CF cells compared to corrected CF and non-CF cells (Fig. 2B and D). These data suggest that the passive Ca^{2+} permeability of the ER is also affected in CF epithelial cells or just reflect a change of the electrochemical gradient for Ca^{2+} ions between the ER and the cytoplasm. Similar differences were observed after TG-mediated ER-Ca^{2+} depletion between CF and non-CF cells when stores were depleted with an extracellular medium containing 1.8 mM Ca^{2+} (Supplemental Fig. 1C and D). VX-809 treatment of CFBE cells restored ER-Ca^{2+} release amplitude and rate to values similar to what observed in 16HBE cells (Fig. 2C and D). It has to be noticed that restoration of F508del-CFTR activity, even if significant, was less effective when ER calcium depletion was recorded in the presence of external Ca^{2+} (data not shown).

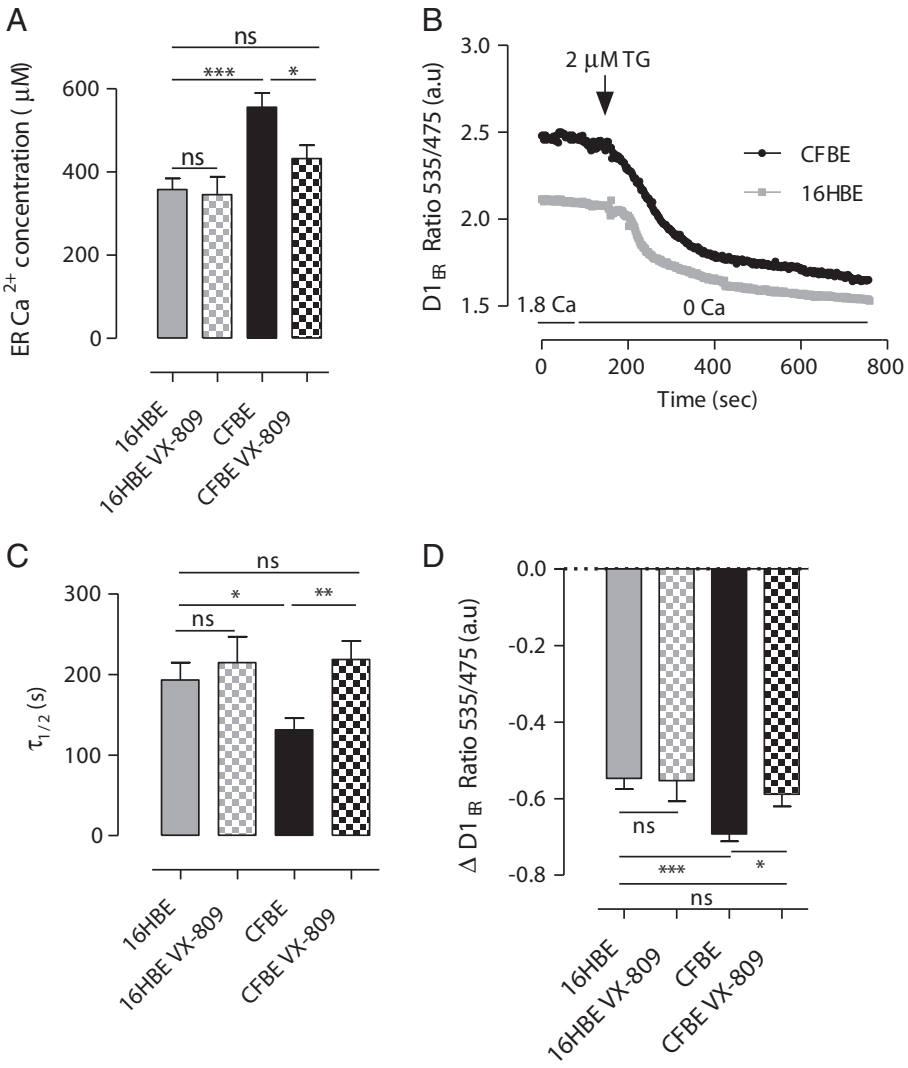

Fig. 2. Evaluation of ER Ca^{2+} concentration and release, with the $D1_{ER}$ cameleon probe, in untreated 16HBE or CFBE and in VX-809 treated cells. CFBE and 16HBE were transiently transfected with the ER-targeted cameleon probe $D1_{ER}$. A, To evaluate ER Ca^{2+} concentration, a min and a max $D1_{ER}$ fluorescence value were measured at the end of each experiment, to evaluate the resting ER Ca^{2+} concentration after calculation. Bar graph represents mean values of the ER Ca^{2+} concentration (µM) in VX-809 treated or untreated CF and non-CF cells (n = 61 for CFBE, n = 75 for 16HBE, n = 102 for VX-809 treated CFBE and n = 61 for VX-809 treated 16HBE in 4 to 9 different experiments). B, Representative recordings of ER Ca^{2+} variation in CFBE and 16HBE stimulated with 2 µM thapsigargin in Ca^{2+}-free medium. C, Bar graph corresponding to mean values of TG-induced ER Ca^{2+} depletion amplitude in each experimental condition (n = 84 for CFBE, n = 64 for 16HBE, n = 41 for VX-809 treated CFBE and n = 30 for VX-809 treated 16HBE, in 5 to 15 different experiments). D, Mean values of the TG-mediated ER Ca^{2+} release kinetic ($\Gamma_{1/2}$) measured in VX-809 treated or untreated CF and non-CF cells (n = 87 for CFBE, n = 75 for 16HBE, n = 33 for VX-809 treated CFBE and n = 21 for VX-809 treated 16HBE).

These data obtained by directly monitoring Ca^{2+} changes in the ER confirm that ER Ca^{2+} content is affected by the abnormal ER accumulation of F508del-CFTR (Fig. 2C).

3.2. F508del-CFTR correction by VX-809 normalized ATP-mediated ER Ca^{2+} depletion in CF bronchial epithelial cells

Then, we analyzed ER Ca^{2+} depletion induced by a more physiological agonist (100 µM ATP) that induces phospholipase C activation, InsP3 (inositol triphosphate) production and the subsequent release of Ca^{2+} from the ER after InsP3 receptor activation (Fig. 3A). In Fura-2 loaded cells exposed to an external Ca^{2+} free solution, ER Ca^{2+} release mediated by ATP stimulation was significantly increased in CF cells (144 AUC ± 5.3 a.u, n = 145) compared to non-CF cells (82 AUC ± 3.7 a.u, n = 178) (Fig. 3B). Interestingly, VX-809 treatment significantly decreased this ATP mediated ER Ca^{2+} release in CF cells (128 AUC ± 3.7 a.u, n = 172) while this pharmacological treatment had no impact in non-CF cells. Identical experiments realized in the presence of 1.8 mM external Ca^{2+} (Supplemental Fig. 1E and F) confirmed the increase in ATP induced ER Ca^{2+} release and the partial correction by VX-809 treatment.

We measured next the ATP-induced ER Ca^{2+} depletion using $D1_{ER}$ probes in cells exposed to an extracellular Ca^{2+} medium. As presented, in Fig. 3C, 100 µM of ATP induced a rapid ER Ca^{2+} store depletion in both human bronchial epithelial cell lines. However, the amplitude of this ER Ca^{2+} depletion was approximately 2 fold smaller than the magnitude of TG mediated depletion. In CF cells, ER Ca^{2+} depletion was significantly increased compared to non-CF cells (Fig. 3D) (CF; −0.3579 ± 0.0152 a.u (Δratio), n = 116 and non-CF; −0.2833 ± 0.0209 a.u (Δratio), n = 86). Interestingly and similarly to what observed with TG stimulation, re-localization of F508del-CFTR out of the ER towards the plasma membrane by VX-809 treatment, significantly normalized ER Ca^{2+} depletion in CF cells. As expected, VX-809 treatment had no impact on ATP induced ER Ca^{2+} depletion in 16HBE cells (Fig. 3D).

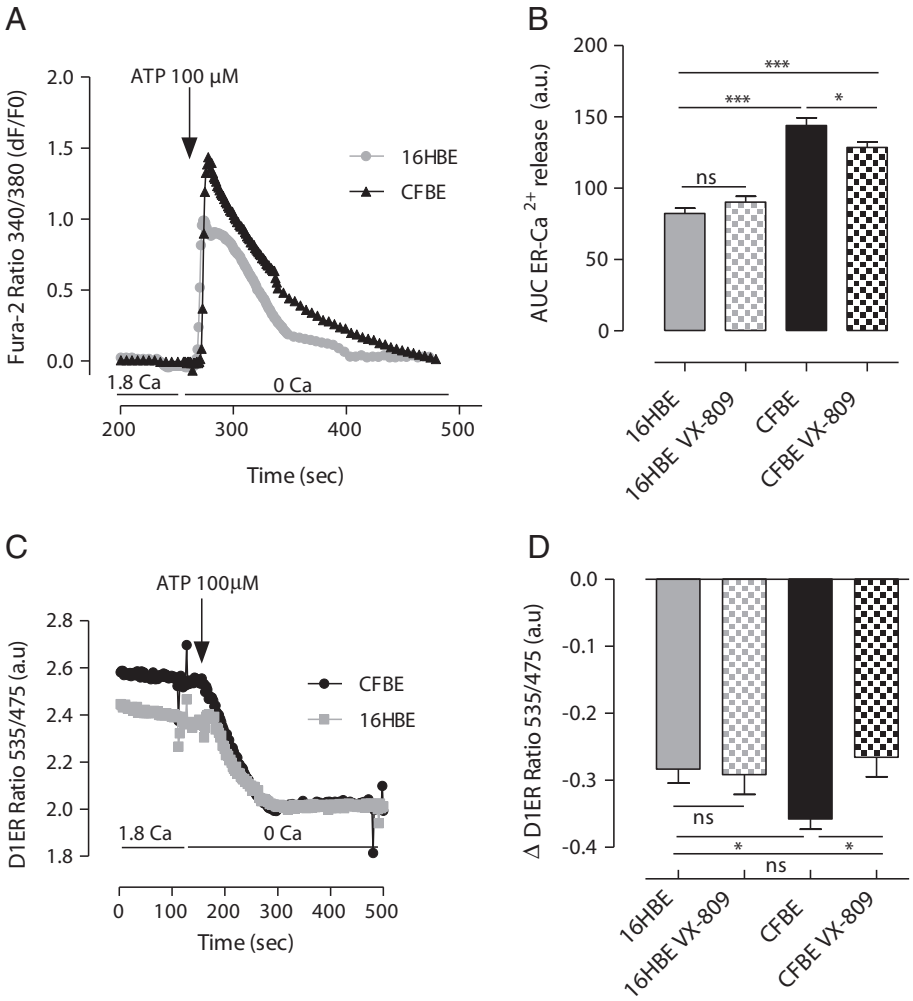

Fig. 3. ATP-mediated ER Ca^{2+} release in CF and non-CF epithelial cells. A, Intracellular Ca^{2+} was measured using Fura-2 in 16HBE and CFBE cells. ER Ca^{2+} release was stimulated by 100 µM ATP in the absence of external Ca^{2+} (1 mM EGTA, Ca^{2+} free). Each trace represents the mean trace of multiple recordings from a representative coverslip. B, Bar graphs display the mean area under the curve (AUC) of ATP-mediated ER Ca^{2+} release in CF, non-CF and VX-809 treated CF epithelial cells. (n = 145 for CFBE, n = 178 for 16HBE, n = 172 for VX-809 treated CFBE and n = 164 for VX-809 treated 16HBE, in 10 to 15 different experiments). C, CFBE and 16HBE were transiently transfected with the ER-targeted cameleon probe $D1_{ER}$. Representative recordings of ER Ca^{2+} concentration variations measured in CFBE and 16HBE cells stimulated with 100 µM ATP in a Ca^{2+}-free medium is shown. D, Mean values of the amplitude of ATP-mediated ER Ca^{2+} depletion in each experimental condition (n = 116 for CFBE, n = 86 for 16HBE, n = 44 for VX-809 treated CFBE and n = 34 for VX-809 treated 16HBE, in 6 to 12 different experiments).

3.3. Differences in ER Ca^{2+} refilling between CF and non-CF bronchial epithelial cells

In F508del-CFTR expressing cells, we have shown that $[Ca^{2+}]_{ER}$ and TG or ATP-mediated ER Ca^{2+} depletion were strongly increased compared to non-CF cells. Moreover, correction of F508del-CFTR trafficking by VX-809 treatment normalized elevated $[Ca^{2+}]_{ER}$ and ER Ca^{2+} release (Figs. 1 to 3). The only known mechanism to refill Ca^{2+} into the ER after depletion is mediated by the Sarcoplasmic Reticulum Ca^{2+} ATPase (SERCA) family protein. To explain the observed increase of $[Ca^{2+}]_{ER}$ in CF cells, we hypothesized that SERCA activity may be modified when F508del-CFTR is retained in the ER. To verify our hypothesis, we next estimated SERCA pump activity in CF and non-CF cells using $D1_{ER}$ cameleon probe. After ER Ca^{2+} depletion by ATP stimulation (100 µM) in the absence of extracellular Ca^{2+}, refilling of ER Ca^{2+} stores was induced by addition of external Ca^{2+} (1.8 mM). As represented in Fig. 4A, addition of Ca^{2+} induced a rapid increase of $[Ca^{2+}]_{ER}$ and a return to its baseline level. Quantification of ER Ca^{2+} refilling rate (Fig. 4B) approximated by the slope of ER Ca^{2+} increase, strongly suggests that SERCA pump activity is increased in CF compared to non-CF cells (CF; 0.007710 ± 0.0004164 ratio/sec, n = 111 and non-CF; 0.005773 ± 0.0006337 ratio/sec, n = 64). Correction of F508del-CFTR abnormal trafficking by VX-809 treatment also fully normalizes this change in SERCA activity in CFBE cells as we observed for ER Ca^{2+} content and release (Fig. 4B).

The significant increase in ER Ca^{2+} refilling amplitude observed in CF cells compared to non-CF-cells confirmed that $[Ca^{2+}]_{ER}$ is increased as suggested by our previous experiments. Increase in the ER Ca^{2+} refilling is normalized in CF cells treated with VX-809 (Fig. 4C).

We next measured ER Ca^{2+} refilling after ER Ca^{2+} depletion induced by tBHQ (tertiary butylhydroquinone), a reversible SERCA pump inhibitor. After ER Ca^{2+} depletion, tBHQ (15 µM) was washed away and ER Ca^{2+} refilling was then induced by addition of 1.8 mM Ca^{2+} in the extracellular medium. In these conditions, we observed similar results to what obtained with an ATP stimulation confirming the increase in SERCA pump activity in CF cells compared to non-CF (Fig. 4D–F). However, correction by VX-809 in CF cells was only observed for the amplitude of the ER Ca^{2+} refilling and not for its rate. Moreover, the expression level of SERCA2b protein is similar in 16HBE and CFBE and is not affected by VX-809 treatment (Supplemental Fig. 2A). This apparent up-regulation

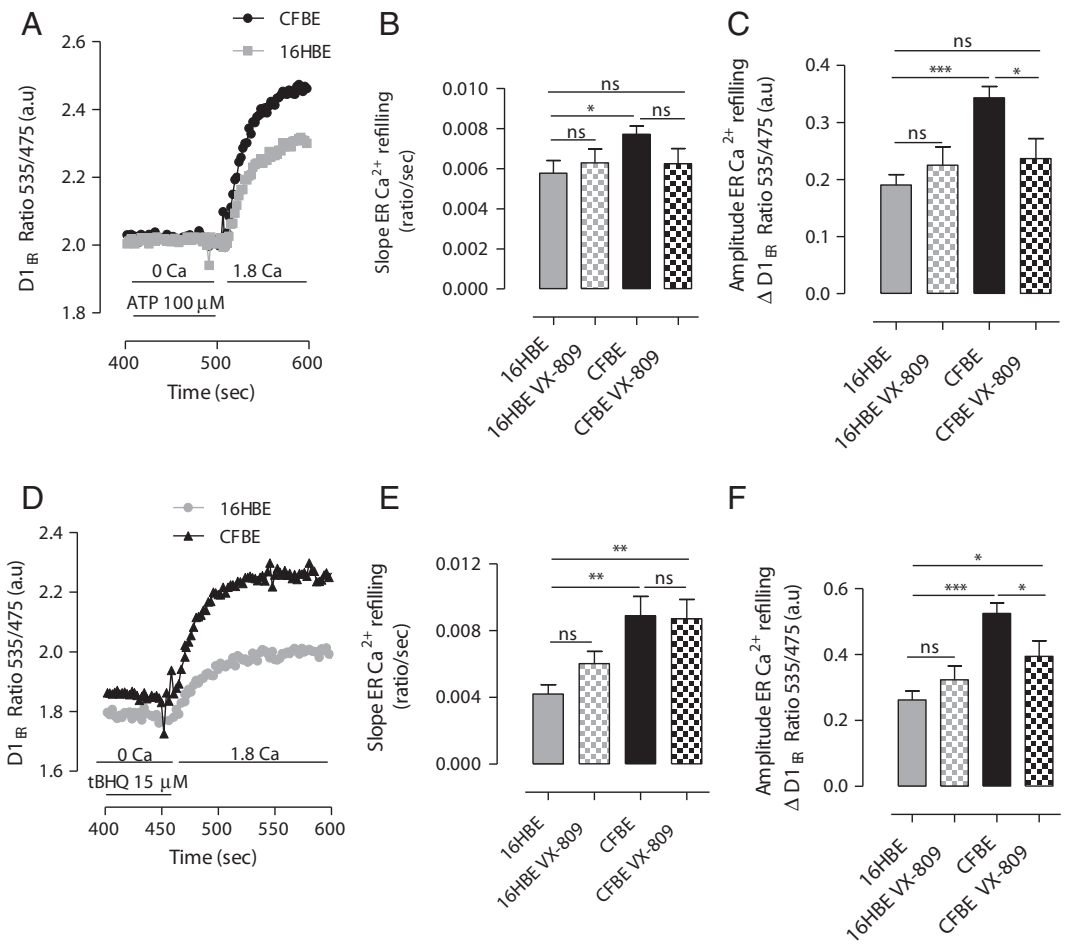

Fig. 4. ER Ca^{2+} refilling in CFBE and 16HBE cells. CFBE and 16HBE were transiently transfected with the ER-targeted cameleon probe $D1_{ER}$. A–C, Representative recordings of ER Ca^{2+} concentration variations measured in CFBE and 16HBE cells stimulated with 100 µM ATP in a Ca^{2+}-free medium. After ER Ca^{2+} depletion induced by addition of 100 µM ATP in a Ca^{2+} free extracellular medium, ER Ca^{2+} refilling was evaluated by changing to a 1.8 mM Ca^{2+} extracellular solution. B–C, Bar graphs represent average values of ER Ca^{2+} refilling rate (B) and amplitude (C) in each condition (n = 111 for CFBE, n = 64 for 16HBE, n = 29 for VX-809 treated CFBE and n = 29 for VX-809 treated 16HBE, in 5 to 12 different experiments). D–F, After ER Ca^{2+} depletion induced by tBHQ (10 min at 15 µM), a reversible inhibitor of the SERCA pump, ER Ca^{2+} refilling was evaluated by changing the Ca^{2+} free extracellular medium by a solution containing 1.8 mM Ca^{2+}. E–F, Bar graphs represent average values of ER Ca^{2+} refilling rate (B) and amplitude (C) in each condition (n = 29 for CFBE, n = 21 for 16HBE, n = 26 for VX-809 treated CFBE and n = 22 for VX-809 treated 16HBE, in 4 different experiments).

of SERCA pump activity in CF cells may also be reflected by the significant decrease of basal Fura-2 ratio value measured in CFBE cells compared to 16HBE cells (16HBE; 1.058 ± 0.004 ratio 340/380, n = 529 and CFBE; 1.002 ± 0.004 ratio 340/380, n = 532). Since our experimental conditions were always the same for Fura-2 fluorescence acquisition, this ratio difference suggests that basal cytosolic Ca^{2+} concentration is lower in CF cells. Moreover VX-809 treatment of CF cells partially restores the difference with non-CF cells (Supplemental Fig. 2B).

3.4. PMCA activity and mitochondrial Ca^{2+} uptake are modified in CF cells

In the following experiments, we compared PMCA activity in CF and non-CF treated or not with VX-809. PMCA activity was evaluated in Fura-2 loaded cells. After SOCE activation with TG, the external solution containing 1.8 mM Ca^{2+} was replaced by a free Ca^{2+} medium when SOCE reaches its maximal amplitude. In these experimental conditions, SERCA pump activity was completely abolished by TG (irreversible SERCA pump blocker) and the decrease in cytosolic [Ca^{2+}], is mainly due to PMCA activities and mitochondrial Ca^{2+} uptake. As suggested on Fig. 5A and pointed out by time constant values of the decrease (Fig. 5B), a significantly lower Ca^{2+} extrusion rate is surprisingly observed in CF cells compared to non-CF cells (CFBE: 185.4 ± 13.14 sec, n = 108; 16HBE: 99.73 ± 5.287 sec, n = 103). Interestingly, the pharmacological F508del-CFTR trafficking correction by VX-809 treatment induced a full normalization of Ca^{2+} extrusion rate, while VX-809 had no impact in non-CF cells (Fig. 5B).

We evaluated next the PMCA activity in the absence of mitochondrial Ca^{2+} uptake. We performed the same previously described experiments in the presence of 2.5 µM CCCP, a mitochondrial protonophore that uncouples mitochondria and abolishes Ca^{2+} uptakes in these organelles. As expected and observed in the absence of CCCP, the rate of Ca^{2+} extrusion was also reduced in CF cells when both mitochondrial and ER Ca^{2+} uptake are abolished (Fig. 5C–D). Moreover, we observed in CF cells that VX-809 restores Ca^{2+} extrusion due to PMCA activity to a level almost similar to non-CF cells. VX-809 treatment had no impact on PMCA activity in 16HBE cells (Fig. 5D). We confirmed by Western blot analysis that PMCA protein expression level was not significantly different between CF and non-CF cells and in VX-809 treated cells (Supplemental Fig. 2A).

To explore the influence of F508del-CFTR expression on Ca^{2+} mitochondrial uptake, we measured this uptake after SOCE activation by directly following changes in mitochondria Ca^{2+} concentration with 4mitD3cpv, a genetically encoded cameleon probe specifically targeted into mitochondria [42]. ER Ca^{2+} store was depleted by TG treatment in 4mitD3cpv transfected epithelial cells and the extracellular Ca^{2+} free medium was next changed to a solution containing 1.8 mM Ca^{2+} to

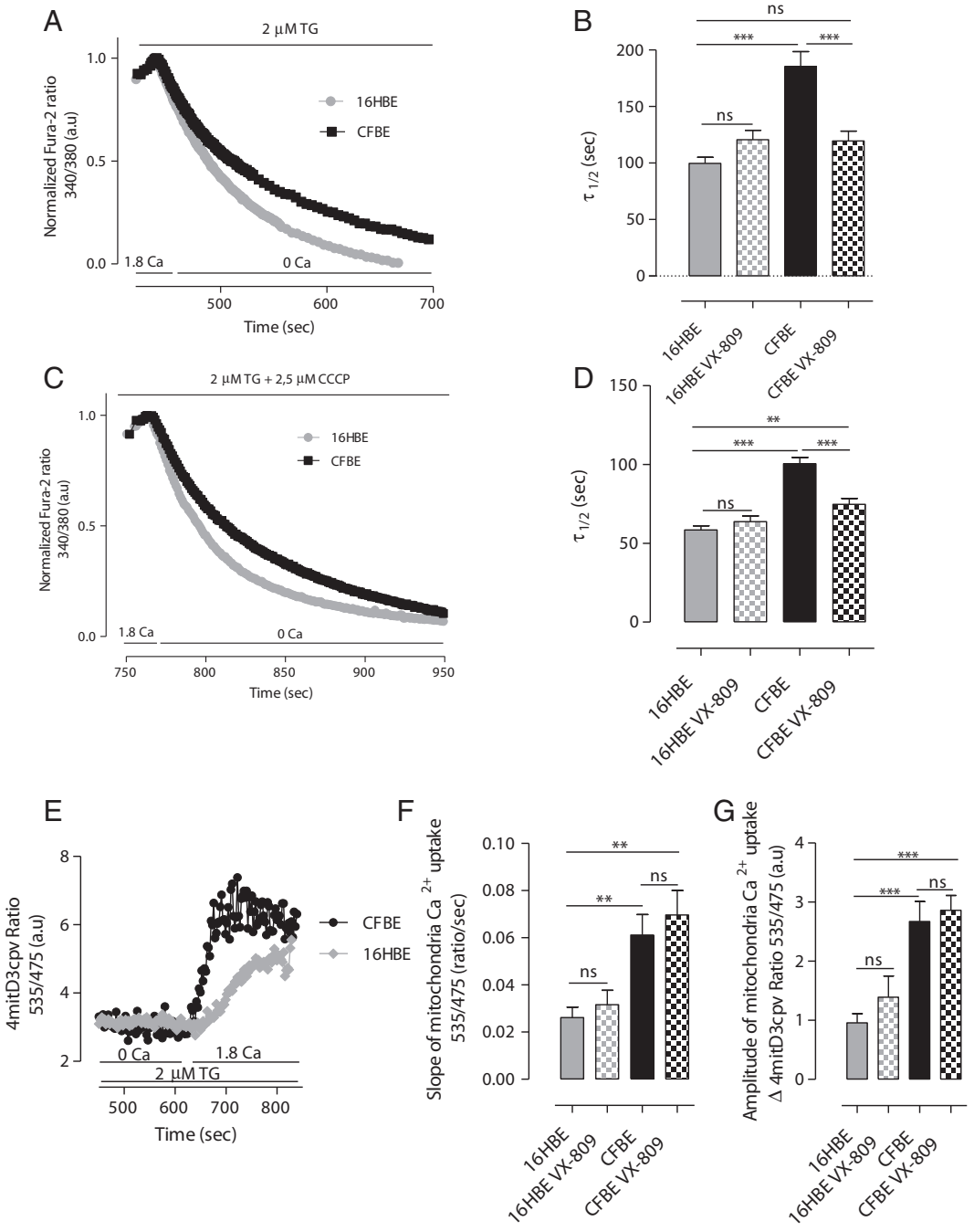

Fig. 5. Evaluation of PMCA activity and mitochondrial Ca^{2+} uptake in CF vs non-CF bronchial epithelial cells. A–D, PMCA activities evaluated after application of 2 µM TG in 1.8 mM Ca^{2+} solution in the absence (A–B) or presence (C–D) of 2.5 µM of CCCP (mitochondrial protonophore) that abrogates mitochondrial Ca^{2+} uptake. Cytosolic Ca^{2+} variations were measured in Fura-2 loaded epithelial cells. At the peak of the TG response, 1.8 mM Ca^{2+} external medium was replaced by a Ca^{2+} free solution to indirectly measure Ca^{2+} extrusion. A, Representative trace of PMCA-mediated Ca^{2+} extrusion in 16HBE and CFBE cells. B, Average values of the rate of intracellular Ca^{2+} concentration decrease, reflecting PMCA activity in untreated 16HBE, CFBE and in VX-809 treated CFBE and 16HBE cells (n = 108 for CFBE, n = 103 for 16HBE, n = 89 for VX-809 treated CFBE and n = 100 for VX-809 treated 16HBE, in 5 different experiments). C, Representative trace of PMCA-mediated Ca^{2+} extrusion in 16HBE and CFBE cells in the presence of 2.5 µM of CCCP. D, Average values of PMCA mediated Ca^{2+} extrusion in the presence of 2.5 µM CCCP (n = 102 for CFBE, n = 100 for 16HBE, n = 73 for VX-809 treated CFBE and n = 54 for VX-809 treated 16HBE, in 5 different experiments). E–G, Bronchial epithelial cells were transiently co-transfected with the mitochondrial Ca^{2+} probe 4mitD3cpv. Mitochondrial Ca^{2+} uptake was evaluated after SOCE stimulation by ER depletion with 2 µM TG, in the absence of external Ca^{2+} and then addition of 1.8 mM Ca^{2+} outside the cells. E, Variation of $[Ca^{2+}]_{mit}$ in CF and non-CF cells after SOCE activation (Average recording of one representative coverslip). F–G, Average values of the rate and amplitude of mitochondrial Ca^{2+} uptake in each conditions (n = 35 for CFBE, n = 20 for 16HBE, n = 22 for VX-809 treated CFBE and n = 21 for VX-809 treated 16HBE, in 4 different experiments).

induce Ca^{2+} entry through SOC channels. As observed in Fig. 5, mitochondrial Ca^{2+} uptake was strongly increased in CFBE cells compared to non-CF cells (CFBE; 0.06115 ± 0.008715 ratio/sec, n = 35 and 16HBE: 0.02615 ± 0.004465 ratio/sec, n = 20) (Fig. 5E–G). Surprisingly, the correction of F508del-CFTR abnormal trafficking by VX-809 treatment was not able to restore normal mitochondrial Ca^{2+} uptake suggesting that deregulation of mitochondrial Ca^{2+} uptake may not be directly related to F508del-CFTR retention in the ER.

3.5. SERCA2b and PMCA interact differently with Wt-CFTR and F508del-CFTR

As described previously in this study, SERCA and PMCA pump activities appear to be strongly modified when F508del-CFTR accumulates in the ER. As well demonstrated in the literature, CFTR is a cargo protein at the cell surface which interacts with several different proteins (named the CFTR interactome) [44]. The mislocalization of F508del-CFTR in CF modifies this interactome and induces perturbations of protein activities. We investigated by a co-immunoprecipitation approach the hypothesis that CFTR protein can interact with SERCA and PMCA pumps and therefore modify their activities (Fig. 6).

As showed in Fig. 6A, we were able to immunoprecipitate CFTR with SERCA. Both WT CFTR and F508del-CFTR are able to interact with SERCA2b in CFBE and 16HBE cells. However, as shown by the differences in band intensities, CFTR interaction with SERCA2b seems to be stronger for WT CFTR than for F508del-CFTR (Fig. 6A). To confirm the interaction

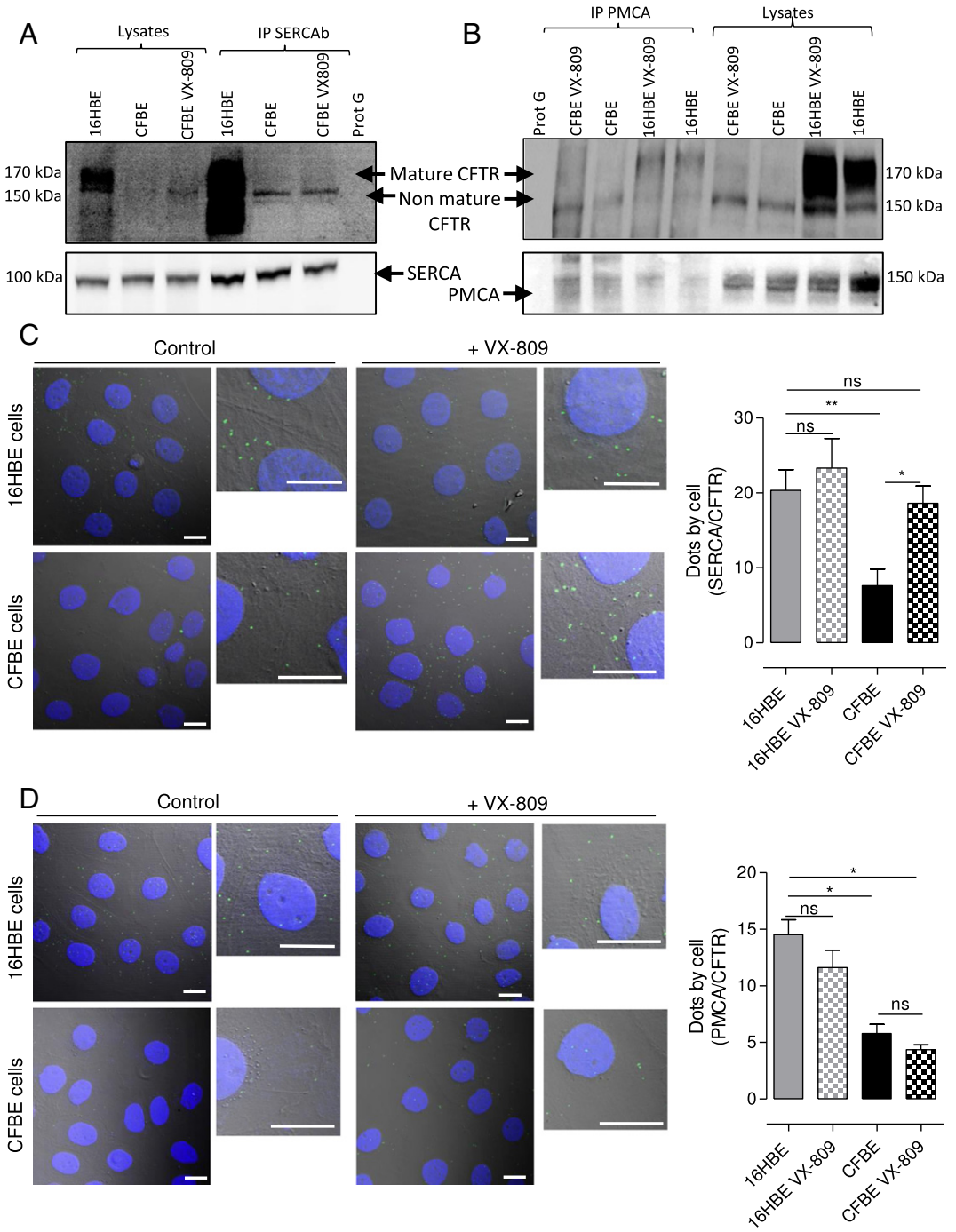

Fig. 6. PMCA and SERCA2b interaction with wild-type or F508del-CFTR in human bronchial epithelial cells. A, Co-immunoprecipitation between SERCA2b and CFTR. Western blot revelation of CFTR protein after immunoprecipitation of the SERCA pump in endogenous conditions. B, Co-immunoprecipitation between PMCA and CFTR. Western blot revelation of the CFTR protein after immunoprecipitation of the PMCA pump in endogenous conditions. C–D, Co-immunoprecipitation by Duolink assays. C, Left panel: Representative images of a Duolink experiment with SERCA2b and CFTR antibodies in CFBE and 16HBE treated or not with VX-809 (24 h at 10 μM). Green dots correspond to an interaction between SERCA2b and CFTR proteins. Scale bar 10 μM. Right panel, average values of the number of dots per cell in each experimental cell condition (n = 4–5). D, Left panel: typical images of a Duolink experiment with PMCA and CFTR antibodies in CFBE and 16HBE treated or not with VX-809 (24 h at 10 μM). Green dots correspond to an interaction between PMCA and CFTR proteins. Scale bar 10 μM. Right panel, mean values of the number of dots per cell in each experimental cell condition (n = 4–5).

and validate this latter observation, we realized Duolink assays to quantify these differences. We confirmed with this approach firstly the CFTR/SERCA2b interaction in each cellular model, and secondly a higher level of interaction between CFTR and SERCA2b in 16HBE cells compared to CFBE cells (Fig. 6C). Even if we were not able to obtain an effect of VX-809 treatment when interaction was evaluated by the IP approach, it appears that restoration of F508del-CFTR localization normalized the difference observed in interaction intensity (Fig. 6C). Additionally, we verified the interaction between CFTR/SERCA2 in BHK cells stably overexpressing WT CFTR or F508del-CFTR (Supplemental Fig. 3A and B). Both mature and non-mature forms of CFTR seem to be able to interact with the SERCA pump.

Similar experiments were next performed to investigate PMCA/CFTR interaction. As showed in Fig. 6B, we were able to detect CFTR after immuno-precipitating PMCA. Both WT CFTR and F508del-CFTR are able to interact with PMCA. However CFTR/PMCA interaction level seems to be lower in CF cells (Fig. 6B). Duolink assays confirmed the CFTR/PMCA interaction and the significantly higher interaction level between these two proteins in non-CF cells compared to CF cells. Surprisingly, VX-809 treatment was not able to correct this difference (Fig. 6D). Interaction between CFTR and PMCA was also explored in BHK cells (Supplemental Fig. 3). In WT CFTR overexpressing BHK cells, we observed a clear interaction between the mature form of CFTR (band C) and PMCA in accordance with the localization of these two proteins at the plasma membrane (Supplemental Fig. 3C). We were not indeed able to detect interaction between CFTR and PMCA in overexpressed F508del-CFTR BHK cells (Supplemental Fig. 3C).

4. Discussion

In this study, we try to decipher the deregulation of Ca^{2+} homeostasis in CF cells at the different hot spots of Ca^{2+} signaling (ER, Mitochondria, and Plasma membrane) in bronchial airway epithelial cells. Using a combination of imaging and biochemical approaches we showed that (i) ER Ca^{2+} concentration is increased in CF cells compared to CF cells, (ii) SERCA pump activity is abnormally increased and PMCA activity decreased in CF cells, (iii) CFTR interacts with SERCA2b and PMCA and these interactions may regulate their pump activities, and (iv) treatment of CF cells with the new CFTR corrector VX-809 is able to correct most of the calcium homeostasis deregulations observed.

Previous works have already reported a severe deregulation of Ca^{2+} homeostasis in CF human primary bronchial epithelial cells [13,19] and in several human epithelial cell lines [14,16,23,45]. At the plasma membrane level, the SOCE channel Orai1 and the non-SOCE Ca^{2+} channel TRPC6 activities were demonstrated to be up-regulated in 2 different CF epithelial cell models [17,19]. These defects were corrected by a pharmacological or low temperature treatment. We also observed in the present study that SOCE is abnormally increased in CFBE compared to 16HBE cells, and we demonstrated for the first time that correction of F508del-CFTR abnormal trafficking by the VX-809 corrector normalized SOCE in CF cells (Fig. 1) as observed in CF cells corrected by low temperature or miglustat exposure [14]. These results obtained in the absence of infectious factors confirm that the presence of CFTR itself at the plasma membrane down-regulates SOCE channel activity.

Concerning intracellular Ca^{2+} pools in CF cells, it was previously reported that increase in Ca^{2+} release from the ER is linked to an expansion of the ER and to an InsP3 receptor (InsP3R) hyperactivity [13,14]. InsP3R deregulation was directly correlated to the F508del-CFTR retention in the ER [20]. However consequences on ER Ca^{2+} content of F508del-CFTR accumulation in this organelle had never been directly characterized using specific targeted Ca^{2+} probes. In this context, we measured specifically $[Ca^{2+}]_{ER}$, ER Ca^{2+} refilling and depletion by a FRET approach using the ER targeted $D1_{ER}$ cameleon Ca^{2+} probe [42]. Interestingly, in CF bronchial epithelial cells, the abnormal ER retention of F508del-CFTR strongly increased $[Ca^{2+}]_{ER}$ (Fig. 2). This deregulation of $[Ca^{2+}]_{ER}$ seems to be linked to the accumulation of F508del-CFTR in the ER as suggested by the recovery in $[Ca^{2+}]_{ER}$ observed in VX-809 treated cells.

ER calcium content is mainly regulated by the SERCA pump activity and a change in its activity is a likely hypothesis to explain a change in $[Ca^{2+}]_{ER}$. By looking at the ER Ca^{2+} refilling in intact cell after store depletion, we observed indeed an increase in SERCA pump activity in CF cells compared to non-CF cells. SERCA pump is encoded by a family of three genes, SERCA1, 2, and 3. SERCA1 is expressed in fast-twitch skeletal muscle [46,47]. SERCA2 encodes SERCA2a, which is expressed predominantly in cardiac and slow-twitch skeletal muscle [48–50]. SERCA2b is ubiquitously expressed [49]. In bronchial and bronchiolar epithelial cells of CF subjects and in different CF cell lines, SERCA2a expression was shown to be significantly decreased compared to non-CF patients [51]. This decrease was linked to a lower SERCA pump activity in CF pulmonary epithelial cells. However Ca^{2+} ATPase activity was indirectly evaluated in microsomal membranes of normal and CF cells. On the contrary, we observed a similar expression of SERCA2b in CF (CFBE) and non-CF (16HBE) human bronchial epithelial cells and SERCA3 expression was poorly expressed in CFBE and 16HBE cells (Supplemental Fig. 3A). Moreover, modification of F508del-CFTR trafficking by VX-809 treatment does not affect SERCA2b expression in our cellular models (Supplemental Fig. 2) and restores SERCA pump activity in CF cells close to normal level. Altogether, our data clearly show an increase in ER Ca^{2+} release correlated with an the increase in $[Ca^{2+}]_{ER}$ consequent to an enhanced SERCA pump activity.

Free Ca^{2+} in the ER lumen is tightly regulated and is crucial for protein synthesis, recycling, and post-traductional modifications. Several Ca^{2+}-dependent chaperone proteins are localized into ER lumen [52]. Among them, calnexin (CNX) and calreticulin (CALR) have been described to interact with CFTR [44] and involved in CFTR folding and traffic. These ER proteins are strongly regulated by ER Ca^{2+} and participated to calcium homeostasis [6,33,53,54]. An ER Ca^{2+} increase contributes to the abnormal F508del-CFTR ER retention. Maintaining low Ca^{2+} level in the ER with SERCA pump inhibitors such as curcumin or thapsigargin also restores abnormal endogenous F508del-CFTR trafficking in airway epithelial cells due to a decrease interaction between F508del-CFTR and the chaperone protein calnexin [33,55,56].

CALR was shown also to bind SERCA2b and to inhibit Ca^{2+} ATPase activity [57]. Similar observation was done with CNX and SERCA [58]. Calumenin is another example of a CFTR interacting protein [59] that can inhibit SERCA2 pump function [60].

All these ER resident proteins interacting with CFTR may constitute a protein complex with SERCA pump regulating its activity. Situations such as CFTR retention in the ER will change the level of interaction between these proteins and therefore impact ER Ca^{2+} signaling.

Interestingly, in the present study we demonstrated a close interaction between CFTR and SERCA2b, and an enhancement of SERCA pump activity in CF cells that is partially normalized by the pharmacological relocalization of F508del-CFTR.

We would predict that increased CFTR protein in the ER when F508del-CFTR is expressed coupled to an increase in $[Ca^{2+}]_{ER}$ may lead to more interaction between CFTR and SERCA.

However, Co-IPs obtained between CFTR and SERCA2b suggest that the level of interaction between these two proteins is reduced in CF cells. Using a Duolink assay, we confirmed SERCA2 and CFTR interaction and the higher level of interaction between CFTR and SERCA2b in non-CF cells compared to F508del-CFTR expressing cells (Fig. 6C). Correction of aberrant localization of F508del-CFTR protein by VX-809 treatment reduced ER Ca^{2+} concentration and SERCA pump activity probably by allowing more SERCA2/CFTR interaction.

These results demonstrate that CFTR localization strongly regulates SERCA pump activity and that the deregulation of its Ca^{2+} ATPase activity contributes to the abnormal regulation of Ca^{2+} homeostasis in CF epithelial cells.

In the present study, we highlight the idea that SERCA activity is strongly up-regulated in CF epithelial cells inducing an increase of

$[Ca^{2+}]_{ER}$ in CF cells. This increase can explain the enhanced IP3R-mediated Ca^{2+} release reported in previous works [20,21] and possibly the increase in SOCE amplitude.

We also report a lower PMCA activity in CF cells that could be corrected by VX-809 treatment. CFTR was previously reported to interact with plasma membrane ion channel. CFTR was shown to be functionally and reciprocally coupled to TRPC6 channels within a molecular complex in airway epithelial human cells [17,18]. Even if somehow controversial, CFTR indirectly interacts with the epithelial sodium channel ENaC and down-regulates its activity [61,62]. Our present results suggest that PMCA is another plasma membrane ion transporter under the control of CFTR.

Here, we demonstrated for the first time and by two different approaches, a clear interaction between CFTR and PMCA and a deregulation of PMCA activities in F508del-CFTR epithelial cells. PMCA was found in caveolae and lipid rafts in different cellular types [63,64] like CFTR protein [65], suggesting that glycosylated CFTR form interacts preferentially to PMCA compared to non-CFTR mature form. Here, we showed by Co-IP that PMCA seems to interact preferentially to the glycosylated CFTR form (Fig. 6). This interaction between mature CFTR and PMCA can explain the PMCA activity decrease observed in CF cells (when mature CFTR is absent) contributing to the deregulation of Ca^{2+} homeostasis in CF epithelial cells [14].

Another important aspect in the control of global Ca^{2+} homeostasis is the role played by mitochondria in ER Ca^{2+} release and Ca^{2+} influx. Mitochondrial Ca^{2+} buffering appears also deregulated in CF. Mitochondria from CF fibroblasts are dysfunctional with an increase of Ca^{2+} signal in CF mitochondria [24]. Il-1b induced mitochondrial Ca^{2+} uptake was absent in CFTR-deficient IB3-1 cells compared to CFTR-corrected S9 cells [23]. In response to histamine stimulation, the CF mitochondria Ca^{2+} uptake is reduced compared to non-CF cells (human tracheal serous gland cells) [22]. Interestingly, in the present study, we demonstrated using a mitochondria cameleon Ca^{2+} probe (4mitD3cpv) that mitochondria Ca^{2+} uptake during SOC entry (TG stimulation) is increased in CF epithelial cells.

Surprisingly, VX809 was not able to normalize this increase in CF cells suggesting that this deregulation was not due to the mislocalization of F508delCFTR. This aspect has to be further investigated to decipher direct and indirect consequences of mutated CFTR expression and localization on Ca^{2+} homeostasis CF cells.

The proximity between ER–mitochondria and plasma membrane–mitochondria creates Ca^{2+} microdomains that play a key role for the function of both organelles particularly for mitochondria bioenergetics. In several cellular models, mitochondria–plasma membrane Ca^{2+} microdomains and the mitochondrial Ca^{2+} uptake levels regulate amplitude of CRAC currents [66–68]. More mitochondrial Ca^{2+} uptake leads to a decrease of CRAC dependent Ca^{2+} inactivation and consequently to an increase of SOCE. In line with these results, SOCE increase in CF cells [19] could be partially explained by an enhanced mitochondrial Ca^{2+} uptake as observed in our study.

We provided here a potential global vision of Ca^{2+} homeostasis deregulation in epithelial cells expressing a mutated F508del-CFTR.

Moreover, using several in vitro experimental approaches we have shown for the first time two novel CFTR protein partners: SERCA2b and PMCA. Both proteins are involved in the regulation of cellular Ca^{2+} homeostasis. A decrease in the interaction between CFTR/SERCA2b in CF cells is correlated to an enhanced activity of SERCA pump leading to the abnormal increased of $[Ca^{2+}]_{ER}$. The absence of CFTR to the plasma membrane in CF cells leads to a decrease in CFTR/PMCA interaction resulting in a lower activity of the PMCA pump. Moreover, we are also showing for the first time that in CF cells, re-localization of F508del-CFTR to the plasma membrane by the corrector VX-809 is able to correct defects in SOCE, $[Ca^{2+}]_{ER}$, ER Ca^{2+} refilling and Ca^{2+} extrusion but does not modify mitochondrial Ca^{2+} uptake perturbations. Our data confirmed that CFTR protein is more than a Cl^- channel and that its mislocalization controls numerous proteins including SERCA2b and PMCA.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.01.010.

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