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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