Biochimica et Biophysica Acta xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis☆

Joana Raquel Martins [a,b], Patthara Kongsuphol [a], Eva Sammels [d], Shehrazade Daimène [b], Fadi AlDehni [a], Luka Clarke [b], Rainer Schreiber [a], Humbert de Smedt [d], Margarida D. Amaral [b,c], Karl Kunzelmann [a,*]

[a] Institut für Physiologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany
[b] University of Lisboa, Faculty of Sciences, BioFIG, Centre for Biodiversity, Functional and Integrative Genomics, Portugal
[c] Centre of Human Genetics, National Institute of Health, Lisboa, Portugal
[d] Department Molecular Cell Biology, K.U. Leuven, Leuven, Belgium

ARTICLE INFO

Article history:
Received 8 May 2011
Received in revised form 9 August 2011
Accepted 23 August 2011
Available online xxxx

Keywords:
TMEM16A
Bestrophin 1
Ca^{2+} activated Cl^- current
CaCC cystic fibrosis
CF inflammation
Purinergic receptor
LPS
Endoplasmic reticulum
Counter-ion channel
Cl^- channel

ABSTRACT

In many cells, increase in intracellular calcium ($[Ca^{2+}]_i$) activates a Ca^{2+}-dependent chloride (Cl^-) conductance (CaCC). CaCC is enhanced in cystic fibrosis (CF) epithelial cells lacking Cl^- transport by the CF transmembrane conductance regulator (CFTR). Here, we show that in freshly isolated nasal epithelial cells of F508del-homozygous CF patients, expression of TMEM16A and bestrophin 1 was unchanged. However, calcium signaling was strongly enhanced after induction of expression of F508del-CFTR, which is unable to exit the endoplasmic reticulum (ER). Since receptor-mediated $[Ca^{2+}]_i$ increase is Cl^- dependent, we suggested that F508del-CFTR may function as an ER chloride counter-ion channel for Ca^{2+}. This was confirmed by expression of the double mutant F508del/G551D-CFTR, which remained in the ER but had no effects on $[Ca^{2+}]_i$. Moreover, F508del-CFTR could serve as a scavenger for inositol-1,4,5-trisphosphate [IP3] receptor binding protein released with IP_3 (IRBIT). Our data may explain how ER-localized F508del-CFTR controls intracellular Ca^{2+} signaling.

1. Introduction

There is a well described relationship between the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated Cl^- channels (CaCCs). Enhanced Ca^{2+} activated Cl^- conductance has been detected in the airways of cystic fibrosis (CF) patients and in cultured airway cells [1–4]. Other studies demonstrated an inhibitory effect of CFTR on CaCCs [5,6]. Yet, the reasons for enhanced CaCC in CF cells remained unclear, although polarized primary cultures, exhibit an expansion of the endoplasmic reticulum (ER) compartment, which correlated with enhanced Ca^{2+}-dependent activation of luminal Cl^- channels [7].

Pro-inflammatory pathways which appear to be constitutively active in CF airways, may be the cause for the observed $Ca^{2+}{}_i$ mobilization from ER Ca^{2+} stores and the augmented Ca^{2+}-activated Cl^- conductance. However, it is currently under debate whether upregulation of pro-inflammatory pathways in CF airway epithelial cells leading to those ER/Ca^{2+} effects, is due to exogenous infection or if this is an intrinsic property of CF cells, caused by misfolded F508del-CFTR, unfolded protein response (UPR) and ER-stress [8]. Increased ER mass and ER-derived $Ca^{2+}{}_i$ signals revert to normal in primary cultures of F508del-CF bronchial epithelia maintained in the absence of luminal infection, while primary cultures of non-CF cells exhibit the same ER/Ca^{2+} effects as CF cells, when exposed to supernatant purulent material (SMM) collected from lungs of CF patients [9]. Moreover, patients with other non-F508del mutants not retained in the ER may also have enhanced CaCCs [2]. Thus, the current view is that the ER expansion observed in CF cells is, in fact, an acquired epithelial response to chronic airway infection/inflammation. Still, the ER/Ca^{2+} effects in CF cells may be accentuated by the absence of wtCFTR expression in epithelial cells [7,10–13]. However, how CFTR plays a role in such events leading to enhanced Ca^{2+} signaling remains unclear.

Two Cl^- channels have been shown to contribute to Ca^{2+}-dependent Cl^- conductance in the airways [14]. Bestrophin 1 (Best-1) facilitates Ca^{2+} activated Cl^- conductance, as airway epithelial cells from Best-1 knockout mice have reduced ATP-dependent Cl^- conductance [15]. Recent data suggest that ER-localized Best-1 facilitates receptor-mediated intracellular Ca^{2+} signaling, probably by serving as

☆ This work has not been supported by the tobacco industry.

* Corresponding author. Tel.: +49 941 943 4302; fax: +49 941 943 4315.
E-mail address: karl.kunzelmann@vkl.uni-regensburg.de (K. Kunzelmann).

0925-4439/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.bbadis.2011.08.008

a Cl^- counter-ion channel in the ER [16]. In contrast TMEM16A was identified as the luminal membrane localized Ca^{2+}-activated Cl^- channel [14,17–19]. By analyzing Cl^- transport in TMEM16A null mice, we demonstrated that TMEM16A is essential for Ca^{2+}-dependent Cl^- secretion as well as mucociliary clearance of mouse airways [20,21]. Therefore we further investigated whether expression of Best-1 and TMEM16A is different in CF and non-CF epithelial cells, and also whether expression of these two CaCCs changes upon exposure to bacterial lipopolysaccharide (LPS). We were unable to detect significant differences in expression of TMEM16A and Best-1 and propose a mechanism for increased receptor-mediated Ca^{2+} signaling in F508del-CFTR expressing cells.

2. Methods

2.1. Cell culture

CFBE cells stably expressing wtCFTR or F508del-CFTR [22] were a generous gift from Dr. J.P. Clancy (University of Alabama at Birmingham, Birmingham, Alabama). Baby hamster kidney (BHK) cells were transfected with wtCFTR or F508del-CFTR. A549 cells were grown on glass cover slips and expression of wtCFTR or F508del-CFTR was induced with 1 μg/ml doxycycline (Sigma-Aldrich, Taufkirchen, Germany). Calu3 cells were grown on permeable supports under air–liquid interface (ALI) conditions. For detection of IL-8 secretion, media from CFBE cells were collected following exposure to LPS. Interleukin (IL)-8 was measured by ELISA (R&D Systems) according to manufacturer instructions.

2.2. cDNAs, siRNAs and transfection

cDNA for humanTMEM16A was inserted with a FLAG tag into pcDNA3.1V5-His (Invitrogen, Karlsruhe, Germany). The pRK5 vector carrying cDNA for human bestrophin-1 (hbest1, NM_004183) was kindly provided by Dr. Hugh Cahill, (John Hopkins University, USA). The N-glycosylation (Asn-Ala-Thr) site was inserted into the N-terminus of hBest1. Mouse IRBIT (NM_145542) was cloned into PEXPR-IBA103.

2.3. Participant selection and nasal cell collection

Following approval by the Ethical Review Board of the University of Lisbon and written consent by patients, samples were collected from F508del-homozygous CF through nasal scraping, and RNA was isolated as described earlier [23]. Real-time PCR was used to quantify the amount of a target sequence in a cDNA sample using an ABI 7000 Sequence detection System (Applied Biosystems) and primers: hTMEM16A (NM_018043.4) 5′-CCTCACGGGCTTTGAAGAG-3′, 5′-CTCCAAGACTCTGGCTTCGT-3′; hBest1 (NM_004183) 5′-TCTTCACGTTCCTGCAGTTCT-3′, 5′ TCCTCTCCAAAGGGGTTGAT 3′ and β-actin (NM_001101) 5′-CAACGGCTCCGGCATGTG-3′, 5′-CTTGCTCTGGGCCTCGTC-3′.

2.4. Western blotting, co-immunoprecipitation, antibodies, N-glycosylation assay, immunofluorescence

Expression of hbest1-NG was analyzed by Western blotting as described earlier [24,25]. Co-immunoprecipitation experiments were performed in A549 cells overexpressing wtCFTR or F508del-CFTR and in baby hamster kidney (BHK) cells transiently expressing wtCFTR or F508del-CFTR according to Refs. [24,25]. For immunofluorescence BHK cells were transfected with hbest1-FLAG or TMEM16A- FLAG-His and fixed with 4% (v/v) formaldehyde. Permeabilized cells were treated with Triton X-100 (0.25% w/v) for 20 min and incubated with the ANTI-FLAG® M2 antibody for 45 min. Cells were observed using an Axioskop fluorescence microscope (Zeiss, Jena, Germany).

2.5. Iodide quenching, Ca^{2+} measurement

Quenching of intracellular fluorescence generated by the iodide sensitive YFP (EYFPI152L) was used to measure anion conductance as described earlier [26]. For measurement of intracellular Ca^{2+} concentrations, cells were loaded with 2 μM Fura-2/AM (Molecular Probes, Eugene, OR) and Ca^{2+} measurements were performed as described earlier [16]. For measurements under polarized conditions, CFBE cells were grown on 12-mm Snapwell coated permeable supports (300,000 cells/well, Costar). The media from the apical compartment was removed to enable an air–liquid interface (ALI). Measurements were performed after 3 days.

2.6. Double electrode voltage clamp (DEVC)

cRNAs encoding IRBIT, $P2Y_2$-receptors or F508del-CFTR were injected and oocytes from *Xenopus laevis* female frogs were measured in DEVC as described previously [16].

3. Results

3.1. Cellular localization of TMEM16A and Best-1

TMEM16A has been identified as a CaCC showing all typical features described previously for endogenous channels [18,27]. Previous work indicated that endogenous TMEM16A is membrane localized while Best-1 resides in the ER [14,16]. We reexamined membrane expression using fluorescent labeled protein constructs overexpressed in BHK cells. With the help of an extracellularly accessible Flag-tag, TMEM16A can be clearly localized in the cell membrane (Supplement 1A). In contrast to TMEM16A, Best-1 was found in an intracellular compartment (Supplement 1B). Moreover, by introducing glycosylation sites, we demonstrate that Best-1 remains in a core-glycosylated form as shown by incubation of cell lysates with N-glycosidase F or Endo H which similarly produced only a single band of lower mobility, indicating that the low-mobility form is endoH-sensitive and hence ER-specific (Supplement 1C). These data reinforce that indeed Best-1 does not move out of the ER and therefore does not reach the cell membrane.

The present result is in complete agreement with recent findings, which locate Best-1 in the ER, where it augments intracellular Ca^{2+} signaling due to its function as a counter-ion channel [16]. In contrast, TMEM16A is a strictly membrane localized Ca^{2+}-activated Cl^- channel that forms the luminal exit pathway for Cl^-[14]. Both ion channels are expressed endogenously in Calu3 cells where both contribute to Ca^{2+}-dependent Cl^- secretion. As expected activation of CaCC in Calu3 cells was inhibited by siRNA-suppression of both TMEM16A and Best-1 (Fig. 1) [14]. The effect of siRNA for Best-1 was even more pronounced when compared with siRNA-TMEM16A, which is probably due to higher efficacy (80% vs. 50%) in inhibiting mRNA.

3.2. Expression of TMEM16A and bestrophin 1 in CF and Non-CF airway epithelial cells and effects of LPS

Since enhanced Ca^{2+}-activated Cl^- conductance has been reported earlier in CF nasal epithelial cells [2], we investigated here whether this is due to increased transcript levels for TMEM16A and Best-1. To this end, we quantitatively analyzed by real-time qRT-PCR levels of transcripts from these two genes in freshly isolated nasal epithelial cells from 5 non-CF healthy individuals (mean age = 14.8 years, 2 male and 3 female) and 5 patients homozygous for F508del-CFTR (mean age = 15.8 years; 3 male and 2 female), as well as in isogenic human airway epithelial cell lines expressing wt- or F508del-CFTR. In native nasal cells, transcripts for TMEM16A were abundant in contrast to those from Best-1, which were expressed at very low levels. However, no significant differences between CF

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

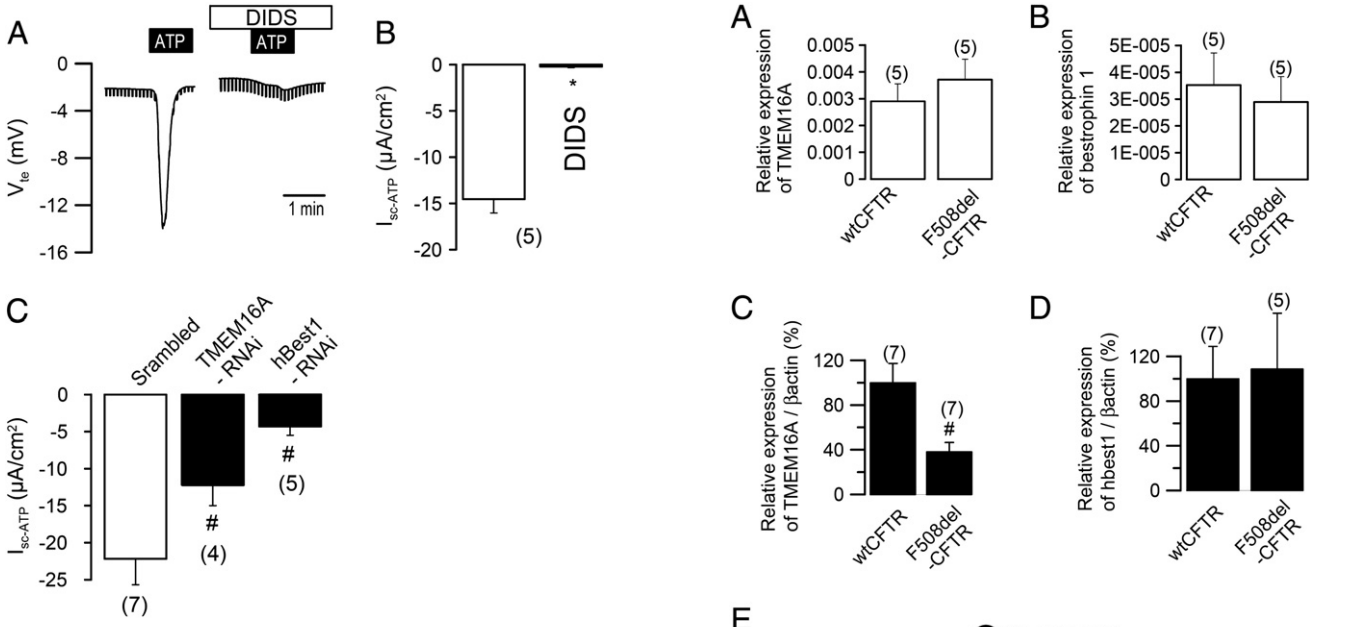

Fig. 1. Contribution of TMEM16A and bestrophin 1 to Ca^{2+} activated Cl^- conductance in polarized Calu3 cells. A) Original traces of the transepithelial voltage measured in Calu3 cells grown on permeable supports. Activation of Ca^{2+} activated Cl^- conductance (CaCC) by ATP (100 μM) as indicated by negative voltage deflection. Activation of CaCC by ATP was inhibited by the CaCC-inhibitor DIDS (200 μM). B) Summary of the activation of short circuit currents by ATP in the absence and presence of DIDS. C) Summary of ATP-activated equivalent short circuit currents (I_{sc-ATP}) in Calu3 cells treated with scrambled siRNA and siRNA for TMEM16A or bestrophin 1. Mean ± SEM, (n) = number of monolayers measured. *Significant effect of DIDS on I_{sc-ATP} (paired *t*-test). # Significant difference between control cells (scrbld) and siRNA treated cells (unpaired *t*-test).

Fig. 2. Expression of TMEM16A and bestrophin 1 in CF and Non-CF airway epithelial cells and effect of LPS. A) Real-time RT-PCR analysis of TMEM16A-expression in freshly isolated nasal epithelial cells from non-CF volunteers and patients homozygous for F508del-CFTR. B) Real-time RT-PCR analysis of bestrophin 1-expression in freshly isolated nasal epithelial cells from non-CF volunteers and patients homozygous for F508del-CFTR. C,D) Real-time RT-PCR analysis of expression of TMEM16A and bestrophin 1 in CFBE cells stably expressing wtCFTR or F508del-CFTR. E) Time course of IL-8 secretion from CFBE cells following exposure to two different concentrations of LPS. F,G) Real-time RT-PCR analysis of expression of TMEM16A and Best-1 in CFBE cells stably expressing wtCFTR or F508del-CFTR. Change of expression (%) induced by incubation of cells with LPS (10 μg/ml, 18 h). No significant changes were induced by LPS. Mean ± SEM, (n) = number of experiments. # Significant difference between wtCFTR and F508del-CFTR and significant increase in IL-8 secretion (unpaired *t*-test).

and non-CF cells were found in the levels of transcripts from both ion channels neither in native cells (Fig. 2A,B) nor in cell lines (Fig. 2C,D). Reduced levels of mRNA for TMEM16A in F508del-expressing CFBE cells are probably due to a clonal drift. Although the number of patients participating in this study is limited, it is probably unlikely that changes in ion channel expression account for enhanced CaCC activity in native CF airway epithelial cells.

We further examined whether exposure to lipopolysaccharides (LPS; 10 μg/ml) for 18 h regulate TMEM16A or Best-1 in wt- and F508del-CFTR CFBE cells. We decided to choose the CFBE cell model in order to have stable cellular background conditions, when comparing the effects of F508del-CFTR and wtCFTR on intracellular Ca^{2+} signalling and Ca^{2+} activated Cl^- currents. For detection of IL-8 secretion, media were collected from CFBE cells following exposure to two different concentrations of LPS. Because we found maximal IL-8 secretion after 18 h incubation with LPS at 10 μg/ml, we used this concentration to analyze transcripts by real-time RT-PCR (Fig. 2E). The data indicate that after incubation with LPS for 18 h, expression of TMEM16A or Best-1 (shown as relative changes in expression) were not significantly changed in CFBE cells expressing either wtCFTR or F508del-CFTR (Fig. 2F,G). Taken together, enhanced Ca^{2+} activated Cl^- conductance observed in airway epithelial cells expressing F508del-CFTR is not explained by an increase in transcripts of known CaCCs. While CFBE/wt-CFTR cells did not show activation of CaCC by stimulation with the purinergic agonist UTP (100 μM), activation of CaCC was readily detectable in CFBE/F508del-CFTR cells (Supplement 2A,B). As expected, substantial activation of a CFTR Cl^--conductance by adenosine (100 μM) was observed in CFBE/CFTR but not in CFBE/F508del-CFTR cells (Supplement 2A,B). Moreover, exposure to LPS (10 μg/ml 18 h) only slightly enhanced activation of CaCC by UTP in both CFBE/wt-CFTR and CFBE/F508del-CFTR cells (Supplement 2C,D). Thus neither the presence of F508del-CFTR nor exposure to pro-inflammatory agents such as LPS increases ion channel expression.

3.3. F508del-CFTR changes intracellular Ca^{2+} signaling

Since expression of Ca^{2+} activated Cl^- channels was not different in cells expressing F508del-CFTR, we examined whether F508del-CFTR has an effect on receptor-mediated Ca^{2+} signaling. An increase in agonist-mediated $[Ca^{2+}]_i$ signals in human CF airway epithelia has

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

been already reported earlier [7]. In the present study, intracellular Ca^{2+} was enhanced by UTP stimulation (100 μM) in both CFBE/wt-CFTR and CFBE/F508del-CFTR cells (Fig. 3). We found that UTP-induced increases in both peak and plateau $[Ca^{2+}]_i$ were significantly enhanced in CFBE/F508del-CFTR cells, while baseline $[Ca^{2+}]_i$ was identical in both CFBE/wt-CFTR and CFBE/F508del-CFTR cells (Fig. 3A–C). We also examined whether exposure to LPS (10 μg/ml, 18 h) changed intracellular Ca^{2+} signals induced by UTP (100 μM) in CFBE/wt-CFTR and CFBE/F508del-CFTR cells. The summary of these experiments indicates a 10% increase of the UTP-induced peak $[Ca^{2+}]_i$ but a 40% decrease of the Ca^{2+} plateau after exposure to LPS, which was however only observed in F508del-CFTR expressing cells (Fig. 3D). We propose that the small increase in peak Ca^{2+} is due to slightly augment Ca^{2+} release from the ER-store, while reuptake of Ca^{2+} into the ER-store may be enhanced under LPS treatment, thus reducing plateau Ca^{2+}. It remains currently unclear why these effects were only observed in F508del-CFTR cells. Finally, Ca^{2+} signals were also measured in CFBE cells grown under polarized (ALI) conditions, which fully confirm results obtained in cells grown on glass cover slips (Fig. 3E,F).

To further examine how F508del-CFTR changes intracellular Ca^{2+} signaling, and to demonstrate that this was not unique to CFBE cells, we created inducible airway epithelial (A549) cell lines, which stably express wtCFTR or F508del-CFTR under an inducible (Tet-ON) promoter (Figs. 4A, 5A). Both cell lines were seeded at exactly the same density and both wtCFTR and F508del-CFTR expressing cells were measured as confluent monolayers. Moreover, for the measurements we discarded cells with excessive CFTR-expression or cells showing excessive loading with Fura2, in order to avoid cell stress and unfolded protein response (UPR). We measured UTP (100 μM) activated Ca^{2+} transients in wtCFTR A549 cells under non-induced (no expression) and induced (expression of wtCFTR) conditions, and found no increase in $Ca^{2+}{}_i$ but rather a significant decrease in both peak and plateau (Fig. 4C). This is due to the depolarization of the membrane voltage by CFTR, which reduces the driving force for Ca^{2+} release and influx [28]. Notably, CFTR is also activated by stimulation with UTP [29]. In contrast, induction of expression of F508del-CFTR significantly increased Ca^{2+} signals elicited by stimulation with UTP (Fig. 5B,C). Thus, expression of ER-localized F508del-CFTR clearly augments intracellular Ca^{2+} signals elicited by stimulation of GTP-coupled membrane receptors.

3.4. ER-trapped F508del-CFTR facilitates Ca^{2+} movements by acting as a Cl^- counter-ion channel

Next, we examined the mechanism by which ER-localized F508del-CFTR possibly augments intracellular Ca^{2+} signals. We hypothesized that F508del-CFTR trapped in the ER could affect receptor-mediated Ca^{2+} release from ER Ca^{2+} stores. One possibility is that F508del-CFTR functions as a Cl^- channel in the ER membrane, thereby allowing Cl^- fluxes in parallel to Ca^{2+} movements. Such a counter-ion channel

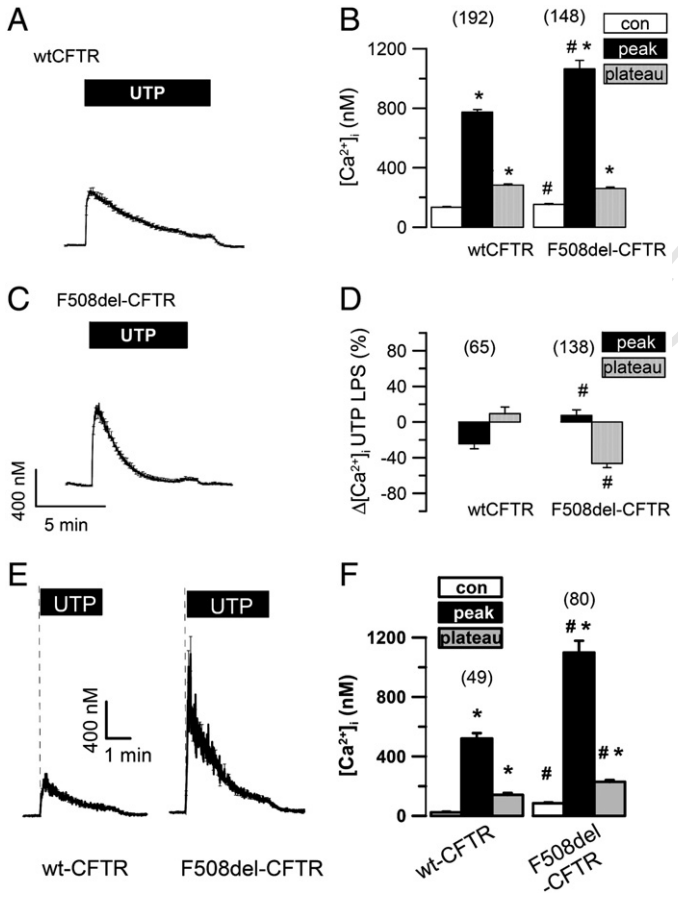

Fig. 3. Ca^{2+} signaling in CFBE/wt-CFTR and CFBE/F508del-CFTR and effect of LPS. A,C) Summary recordings of increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in CFBE/wt-CFTR (A) and CFBE/F508del-CFTR (C) cells activated by stimulation with UTP (100 μM). B) Summary of baseline $[Ca^{2+}]_i$ and UTP-induced increase peak and plateau $[Ca^{2+}]_i$ after stimulation with UTP of CFBE/wt-CFTR and CFBE/F508del-CFTR (D) cells. D) Change (%) of UTP-induced peak and plateau $[Ca^{2+}]_i$ after incubation of CFBE/wt-CFTR and CFBE/F508del-CFTR cells with LPS (10 μg/ml, 18 h). E) Summary recordings of $[Ca^{2+}]_i$ in CFBE/wt-CFTR (left) and CFBE/F508del-CFTR (right) cells. F) Summary of Ca^{2+} levels (right) obtained in polarized grown CFBE cells. Mean ± SEM, (n) = number of cells measured (corresponding to the number of experiments). *Significant effects of UTP (paired *t*-test). #Significant difference between wtCFTR and F508del-CFTR or control and LPS-treatment (unpaired *t*-test).

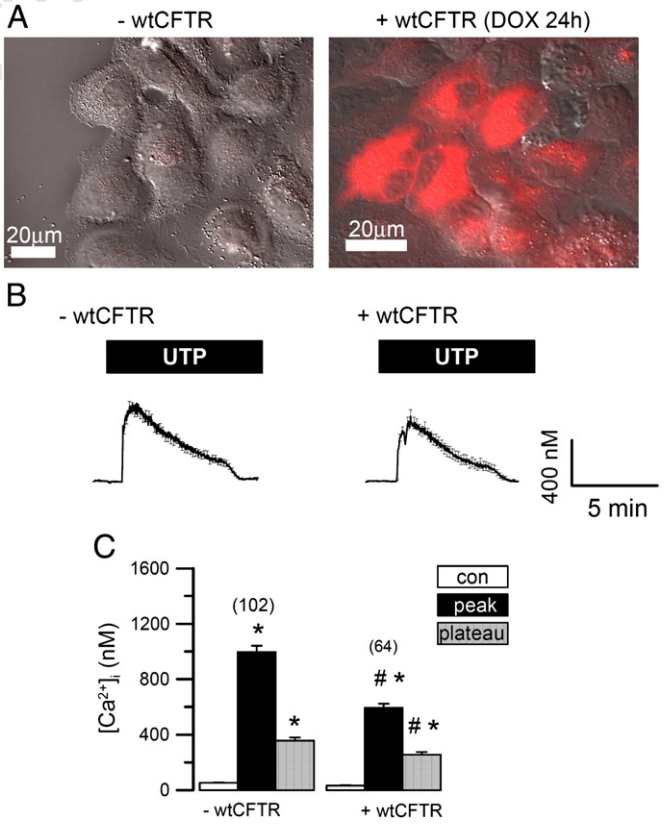

Fig. 4. Induction of expression of wtCFTR does not augment Ca^{2+} signaling. A) Expression of wtCFTR in non-induced (left upper) and induced (right upper) A549 cells. B) Original recordings of intracellular Ca^{2+} $[Ca^{2+}]_i$ signals elicited by UTP (100 μM) in non-induced (no wtCFTR, left) and induced (wtCFTR, right) A549 cells. C) Summary of baseline $[Ca^{2+}]_i$ (con) and peak and plateau $[Ca^{2+}]_i$ after stimulation with UTP. *Significant effects of UTP (paired *t*-test). #Significant difference between +/− wtCFTR and F508del-CFTR (unpaired *t*-test).

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

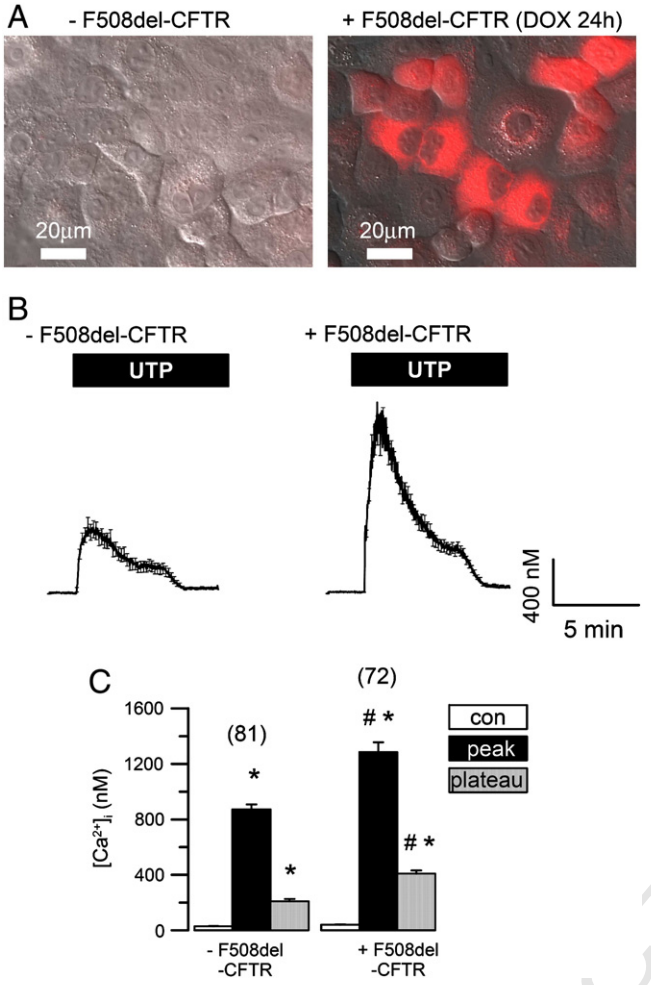

Fig. 5. Induction of expression of F508del-CFTR augments Ca^{2+} signaling. A) Expression of F508del-CFTR in non-induced (left upper) and induced (right upper) A549 cells. B) Original recordings of intracellular Ca^{2+} ($[Ca^{2+}]_i$ signals elicited by UTP (100 μM) in non-induced (no F508del-CFTR, left) and induced (F508del-CFTR, right) A549 cells. C) Summary of baseline $[Ca^{2+}]_i$ (con) and peak and plateau $[Ca^{2+}]_i$ after stimulation with UTP. Bar = 20 μm. Mean ± SEM, (n) = number of cells measured. *Significant effects of UTP (paired *t*-test). #Significant difference between wtCFTR and F508del-CFTR (unpaired *t*-test).

function has been discussed for a long time and was recently proposed to be the Ca^{2+}-activated Cl^- channel Best-1 [16]. We therefore, tested whether UTP-induced Ca^{2+} signaling in A549 cells was Cl^- dependent. To that end, we measured $Ca^{2+}{}_i$ transients in induced A549/wtCFTR and A549/F508del-CFTR cells in Cl^--depleted cells (0 mM extracellular Cl^-; Fig. 6A). UTP-induced increase in $[Ca^{2+}i]$ was largely reduced in the absence of extracellular Cl^- and when compared to Ca^{2+} increase in physiological Ringer (145 mM extracellular Cl^-) solution (Fig. 3B). Notably, attenuation of Ca^{2+} transients in Cl^- free solution was more pronounced in F508del-CFTR expressing cells (Figs. 3B, 6A) and increased Ca^{2+} signals in F508del-CFTR expressing cells were no longer observed under Cl^- free conditions (Fig. 6A).

In order to further examine whether the ability of F508del-CFTR to produce a Cl^- conductance in the ER membrane may influence the Ca^{2+} signal, we expressed the double mutant F508del/G551D-CFTR that i) does not traffic to the cell membrane but remains in the ER (caused by the F508del mutation; Fig. 6C) and ii) does not operate as a Cl^- channel, due to defective channel gating (caused by the G551D mutation; data not shown). The experiments were performed on confluent monolayers. In fact, A549 cells expressing the double mutant F508del/G551D-CFTR cells did no longer produce enhanced Ca^{2+} signals (Fig. 6B). These results suggest that the presence of F508del-CFTR in the ER, which has been shown earlier to be functional when retained in endoplasmic reticulum [30], may affect intracellular Ca^{2+} signaling by acting as a Cl^- counter-ion channel, thereby facilitating Ca^{2+} movement over the ER membrane.

3.5. Expression of IRBIT antagonizes enhanced Ca^{2+} signals in *Xenopus* oocytes

It was recently reported, that the protein inositol-1,4,5-trisphosphate [IP3] receptor binding protein released with IP_3 (IRBIT) binds to CFTR when released from the IP_3-receptor upon binding of inositol-1,4,5-trisphosphate [IP3] [25]. Because F508del-CFTR accumulates in the ER being thus in close proximity to the IP_3-receptor, we speculated that F508del-CF may compete with IP_3receptors for binding to IRBIT [31]. Thus reduced binding of IRBIT to the IP_3receptor would enhance agonist-induced Ca^{2+} release from the ER. To test this hypothesis, we made use of the expression system in *Xenopus* oocytes since it allows parallel expression of several proteins. Although oocytes express at low temperatures (18 °C), the amount of mature F508-CFTR in the cell membrane is probably low as whole cell conductance in these cells were only 12 ± 2.1 μS (n = 12), which is low compared to wtCFTR-expressing oocytes (123 ± 10.7 μS; n = 14). When $P2Y_2$-receptors were expressed in oocytes, stimulation by UTP (100 μM) activated endogenous Ca^{2+}-dependent TMEM16A channels and produced a transient and outwardly rectifying whole cell Cl^- current (Fig. 7A). Notably, co-expression of wtCFTR with $P2Y_2$-receptors slightly, but significantly augmented the Ca^{2+}-activated Cl^- current, while co-expression of F508del-CFTR induced a significantly larger Ca^{2+} activated Cl^- current (Fig. 7B–D). Additional co-expression of IRBIT had little effects on Ca^{2+} activated currents in wtCFTR-expressing cells, but dramatically inhibited UTP-induced currents in F508del-CFTR co-expressing cells (Fig. 7D). These results would be in agreement with the idea that F508del-CFTR accumulating in the ER may bind IRBIT thereby facilitating agonist-induced IP_3 binding to the IP_3 receptor and further increase in intracellular Ca^{2+}, or it could simply be a non-specific antagonistic effect of IRBIT. To examine binding of IRBIT to CFTR we performed co-immunoprecipitation experiments in two different cell lines: A549 cells stably expressing either wtCFTR or F508del-CFTR, and baby hamster kidney (BHK) cells transiently expressing wtCFTR or F508del-CFTR. Moreover, two different co-immunoprecipitation protocols [24,25] were used and either IRBIT or CFTR were immunoprecipitated. Under no conditions we could observe a co-immunoprecipitation, as observed in [25], and we have therefore no evidence that IRBIT binds directly to CFTR when expressed in these two different cell lines (Supplement 3). Taken together, the present results suggest that enhanced Ca^{2+}-activated Cl^- conductance in CF epithelial cells is due to augmented intracellular Ca^{2+} signaling, caused by ER-localized F508del-CFTR. F508del-CFTR may probably function as an ER-located counter-ion channel which facilitates Ca^{2+} release from ER stores. In this regard it is probably interesting to note that in an earlier study we were unable to detect enhanced Ca^{2+} activated Cl^- transport in epithelial tissues of transgenic G551D mice [32].

4. Discussion

4.1. Inflammation in CF

Although CFTR is known for more than 20 years, it remains enigmatic how abnormalities in CFTR can cause chronic and persistent pulmonary inflammation. There is agreement that loss of functional CFTR results in activation of neutrophils that produce large amounts of proteases and reactive oxygen species (ROS). These changes are associated with reduced mucociliary clearance of bacteria, and induction of hyperinflammatory responses in CF airways. The NF-κB pathway and Ca^{2+} mobilization in airway epithelial cells are

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

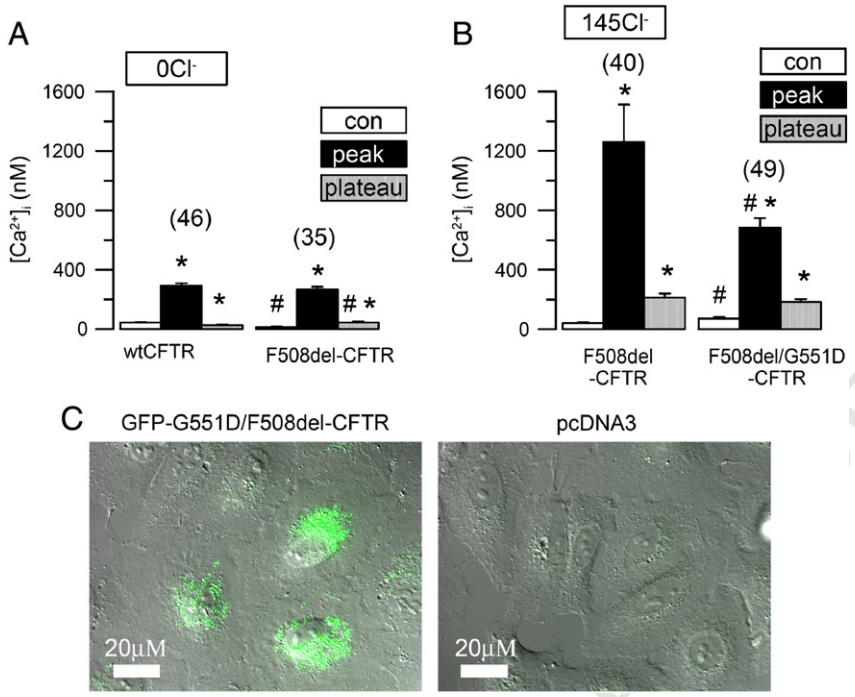

Fig. 6. Intracellular Ca^{2+} signaling is Cl^- dependent. A) Summary of intracellular Ca^{2+} $[Ca^{2+}]_i$ signals elicited by UTP (100 µM) stimulation of A549 cells expressing wt-CFTR or F508del-CFTR in Cl^- depleted cells (0 mM extracellular Cl^- concentration). Peak and plateau Ca^{2+} increase were largely attenuated in both A549/wt-CFTR and A549/F508del-CFTR cells in the absence of extracellular Cl^-. B) Summary of $[Ca^{2+}]_i$ increase induced by UTP (100 µM) in A549 cells expressing F508del-CFTR or the double mutant F508del/G551D-CFTR in the presence of 145 mM extracellular Cl^- concentration. C) Expression of F508del/G551D-CFTR (left panel) and pcDNA3 control plasmid (right panel) in A549 cells. Mean ± SEM, (n) = number of cells measured. *Significant effects of UTP (paired *t*-test). #Significant difference between wtCFTR and F508del-CFTR or F508del-CFTR and the double mutant (unpaired *t*-test).

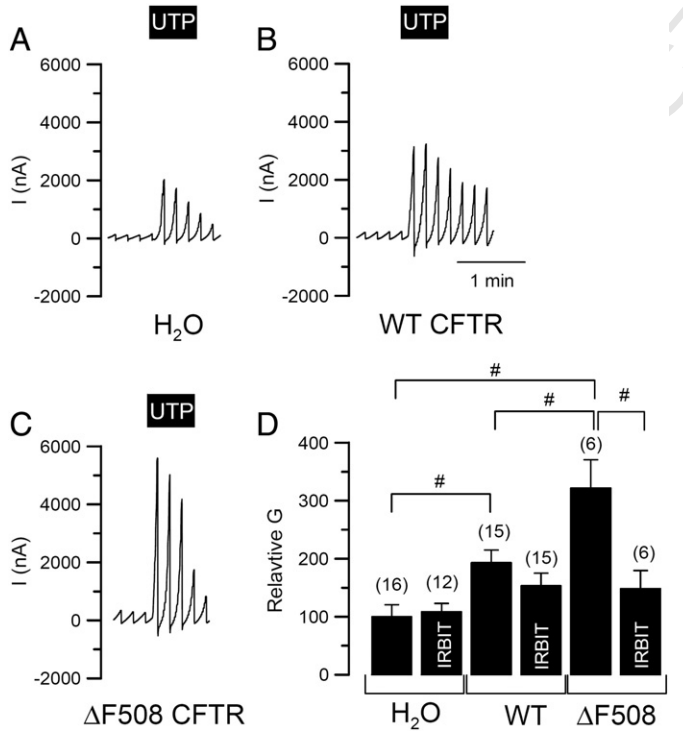

Fig. 7. Potential role of IRBIT for enhanced Ca^{2+} activated Cl^- conductance in F508del-CFTR expressing oocytes. A) Original recording of whole cell Cl^- currents activated by UTP (100 µM) in $P2Y_2$-receptor expressing *Xenopus* oocytes. B) Original recording of whole cell Cl^- currents activated by UTP in $P2Y_2$-receptor and wtCFTR co-expressing *Xenopus* oocytes. C) Original recording of whole cell Cl^- currents activated by UTP in $P2Y_2$-receptor and F505del-CFTR co-expressing *Xenopus* oocytes. D) Summary of calculated relative whole cell conductances activated by UTP in the absence or presence of coexpressed IRBIT. Mean ± SEM, (n) = number of cells measured. *Significant effects of UTP (paired *t*-test). #Significant difference between different batches (unpaired *t*-test).

believed to be of key importance for lung inflammation, through release of mediators such as interleukin-8 [33]. Evidence suggests that CFTR mutations, most importantly F508del-CFTR itself can produce a pro-inflammatory milieu in the airways that precedes infection. Thus F508del-CFTR has been demonstrated to accumulate in the ER and to trigger a stress response that leads to NFκB activation and IL8 production [12,13,34]. Another study found that inhibition of CFTR in airway epithelial cells mimicked the CF-typical inflammatory profile with increase in nuclear NFχB and IL-8 secretion [11], while others showed that expression of functional CFTR on the cell surface negatively regulates NFκB mediated innate immune response [35].

Other studies demonstrated increased secretion of pro-inflammatory cytokines by CF airway cells only after exposure to pathogenic bacteria [7,10,36]. Thus Ribeiro and collaborators demonstrated expansion of an ER compartment close to the luminal membrane of chronically infected and inflamed airway epithelia, like those affected by cystic fibrosis [7]. Large luminal ER pools lead to enhanced apical Ca^{2+} signaling, which explains the augmented Ca^{2+} activated Cl^- secretion observed in cystic fibrosis airways. In contrast to these studies, we were not able to detect any significant effects of the major bacterial component LPS. Moreover, in preliminary experiments we incubated airway cells with purulent sputum from CF patients (kindly provided by Dr. Carla Ribeiro, UNC, Chapel Hill, USA) in 1:200 and 1:1 dilutions, 16 h) but did not see a change in expression of Best-1 or TMEM16A (data not shown).

4.2. Enhanced Ca^{2+}-dependent activation of TMEM16A

Interestingly, pro-inflammatory interleukins have been shown to stimulate both Ca^{2+}-activated Cl^- and SK4 K^+ channels [37]. Galietta and colleagues actually identified TMEM16A as a Ca^{2+} activated Cl^- channel because Ca^{2+} activated Cl^- secretion in bronchial epithelial cells was upregulated by IL4 [18,37]. Although Best-1 was found to be upregulated during renal inflammation [16,27] it was only minimally

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

enhanced in F508del-CFTR expressing cells upon exposure of the cells to bacterial LPS. The fact that retention of misfolded F508del-CFTR leads to imbalances in Ca^{2+} homeostasis is a well recognized fact, however, the reasons for the Ca^{2+} increase are poorly understood [13]. Our present data suggest a direct link between F508del-CFTR and intracellular Ca^{2+} signaling, thereby connecting F508del-CFTR to enhanced Ca^{2+}-dependent Cl^- secretion in CF. Notably; we also found recently that baseline Cl^- currents mediated by the Cl^- channel SLC26A9 are enhanced in wtCFTR-expressing cells when compared to airway cells expressing F508del-CFTR [38].

Our data would explain why conversely Ca^{2+} signaling is affected by F508del-CFTR, as this defective, but still partially active CFTR channel may provide a Cl^- conductance in the ER membrane and thus facilitate Ca^{2+} movement by allowing transport of the counter-ion Cl^-[30]. The concept of a counter-ion channel in the ER to balance negative charges occurring through Ca^{2+} release and reuptake into the ER-store has long been proposed [39]. In the sarcoplasmic reticulum (SR), Cl^- channels play an essential role in excitation–contraction coupling, by balancing charge movement during calcium release and reuptake [40,41]. This is also known from airway smooth muscle cells. SR-localized Cl^- channels in the SR membrane allow for neutralization of electrostatic charges that would otherwise build up during Ca^{2+} movement [42]. Blockage of these Cl^- channels in airway smooth muscle cells might be an effective way in inhibiting airway smooth muscle hyperresponsiveness observed in asthma [43]. Also for Best-1, a function as a counter-ion channel in the ER of epithelial cells has been proposed recently [16].

4.3. Enhanced Ca^{2+} signaling, proliferation and the role of IRBIT

Our present data also provide an explanation for the enhanced proliferation observed for CF epithelial cells. It has been shown earlier that cell proliferation in bronchial epithelium and submucosal glands of cystic fibrosis patients is much increased when compared to non-CF cells [44]. The high proliferation rate of CF airway epithelial cells has been explained by the chronic inflammatory process that takes place in CF airways. However, it is also observed under *in vitro* conditions and in the absence of exogenous pro-inflammatory factors [45]. A change in intracellular Ca^{2+} signaling in cells expressing F508del-CFTR could explain enhanced proliferative activity and delayed cellular differentiation. Finally, the present experiments also demonstrate a functional interference of CFTR with the IP_3-receptor binding protein IRBIT. IRBIT has been shown to suppress the activity of IP3 receptors by competing with IP3 for a common binding site [31]. A recent report showed that IRBIT coordinates epithelial fluid and HCO3-secretion due to stimulation of the Na^+/HCO_3^- co-transporter and CFTR [25]. However, in contrast to this study we were unable to coimmunoprecipitate CFTR and IRBIT in the present study (Supplement 3) and therefore propose that F508del-CFTR affects Ca^{2+} signaling in an IRBIT and Cl^- dependent manner, due to its ability to operate as an ER-trapped ion channel.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2011.08.008.

Acknowledgements

The work was supported by DFGSFB699A7, DFGKU 756/8-2, and TargetScreen2 (EU-FP6-2005-LH-037365) and pluriannual funding of CIGMH (FCT, Portugal). JR Martins is a recipient of doctoral fellowship SFRH/BD/28663/2006 (from FCT, Portugal). The authors would like to thank Dr. Celeste Barreto (Hospital de Sta Maria, Lisboa, Portugal), CF patients and their families for access to nasal cells and Carla Ribeiro (UNC, Chapel Hill, NC, USA) for SMM lavage fluid. E.S. obtained a doctoral grant from the "Excellentiefinanciering", (EF KULeuven, 2005–2009). We thank Kirsten Welkenhuyzen for excellent technical assistance.

References

[1] M.R. Knowles, L.L. Clarke, R.C. Boucher, Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis, N. Engl. J. Med. 325 (1991) 533–538.
[2] M. Mall, T. Gonska, J. Thomas, R. Schreiber, H.H. Seydewitz, J. Kuehr, M. Brandis, K. Kunzelmann, Modulation of Ca2+ activated Cl− secretion by basolateral K+ channels in human normal and cystic fibrosis airway epithelia, Pediatr. Res. 53 (2003) 608–618.
[3] B.R. Grubb, R.N. Vick, R.C. Boucher, Hyperabsorption of Na^+ and raised Ca^{2+} mediated Cl^- secretion in nasal epithelia of CF mice, Am. J. Physiol. 266 (1994) C1478–C1483.
[4] A.M. Paradiso, C.M. Ribeiro, R.C. Boucher, Polarized signaling via purinoceptors in normal and cystic fibrosis airway epithelia, J. Gen. Physiol. 117 (2001) 53–68.
[5] K. Kunzelmann, M. Mall, M. Briel, A. Hipper, R. Nitschke, S. Ricken, R. Greger, The cystic fibrosis transmembrane conductance regulator attenuates the endogenous Ca^{2+} activated Cl^- conductance in *Xenopus* ooyctes, Pflügers Arch. 434 (1997) 178–181.
[6] L. Wei, A. Vankeerberghen, H. Cuppens, J. Eggermont, J.J. Cassiman, G. Droogmans, B. Nilius, Interaction between calcium-activated chloride channels and the cystic fibrosis transmembrane conductance regulator, Pflugers Arch. 438 (1999) 635–641.
[7] C.M. Ribeiro, A.M. Paradiso, U. Schwab, J. Perez-Vilar, L. Jones, W. O'neal, R.C. Boucher, Chronic airway infection/inflammation induces a Ca2+i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia, J. Biol. Chem. 280 (2005) 17798–17806.
[8] R. Bartoszewski, A. Rab, A. Jurkuvenaite, M. Mazur, J. Wakefield, J.F. Collawn, Z. Bebok, Activation of the unfolded protein response by deltaF508 CFTR, Am. J. Respir. Cell Mol. Biol. 39 (2008) 448–457.
[9] C.M. Ribeiro, The role of intracellular calcium signals in inflammatory responses of polarised cystic fibrosis human airway epithelia, Drugs R.D. 7 (2006) 17–31.
[10] K. Hybiske, Z. Fu, C. Schwarzer, J. Tseng, J. Do, N. Huang, T.E. Machen, Effects of cystic fibrosis transmembrane conductance regulator (CFTR) and delta F508-CFTR on inflammatory response, ER stress and Ca2+ of airway epithelia, Am. J. Physiol. Lung Cell. Mol. Physiol. 293 (2007) L1250–L1260.
[11] A. Perez, A.C. Issler, C.U. Cotton, T.J. Kelley, A.S. Verkman, P.B. Davis, CFTR inhibition mimics the cystic fibrosis inflammatory profile, Am. J. Physiol. Lung Cell. Mol. Physiol. 292 (2007) L383–L395.
[12] F. Antigny, C. Norez, F. Becq, C. Vandebrouck, Calcium homeostasis is abnormal in cystic fibrosis airway epithelial cells but is normalized after rescue of F508del-CFTR, Cell Calcium 43 (2008) 175–183.
[13] M. Rottner, C. Kunzelmann, M. Mergey, J.M. Freyssinet, M.C. Martinez, Exaggerated apoptosis and NF-{kappa}B activation in pancreatic and tracheal cystic fibrosis cells, FASEB J. 21 (2007) 2939–2948.
[14] K. Kunzelmann, P. Kongsuphol, F. AlDehni, Y. Tian, J. Ousingsawat, R. Warth, R. Schreiber, Bestrophin and TMEM16 - Ca2+ activated Cl− channels with different functions, Cell Calcium 46 (2009) 233–241.
[15] R. Barro Soria, R. Schreiber, K. Kunzelmann, Bestrophin 1 and 2 are components of the Ca2+ activated Cl− conductance in mouse airways, BBA 1783 (2008) 1993–2000.
[16] R. Barro Soria, F. AlDehni, J. Almaca, R. Witzgall, R. Schreiber, K. Kunzelmann, ER localized bestrophin1 acts as a counter-ion channel to activate Ca2+ dependent ion channels TMEM16A and SK4, Pflügers Arch. 459 (2009) 485–497.
[17] Y.D. Yang, H. Cho, J.Y. Koo, M.H. Tak, Y. Cho, W.S. Shim, S.P. Park, J. Lee, B. Lee, B.M. Kim, R. Raouf, Y.K. Shin, U. Oh, TMEM16A confers receptor-activated calcium-dependent chloride conductance, Nature 455 (2008) 1210–1215.
[18] A. Caputo, E. Caci, L. Ferrera, N. Pedemonte, C. Barsanti, E. Sondo, U. Pfeffer, R. Ravazzolo, O. Zegarra-Moran, L.J. Galietta, TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity, Science 322 (2008) 590–594.
[19] L. Ferrera, A. Caputo, I. Ubby, E. Bussani, O. Zegarra-Moran, R. Ravazzolo, F. Pagani, L.J. Galietta, Regulation of TMEM16A chloride channel properties by alternative splicing, J. Biol. Chem. 284 (2009) 33360–33368.
[20] J. Ousingsawat, J.R. Martins, R. Schreiber, J.R. Rock, B.D. Harfe, K. Kunzelmann, Loss of TMEM16A causes a defect in epithelial Ca2+ dependent chloride transport, J. Biol. Chem. 284 (2009) 28698–28703.
[21] J.R. Rock, W.K. O'Neal, S.E. Gabriel, S.H. Randell, B.D. Harfe, R.C. Boucher, B.R. Grubb, Transmembrane protein 16A (TMEM16A) is a Ca2+ regulated Cl−-secretory channel in mouse airways, J. Biol. Chem. 284 (2009) 14875–14880.
[22] Z. Bebok, J.F. Collawn, J. Wakefield, W. Parker, Y. Li, K. Varga, E.J. Sorscher, J.P. Clancy, Failure of cAMP agonists to activate rescued deltaF508 CFTR in CFBE41o-airway epithelial monolayers, J. Physiol. 569 (2005) 601–615.
[23] S. Beck, D. Penque, S. Garcia, A. Gomes, C. Farinha, L. Mata, S. Gulbenkian, K. Gil-Ferreira, A. Duarte, P. Pacheco, C. Barreto, B. Lopes, J. Cavaco, J. Lavinha, M.D. Amaral, Cystic fibrosis patients with the 3272–26A–>G mutation have mild disease, leaky alternative mRNA splicing, and CFTR protein at the cell membrane, Hum. Mutat. 14 (1999) 133–144.
[24] E. Sammels, B. Devogelaere, D. Mekahli, G. Bultynck, L. Missiaen, J.B. Parys, Y. Cai, S. Somlo, H. De Smedt, Polycystin-2 activation by inositol 1,4,5-trisphosphate-induced Ca2+ release requires its direct association with the inositol 1,4,5-trisphosphate receptor in a signaling microdomain, J. Biol. Chem. 285 (2010) 18794–18805.
[25] D. Yang, N. Shcheynikov, W. Zeng, E. Ohana, I. So, H. Ando, A. Mizutani, K. Mikoshiba, S. Muallem, IRBIT coordinates epithelial fluid and HCO3-secretion by stimulating the transporters pNBC1 and CFTR in the murine pancreatic duct, J. Clin. Invest. 119 (2009) 193–202.
[26] L.J. Galietta, P.M. Haggie, A.S. Verkman, Green fluorescent protein-based halide indicators with improved chloride and iodide affinities, FEBS Lett. 499 (2001) 220–224.

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008

[27] F. AlDehni, M. Spitzner, J.R. Martins, R. Barro Soria, R. Schreiber, K. Kunzelmann, Role of bestrophin for proliferation and in epithelial to mesenchymal transition, J. Am. Soc. Nephrol. 20 (2009) 1556–1564.
[28] K.G. Fischer, J. Leipziger, P. Rubini-Illes, R. Nitschke, R. Greger, Attenuation of stimulated Ca^{2+} influx in colonic epithelial ($HT_{29)\ cells\ by\ cAMP}$, Pflugers Arch. 432 (1996) 735–740.
[29] D. Faria, R. Schreiber, K. Kunzelmann, CFTR is activated through stimulation of purinergic P2Y2 receptors, Pflügers Arch. 457 (2009) 1373–1380.
[30] E.A. Pasyk, J.K. Foskett, Mutant (delta F508) cystic fibrosis transmembrane conductance regulator Cl— channel is functional when retained in endoplasmic reticulum of mammalian cells, J. Biol. Chem. 270 (1995) 12347–12350.
[31] H. Ando, A. Mizutani, H. Kiefer, D. Tsuzurugi, T. Michikawa, K. Mikoshiba, IRBIT suppresses IP3 receptor activity by competing with IP3 for the common binding site on the IP3 receptor, Mol. Cell 22 (2006) 795–806.
[32] D. Oceandy, B.J. McMorran, R. Schreiber, B. Wainwright, K. Kunzelmann, GFP-tagged CFTR transgene is functional in the G551D Cystic fibrosis mouse colon, J. Membr. Biol. 192 (2003) 159–167.
[33] J. Jacquot, O. Tabary, P. Le Rouzic, A. Clement, , Airway epithelial cell inflammatory signalling in cystic fibrosis, Int. J. Biochem. Cell Biol. (2008).
[34] D.R. Koehler, G.P. Downey, N.B. Sweezey, A.K. Tanswell, J. Hu, Lung inflammation as a therapeutic target in cystic fibrosis, Am. J. Respir. Cell Mol. Biol. 31 (2004) 377–381.
[35] N. Vij, S. Mazur, P.L. Zeitlin, CFTR is a negative regulator of NFkappaB mediated innate immune response, PLoS One 4 (2009) e4664.
[36] M.N. Becker, M.S. Sauer, M.S. Muhlebach, A.J. Hirsh, Q. Wu, M.W. Verghese, S.H. Randell, Cytokine secretion by cystic fibrosis airway epithelial cells, Am. J. Respir. Crit. Care Med. 169 (2004) 645–653.
[37] L.J. Galietta, P. Pagesy, C. Folli, E. Caci, L. Romio, B. Costes, E. Nicolis, G. Cabrini, M. Goossens, R. Ravazzolo, O. Zegarra-Moran, IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro, J. Immunol. 168 (2002) 839–845.
[38] J. Ousingsawat, R. Schreiber, K. Kunzelmann, Differential contribution of SLC26A9 to Cl(−) conductance in polarized and non-polarized epithelial cells, J. Cell. Physiol. (2011) 10.
[39] M.J. Berridge, The endoplasmic reticulum: a multifunctional signaling organelle, Cell Calcium 32 (2002) 235–249.
[40] J.I. Kourie, ATP-sensitive voltage- and calcium-dependent chloride channels in sarcoplasmic reticulum vesicles from rabbit skeletal muscle, J. Membr. Biol. 157 (1997) 39–51.
[41] J.R. Coonan, G.D. Lamb, Effect of chloride on Ca2+ release from the sarcoplasmic reticulum of mechanically skinned skeletal muscle fibres, Pflugers Arch. 435 (1998) 720–730.
[42] S. Hirota, N. Trimble, E. Pertens, L.J. Janssen, Intracellular Cl— fluxes play a novel role in Ca2+ handling in airway smooth muscle, Am. J. Physiol. Lung Cell. Mol. Physiol. 290 (2006) L1146–L1153.
[43] L.J. Janssen, Asthma therapy: how far have we come, why did we fail and where should we go next? Eur. Respir. J. 33 (2009) 11–20.
[44] M.W. Leigh, J.E. Kylander, J.R. Yankaskas, R.C. Boucher, Cell proliferation in bronchial epithelium and submucosal glands of cystic fibrosis patients, Am. J. Respir. Cell Mol. Biol. 12 (1995) 605–612.
[45] R. Hajj, P. Lesimple, B. Nawrocki-Raby, P. Birembaut, E. Puchelle, C. Coraux, Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis, J. Pathol. 211 (2007) 340–350.

Please cite this article as: J.R. Martins, et al., F508del-CFTR increases intracellular Ca^{2+} signaling that causes enhanced calcium-dependent Cl^- conductance in cystic fibrosis, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.08.008