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Calcium-dependent regulation of NF-$_{\kappa}$B activation in cystic fibrosis airway epithelial cells

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Abstract

Dysregulation of nuclear factor kappa B (NF-$_{\kappa}$B) and increased Ca^{2+} signals have been reported in airway epithelial cells of patients with cystic fibrosis (CF). The hypothesis that Ca^{2+} signaling may regulate NF-$_{\kappa}$B activation was tested in a CF bronchial epithelial cell line (IB3-1, CFTR genotype ΔF508/W1282X) and compared to the CFTR-corrected epithelial cell line S9 using fluorescence microscopy to visualized in situ NF-$_{\kappa}$B activation at the single cell level. Upon stimulation with IL-1β, we observed a slow but prolonged $[Ca^{2+}]_i$ increase (up to 10 min) in IB3-1 cells compared to S9 cells. The IL-1β-induced $[Ca^{2+}]_i$ response was accompanied by an activation of NF-$_{\kappa}$B in IB3-1 but not in S9 cells. Pretreatment of IB3-1 cells with the ER Ca^{2+} pump inhibitor thapsigargin inhibited the IL-1β-induced $[Ca^{2+}]_i$ response. Treatment with either the calcium chelator BAPTA or an inhibitor of $I_{\kappa}B\alpha$ phosphorylation (digitoxin) led to a drastic $[Ca^{2+}]_i$ decrease accompanied by an inhibition of NF-$_{\kappa}$B activation of IL-1β-stimulated IB3-1 cells in comparison to untreated cells. In IB3-1 cells cultured at low temperature (26 °C) for 16 h, the IL-1β-induced $[Ca^{2+}]_i$ response was inhibited and no significant NF-$_{\kappa}$B activation was observed. To our knowledge, this is the first report of visualization of the Ca^{2+}-mediated activation of NF-$_{\kappa}$B in individual living airway epithelial cells. Our results support the concept that $[Ca^{2+}]_i$ is a key regulator of NF-$_{\kappa}$B activation in CF airway epithelial cells.
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Keywords: CF airway epithelial cells; NF-$_{\kappa}$B activation; Calcium; Fluorescence microscopy

1. Introduction

Cystic fibrosis (CF) is a genetic disease caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), which has been shown to be a cAMP-activated Cl^- channel [1,2] and to regulate the activity of other channels in airway epithelial cells [3]. Although the mechanism by which CFTR mutation leads to inflammatory lung disease is only partially understood, several studies point to a mechanism whereby the most common mutant form of CFTR in airway epithelial cells (ΔF508) leads to a cell stress response, resulting in increased and prolonged nuclear factor kappa B (NF-$_{\kappa}$B) activation [4–6]. Recent work [7] described greater baseline expression of nuclear factor p65 and increased activation of NF-$_{\kappa}$B in unstimulated primary airway epithelial cells from patients with CF (ΔF508/ΔF508) compared to non-CF disease patients. This finding was consistent with previously reported results that demonstrated higher constitutive activation of NF-$_{\kappa}$B associated with exaggerated proinflammatory cytokine response by CF human primary bronchial epithelial cells and respiratory epithelial cell lines [5,6,8,9]. NF-$_{\kappa}$B is a central mediator that can rapidly activate transcription of various inflammatory cytokines, chemokines and adhesion molecules in lung epithelial cells [10–12]. NF-$_{\kappa}$B is activated by its release from cytoplasmic

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$I_\kappa B$ proteins such as $I_\kappa B\alpha$ inhibitor protein and subsequently translocates to the nucleus [13]. There is accumulating evidence showing that lung epithelial cells express high levels of pro-inflammatory mediators (TNF-α, IL-6, IL-8) in response to various stimuli (bacteria and cytokines) through NF-$_\kappa$B-dependent transcription [14–16]. Despite the fact that the link between NF-$_\kappa$B and inflammatory gene activation is now (at least partly) established, the upstream events leading to NF-$_\kappa$B pathway activation remain to be elucitated.

Stimulation of cells with various stimuli induces a transient and rapid rise in the intracellular free calcium concentration $[Ca^{2+}]_i$ through mobilization of inositol 1,4,5-triphosphate-sensitive stores as well as from extracellular space [17–19]. Perturbation of airway epithelial cells by bacteria can initiate Ca^{2+} signaling and stimulated NF-$_\kappa$B translocation [20]. Other studies have shown that once activated, CFTR regulates $[Ca^{2+}]_i$ by mediating nucleotide release and activation of cell surface purinoceptors in normal and CF human airway epithelia [21]. Moreover, it has been shown that $P2Y_2$-R activation promotes greater Ca^{2+} mobilization in CF compared to human nasal epithelia. Recently, a study demonstrated that the larger Ca^{2+} signals elicited in CF bronchial epithelial cells compared to normal bronchial epithelial cells resulted from an expansion of the apically-confined ER Ca^{2+} stores [22]. According to the authors, the expansion of ER Ca^{2+} stores observed in CF airway epithelial cells is not the consequence of ER retention of ΔF508 CFTR protein, but may rather result from chronic luminal airway infection/ inflammation.

In the search for pharmacological treatment of CF airway disease, depleting ER Ca^{2+} stores of CF epithelial cell lines by a treatment with thapsigargin was shown to restore functional surface expression of ΔF508-CFTR protein [23], but this effect was not further confirmed by others [24]. More recently, treatment of CF lung epithelial cells with digitoxin mimicked gene therapy with CFTR and was able to suppress hypersecretion of IL-8 by blocking $I_\kappa B\alpha$, the inhibitor of NF-$_\kappa$B [25]. Inflammation in lung is an important factor in the pathogenesis of CF. Studies examining sputum and bronchoalveolar lavage liquids have shown increased levels of pro-inflammatory cytokines such as IL-8 and IL-1β in CF patients [26,27]. Regulation of NF-$_\kappa$B pathway by $[Ca^{2+}]_i$ has been studied in several cell types, including T-cell stimulation [28], B lymphocytes [29], and cerebellar granule neurons [30]. However, we are unaware of studies on lung epithelial cells in which attention had been specifically directed to the Ca^{2+}-mediated activation of NF-$_\kappa$B.

In the present work, we have therefore investigate the changes of $[Ca^{2+}]_i$ and dynamics of in situ NF-$_\kappa$B activation measured by FRET (Fluorescence Resonance Energy Transfer) microscopy on the CF lung IB3-1 cell line and its (wild type) CFTR-corrected S9 cell line in response to IL-1β stimulation. In the present study, we demonstrate that IL-1β induces a prolonged $[Ca^{2+}]_i$ in IB3-1 cells in comparison to CFTR-corrected cells and the Ca_i^{2+} mobilization is a key regulator of NF-$_\kappa$B activation. The treatment of CFTR-deficient IB3-1 cells by either the Ca^{2+} chelator BAPTA or low temperature resulted in a marked $[Ca^{2+}]_i$ reduction associated with an inhibition of NF-$_\kappa$B activation in IL-1β-stimulated CFTR-deficient IB3-1 cells. Our data support the concept that $[Ca^{2+}]_i$ is a key regulator of NF-$_\kappa$B activation in CF airway epithelial cells.

2. Materials and methods

2.1. Materials

Reagents of analytical grade and deionized water were used. The acetoxymethyl esters of the Ca^{2+} indicators Fluo-3, Rhod-FF, Fura-Red and pluronic F127 were from Molecular Probes (Invitrogen, Cergy-Pontoise, France). Minimum Essential Medium (MEM), Optimem, L-Glutamine, fetal bovine serum and penicillin–streptomycin were obtained from Invitrogen (Invitrogen SARL, France). IL-1β was from R and D systems (R and D, Lille, France), BAPTA-/AM, bromo-A23187 ionophore were from VWR (VWR, Fontenay sous bois, France). FuGENE™6 was purchased from Roche (Roche Diagnostic, Meylan, France). Thapsigargin, NaCl, KCl, $MgCl_2$, HEPES, digitoxin, DMSO, and EGTA were from Sigma (Sigma-Aldrich, Saint Quentin Fallavier, France).

2.2. Cell lines and culture conditions

The airway epithelial cell lines used were IB3-1 (CFTR genotype ΔF508/W1282X) and cells derived from IB3-1 that were stably transfected to achieve low-level expression of full-length wild-type CFTR (S9 cells). CFTR-deficient IB3-1 cells are well characterized [31–33]. CFTR-corrected S9 cells have been shown to have phenotypic correction of a wide range of CF phenotypes [6,25,34]. Both cell types were purchased from ATCC (LGC Promochem, France) and cultured in MEM with Eagle's salts and L-glutamine, supplemented by 10% fetal bovine serum and 100 U/ml of penicillin–streptomycin in a humidified CO_2 incubator (37 °C, 5% CO_2). In some experiments, confluent CFTR-deficient IB3-1 cells were maintained at 26 °C for 16 h incubation prior to their IL-1β exposure at 26 °C for an additional 2 h period.

2.3. Plamids, gene transfer and transfection

YFP-p65NF-$_\kappa$B and $I_\kappa B\alpha$-CFP fusion proteins were prepared as described previously [35]. Cells were transiently transfected with 3 μl of FuGENE™6 transfection reagents and 2 μg of DNA mixed in 100 μl of Optimem (Invitrogen GmbH, Karlsruhe, Germany)

according the manufacturer's instructions. Cells were seeded on 35 mm glass bottom culture dishes (Matek Corporation Ashland, MD, USA), transiently transfected with $I_{\kappa}B\alpha$-CFP and YFP-p65NF-$_{\kappa}$B and investigated one day after transfection.

2.4. Fluorescence microscopy

FRET microscopy imaging was performed on a Zeiss Axiovert 135 microscope equipped with a Fluar 63× oil immersion objective (Carl Zeiss AG, Jena, Germany) and modified with a 2-filter wheel (Visitron System, Puchheim, Germany) able to discriminate between the fluorescent emission images of CFP (480±15 nm) and YFP (545±17.5 nm) of fluorescence. For FRET images, the acceptor emission upon specific acceptor excitation, $F_A(i)$ and the fluorescence through the donor filter upon donor excitation, $F_D(i)$, are measured at each position i of an image, as previously described [36,37]. These are used to correct the acceptor emission upon donor excitation, $F_{DA}(i)$, to obtain the sensitized emission. Apparent FRET efficiencies, $E_A(i)$, were calculated: $E_A(i)=(F_{DA}(i)-F_D(i)\cdot R_D-F_A(i)\cdot R_E)/(F_A(i))$, as previously described [38]. Ratio digital images taken at −5 min intervals were then processed by using ImageJ version 1.32 software (NIH, USA).

Preliminary experiments demonstrated an NF-$_{\kappa}$B activation in both IB3-1 and S9 cells using IL-1β concentrations higher to 50 ng/ml. Interestingly, IB3-1, but not S9 cells showed a significant NF-$_{\kappa}$B activation in response to lower concentrations (20 ng/ml IL-1β). Both IB3-1 and S9 cells expressed a similar level of IL-1RI/II receptors at resting state and after 2 h incubation with 20 ng/ml IL-1β (evaluated by Western blotting, data not shown). Therefore, all experiments in the present work were done with 20 ng/ml IL-1β. Furthermore, in some experiments, to simultaneously monitor the change in $[Ca^{2+}]_i$ and in situ dynamics of NF-$_{\kappa}$B activation in the same living cell, we loaded cells with Fura-Red for 30 min. After washing off excess of the dye, cells were stimulated with 20 ng/ml IL-1β and monitored with a long pass emission filter (>610 nm) for a 2 h period.

2.5. Calcium measurement

Relative intracellular calcium $[Ca^{2+}]_i$ levels were estimated by using Fluo-3 (2 μM) and 0.01% pluronic F127. Confluent monolayers of both cell types were maintained for two days on 35 mm glass bottom culture dishes (Matek Corporation, MA, USA) in MEM medium supplemented with 10% FCS. Monolayers were washed and loaded with Fluo-3/AM and/or Rhod-FF/AM (Molecular Probes, Europe BV, Leiden, The Netherlands) for 30 min at 37 °C in MEM without phenol red. Cells were then rinsed and incubated for another 30 min to allow for the hydrolysis of acetoxymethyl ester. After washing off the excess of dye, cells were mounted on a microscope stage for a 2 h period at 37 °C, 5% CO_2 (Perkin Elmer, MA, USA) and imaged at ×63 magnification on a fast spinning disc confocal switching for fast multichannel 4D time-lapse (lasers: 442, 488, 514, 568, 647 nm). Images were recorded every 5 s and were analyzed with ImageJ software, V1.32 (NIH, USA). At the end of the experiment, the maximum signal (F_{max}) was obtained by adding of 10 μM A23187 ionomycin, and the minimum signal (F_{min}) was obtained by adding 10 mM EGTA to the cell monolayer. The $[Ca^{2+}]_i$ was calculated according to the following formula [39]: $[Ca^{2+}]_i$ (nM)$=K_d$ $(F-F_{min})$ / $(F_{max}-F)$, in which K_d was assumed to be 390 nM. Basal $[Ca^{2+}]_i$ levels were not significantly different between CFTR-deficient IB3-1 and CFTR-corrected S9 cells at the beginning of each experiment.

2.6. Data analysis

All data are expressed as means±SEM of at least five experiments. Statistical difference was determined by Student's t test or one-way ANOVA. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Prolonged IL-β-stimulated $[Ca^{2+}]_i$ response in CFTR-deficient cells compared to CFTR-corrected epithelial cells

We investigated how the addition of IL-1β (20.0 ng/ml) modulated $[Ca^{2+}]_i$ in CFTR-deficient IB3-1 and CFTR-corrected S9 cells on a 1200 s period by preloading with the cell-permeant fluorescent dye Fluo-3 AM (Fig. 1A and B). Exposure of both cell types to IL-1β generated a significant $[Ca^{2+}]_i$ increase, however, magnitude and duration of the $[Ca^{2+}]_i$ increases were different (Fig. 1B). Upon addition of IL-1β, CFTR-corrected S9 cells induced a progressive increase of $[Ca^{2+}]_i$ with a $[Ca^{2+}]_i$ peak after 300 s and a return to baseline after 600 s. In CFTR-deficient IB3-1 cells, the addition of IL-1β induced a slower but prolonged $[Ca^{2+}]_i$ increase up to 700 s followed by a return to a 150% value of the $[Ca^{2+}]_i$ baseline which was maintained for a period of 800–1200 s.

3.2. Mitochondrial Ca^{2+} and ER Ca^{2+} mobilization of IL-β-stimulated $[Ca^{2+}]_i$ response

We next addressed how mitochondrial Ca^{2+} and ER Ca^{2+} stores participated in the IL-1β-induced $[Ca^{2+}]_i$ response, both in CFTR-deficient IB3-1 and CFTR-corrected S9 cells (Fig. 2A). Mitochondria are postulated to play a key role in the regulation of capacitative Ca^{2+} entry [40,41]. To specifically characterize the mitochondrial Ca^{2+} storage capacity and mobilization in both cell types, Rhod-FF fluorescence was simultaneously measured with Fluo-3 fluorescence during the IL-1β-induced $[Ca^{2+}]_i$ response.

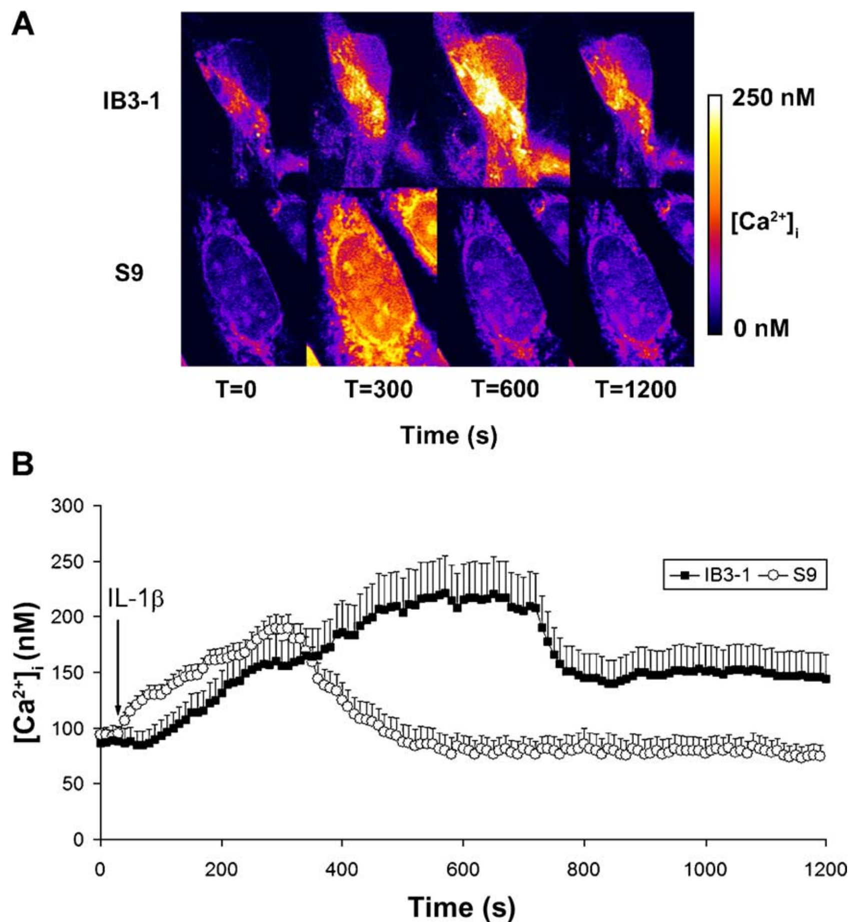

Fig. 1. Effects of IL-1β on $[Ca^{2+}]_i$ response. Pseudocolor images of Ca_i^{2+} fluorescence intensity are shown at 0, 300, 600 and 1200 s time in single CFTR-deficient IB3-1 and CFTR-corrected S9 cells visualized with Fluo-3 every 5 s for a 1200 s period (A), average $[Ca^{2+}]_i$ recorded in CFTR-deficient IB3-1 and CFTR-corrected S9 cells in response to IL-1β stimulation. In CFTR-deficient IB3-1 cells, the addition of IL-1β induced a slower but prolonged $[Ca^{2+}]_i$ increase for a period of 700 s with return to a 150% plateau value of the $[Ca^{2+}]_i$ baseline within 800–1200 s. Experiments ($n=9$ for each cell type) were realized in an optical field of 10 cells/experiment (B).

As depicted in Fig. 2A, a rapid and large discharge of mitochondrial Ca^{2+} was observed in CFTR-corrected S9 cells for a 450 s period following by a progressive Ca^{2+} increase to a 80% level of mitochondrial baseline Ca^{2+} store on the 900–1200 s period. In marked contrast, CFTR-deficient IB3-1 cells exposed to IL-1β manifested a mitochondrial Ca^{2+} increase (with a 30–35% increase relative to baseline fluoresence) for 100 s followed by a slow Ca^{2+} decrease to a 60% level of mitochondrial baseline Ca^{2+} store on the 800–1200 s period.

To support the hypothesis that ER Ca^{2+} stores are, in a large part, involved in the IL-1β-induced $[Ca^{2+}]_i$ response, the effect of 1.0 μM thapsigargin (an ER Ca^{2+}-ATPase inhibitor) on the IL-1β-induced $[Ca^{2+}]_i$ response was tested in both CFTR-deficient IB3-1 and CFTR-corrected S9 cells (Fig. 2B). We first observed that thapsigargin induced a rapid and greater ER $[Ca^{2+}]_i$ rise in CFTR-deficient IB3-1 cells, indicating that the amount of Ca^{2+} sequestered in the ER was slightly higher in CFTR-deficient IB3-1 (a 45–50% Ca^{2+} increase) compared to CFTR-corrected S9 cells. Second, preincubation of both cell types with thapsigargin completely blocked the IL-1β-induced $[Ca^{2+}]_i$ response previously observed in cells for the 1200 s period. We next investigated the role of extracellular Ca^{2+} in the IL-1β-induced $[Ca^{2+}]_i$ response of CFTR-deficient IB3-1 cells. Exposure of CFTR-deficient IB3-1 cells to external Ca^{2+}-free medium (0 mM $CaCl_2$ and 5 mM EGTA) produced a similar profile of IL-1β-induced $[Ca^{2+}]_i$ response compared to controls with extracellular Ca^{2+} (data not shown).

Thus, our data demonstrates that an absence of mitochondrial Ca^{2+} mobilization and a large ER Ca^{2+} mobilization in CFTR-deficient IB3-1 cells compared to CFTR-corrected S9 cells in the IL-1β-induced $[Ca^{2+}]_i$ response.

3.3. *In situ NF-$_\kappa$B activation after the IL-1β-induced $[Ca^{2+}]_i$ response*

To investigate the mechanism by which IL-1β-stimulation activates the NF-$_\kappa$B signaling pathway in both CFTR-deficient IB3-1 and CFTR-corrected S9 cells, we made use of fluorescent fusion constructs expressing the p65Rel/NF-$_\kappa$B protein and the inhibitor $I_kB\alpha$. We monitored the

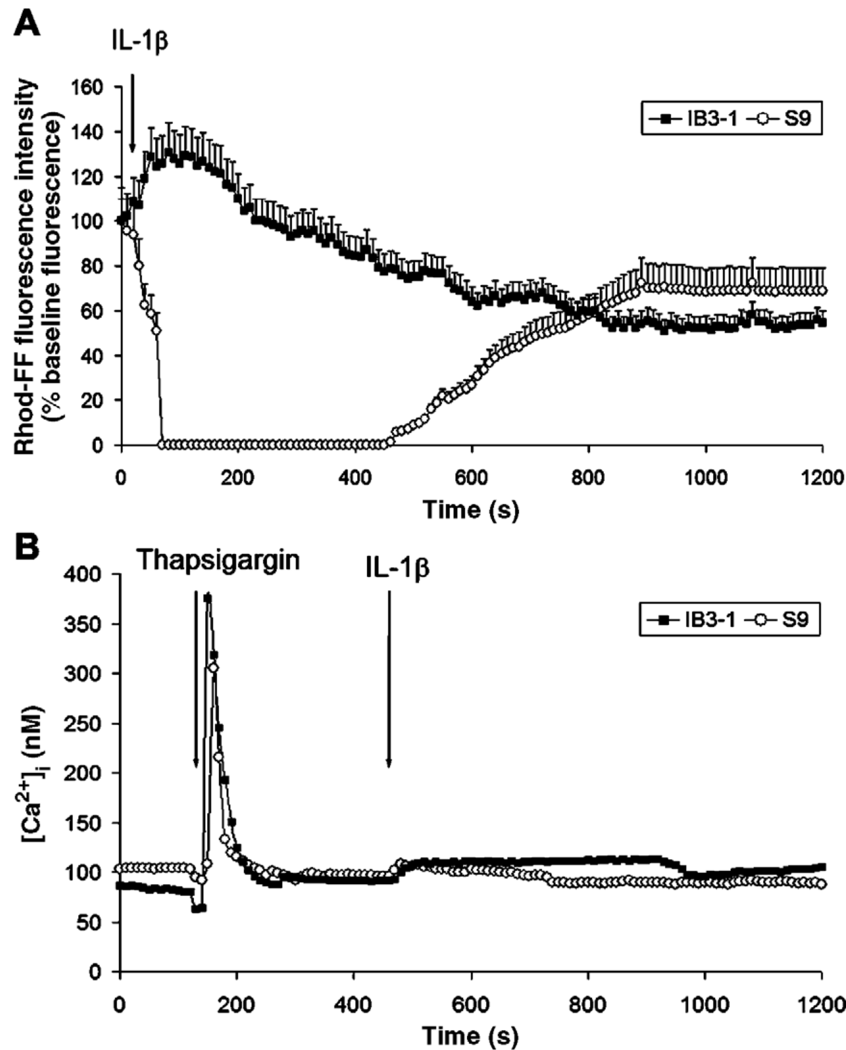

Fig. 2. Changes of mitochondrial Ca^{2+} level (expressed in % baseline fluorescence) visualized every 5 s for a 1200 s period with Rhod-FF dye in single CFTR-deficient IB3-1 and CFTR-corrected S9 cells in response to IL-1β stimulation. Experiments ($n=5$ for each cell type) were realized in an optical field of 10 cells/experiment (A), $[Ca^{2+}]_i$ recordings upon exposure to IL-1β in both cell types treated with 1.0 μM thapsigargin for 1200 s. A greater ER Ca^{2+} release was observed in CFTR-deficient IB3-1 (378.4 ± 43.5 nM, $n=6$) compared to S9 cells (267.2 ± 37.3 nM, $n=6$) in response to thapsigargin. Experiments were realized in an optical field of 10 cells/experiment (B).

YFP-p65NF-$_{\kappa}$B and I$_{\kappa}$Bα-CFP fluorescence ratios in individual living cells exposed to IL-1β on a 2 h period (Fig. 3A). The YFP-p65NF-$_{\kappa}$B/I$_{\kappa}$Bα-CFP dissociation (i.e. NF-$_{\kappa}$B activation) in CFTR-deficient IB3-1 cells upon the IL-1β-stimulation reached a 1 h-peak (175% of the time 0 value) following by a 175% to 155% plateau value for the 1–2 h period. By contrast, no significant modification of YFP-p65NF-$_{\kappa}$B/I$_{\kappa}$Bα-CFP dissociation was observed in CFTR-corrected S9 cells in response to IL-1β−stimulation for the 1–2 h period. Representative fluorescence images acquired with YFP, CFP and FRET filters in individual living CFTR-deficient IB3-1 and CFTR-corrected S9 cells are shown in Fig. 3B. To simultaneously visualize transient Ca^{2+} increases and p65NF-$_{\kappa}$B activation in the same living cell, experiments were performed using Fura-Red. Its fluorescence intensity decreased upon increase of cytoplasm $[Ca^{2+}]_i$. Thus, we observed the strongest reduction of Fura-Red fluorescence (i.e. maximal $[Ca^{2+}]_i$) in CFTR-corrected S9 cells early (300 s) but later in CFTR-deficient IB3-1 cells (600 s) in response to IL-1β-stimulation during the 1200 s period (Fig. 3C). Thus, our data showed that the prolonged IL-1β-induced $[Ca^{2+}]_i$ response was accompanied by an activation of NF-$_{\kappa}$B in IB3-1 cells.

3.4. BAPTA inhibition of IL-1β-induced $[Ca^{2+}]_i$ response and NF-$_{\kappa}$B activation in CFTR-deficient IB3-1 cells

Pretreatment of CFTR-deficient IB3-1 cells with the calcium chelator (BAPTA AM, 10 μM, 15 min) prior to IL-1β stimulation led to a total inhibition of p65NF-$_{\kappa}$B activation in comparison to untreated control cells (Fig. 4A). To verify the role of the Ca^{2+} signal in NF-$_{\kappa}$B activation, the well known Ca^{2+} ionophore bromo-A23187 was used to elevate $[Ca^{2+}]_i$. As shown in Fig. 4A, 2 μM

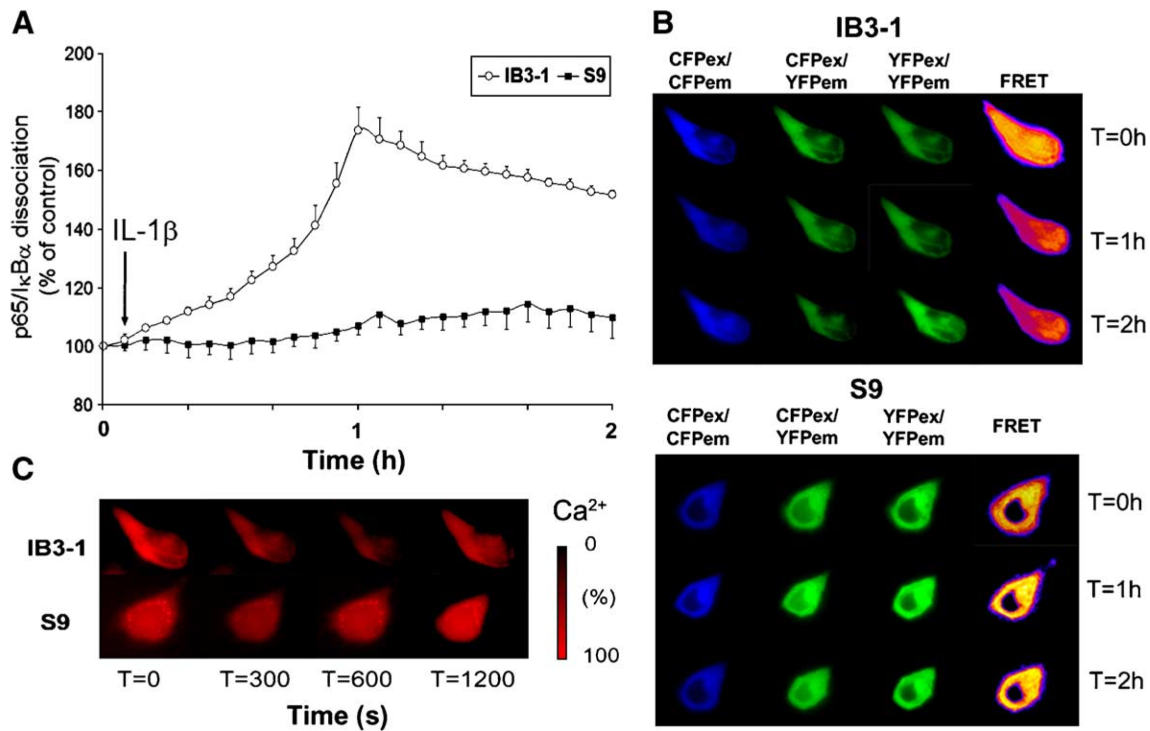

Fig. 3. Dynamics of p65NF-$_{\kappa}$B/I$_{\kappa}$Bα dissociation (expressed in % of the time 0 value) in single CFTR-deficient IB3-1 and CFTR-corrected S9 cells in response to IL-1β for a 2 h period. Experiments ($n = 9$ for each cell type, 15 fields/experiment, $p < 0.001$ in all timepoints from 15 min to 2 h period between the two cell types) were realized in an optical field of 1–2 cells/experiment. Fluorescence images were taken at − 5 min intervals (A). Representative fluorescence images acquired with YFP, CFP and FRET filters in single living CFTR-deficient IB3-1 and CFTR-corrected S9 cells after 0, 1 h and 2 h. Ratio images represent the acceptor filter images divided by donor filter images (B), Ca^{2+} fluorescence intensities visualized with Fura-Red in the same cells for the IL-1β stimulation are shown at 0, 300 s, 600 s and 1200 s (C).

A23187 strongly promoted and sustained an elevated NF-$_{\kappa}$B activation in CFTR-deficient IB3-1 cells for a 2 h period. Representative FRET images in CFTR-deficient IB3-1 cells exposed to either A23187 or IL-1β or BAPTA pretreated-CFTR-deficient IB3-1 cells prior to IL-1β stimulation are shown at timepoints 0, 1 and 2 h (Fig. 4B).

3.5. Temperature sensitivity and effects of digitoxin on the IL-1β-induced $[Ca^{2+}]_i$ response and NF-$_{\kappa}$B activation in CFTR-deficient IB3-1 cells

Based on the hypothesis that temperature reduction facilitates expression of the mutant ΔF508-CFTR protein in the plasma membrane and should therefore restore autocrine functionality of the CFTR in $[Ca^{2+}]_i$ regulation [42], temperature sensitivity of the IL-1β-induced $[Ca^{2+}]_i$ response and p65NF-$_{\kappa}$B activation was measured in CFTR-deficient IB3-1 IL-1β-induced $[Ca^{2+}]_i$ cells (Fig. 5A). When IL-1β was applied to CFTR-deficient IB3-1 cells previously maintained for 16 h at 26 °C and then tested at 26 °C for a 2 h period, a total absence of NF-$_{\kappa}$B activation was observed compared to CFTR-deficient IB3-1 cells cultured and tested at 37 °C.

Digitoxin, a cardiac glycoside, has been recently reported to rescue more than 62% of informative genes which were affected by wild-type CFTR transfection of CFTR-deficient IB3-1 cells. The molecular action of digitoxin was shown to block the NF-$_{\kappa}$B activity through the inhibition of I$_{\kappa}$Bα phosphorylation [25]. Pretreatment of CFTR-deficient IB3-1 cells with digitoxin (10 nM, 15 min) prior to IL-1β addition completely inhibited the NF-$_{\kappa}$B activation (Fig. 5A). As demonstrated in Fig. 5B, only a non-significant reduction of Fura-Red fluorescence (i.e. a $[Ca^{2+}]_i$ increase) was detected in IL-1β-stimulated CFTR-deficient IB3-1 cells exposed to either digitoxin or low temperature (26 °C) compared to IL-1β-stimulated CFTR-deficient IB3-1 cells cultured and tested at 37 °C after 0, 300, 600 and 1200 s, respectively.

Thus, the treatment of CFTR-deficient IB3-1 cells by either the Ca^{2+} chelator BAPTA or digitoxin or low temperature resulted in a marked $[Ca^{2+}]_i$ decrease in IL-1β-stimulated CFTR-deficient IB3-1 cells and was associated with an inhibition of NF-$_{\kappa}$B activation.

4. Discussion

In the lungs, NF-$_{\kappa}$B activation of epithelial cells occurs under physiological and pathological conditions, including during non-apoptotic and apoptotic cell death [43], tolerance to oxidative stress [44,45] and chronic obstructive bronchopulmonary disorders such as in CF [5,7,46]. Because NF-$_{\kappa}$B

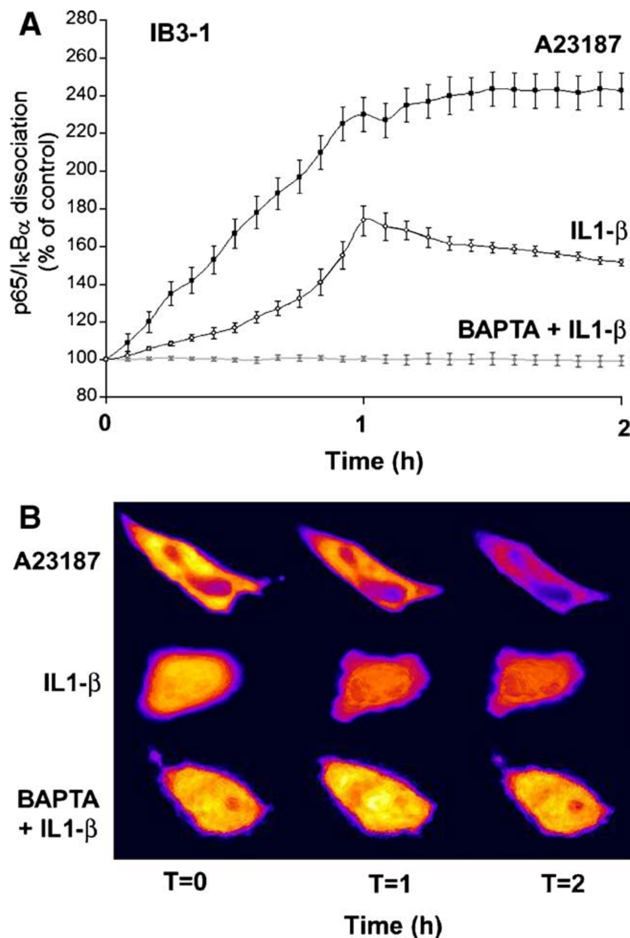

Fig. 4. Dynamics of p65NF-$_κ$B/I$_κ$Bα dissociation (expressed in % of the time 0 value) in single CFTR-deficient IB3-1 exposed to either 2 μM A23187, or 20.0 ng/ml IL-1β or pretreated with BAPTA (10 μM, 15 min) before exposure to 20.0 ng/ml IL-1β for a 2 h period. Experiments (n = 9, 15 fields/experiment, p < 0.001 in all timepoints from 15 min to 2 h) were realized in an optical field of 1–2 cells/experiment. Fluorescence images were taken at −5 min intervals (A), Representative FRET images in CFTR-deficient IB3-1 cells exposed to either A23187 or IL-1β or BAPTA prior to IL-1β stimulation are shown at 0, 1 h and 2 h (B).

plays a critical role in the regulation of immune and inflammatory events in airway epithelium of CF patients, it is important to identify the signaling pathways leading to its activation. Several signals have been shown to mobilize intracellular Ca^{2+} and activate NF-$_κ$B, suggesting that Ca^{2+} is involved as a second messenger for the induction of NF-$_κ$B [30,47,48].

The present study is the first description of the critical role of $[Ca^{2+}]_i$ in NF-$_κ$B activation of CFTR-deficient cells in response to IL-1β. A number of observations led to the conclusion that the prolonged duration of intracellular Ca^{2+} rise and/or the opening of enlarged ER Ca^{2+} stores could be responsible for NF-$_κ$B activation in CF cells. First, the addition of IL-1β in CFTR-deficient IB3-1 cells elicited a more prolonged $[Ca^{2+}]_i$ duration which was accompanied by an activation of NF-$_κ$B but not in CFTR-corrected S9 cells. Second, in CFTR-deficient IB3-1 cells, the IL-1β-induced $[Ca^{2+}]_i$ response was not affected by removal of external Ca^{2+} but completely inhibited by ATPase inhibitor thapsigargin. Third, no significant mobilization of mitochondrial Ca^{2+} stores was observed in CFTR-deficient IB3-1 cells in response to IL-1β, which occurred by contrast, in CFTR-corrected S9 cells. Fourth, treatment of CFTR-deficient IB3-1 cells by either the Ca^{2+} chelator BAPTA or digitoxin resulted in a marked $[Ca^{2+}]_i$ decrease in IL-1β-stimulated CFTR-deficient IB3-1 cells and was associated with an inhibition of NF-$_κ$B activation. The digitoxin effect on the inhibition of NF-$_κ$B activation in IL-1β-stimulated CFTR-deficient IB3-1 cells is in line with a previous report where a marked inhibition of IB3-1NF-$_κ$B activation was reported through an inhibition of I$_κ$Bα phosphorylation associated with a subsequent decrease of IL-8 production using microarray technology [25]. From our data, we believe that the site of digitoxin action in CF epithelial cells appears to be at the interface of the reaction by which activated Ca^{2+}-dependent kinases induce the phosphorylation of I$_κ$Bα.

Intracellular Ca^{2+} plays a central role in numerous cellular functions including cell division, cytoskeletal organization, transport and gene expression [49–51]. Production and

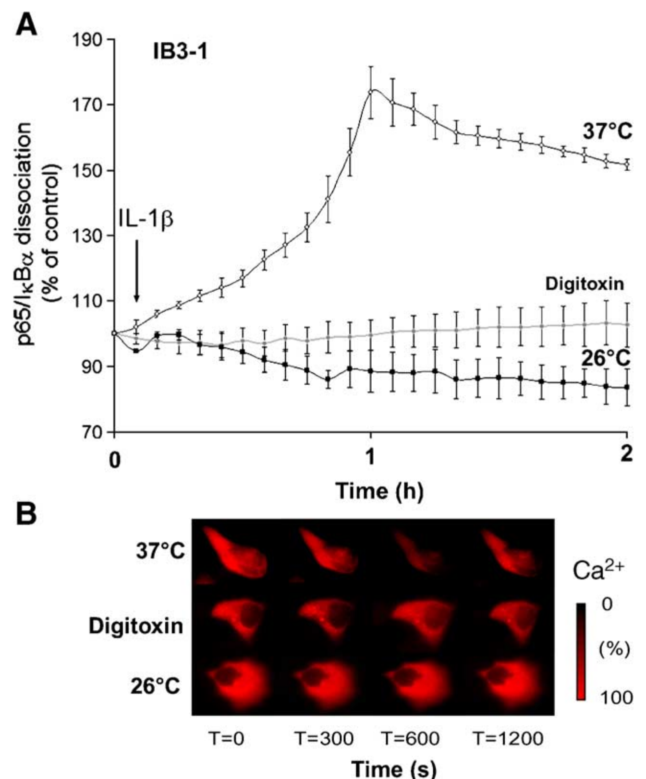

Fig. 5. Dynamics of p65NF-$_κ$B/I$_κ$Bα dissociation (expressed in % of the time 0 value) in single CFTR-deficient IB3-1 cells either cultured and tested at 37 °C or cultured and tested at 26 °C or pretreated with digitoxin (10 nM, 15 min) before exposure to 20.0 ng/ml IL-1β for a 2 h period. Experiments (n = 9, 15 fields/experiment) were realized in an optical field of 1–2 cells/experiment. Fluorescence images were taken at −5 min intervals (A), Ca^{2+} fluorescence intensities, visualized with Fura-Red in the same cells cultured and tested at 37 °C or cultured and tested at 26 °C or pretreated with digitoxin prior to IL-1β stimulation are shown at 0, 300, 600 and 1200 s (B).

secretion of IL-8 by neutrophils stimulated with chemotactic stimuli occurs through Ca^{2+}-dependent mechanisms [52,53]. In T lymphocytes and Jurkat cells, it has been shown that modulation of $[Ca^{2+}]_i$ contributed to the activation of several immune-dependent genes including IL-2 and IL-8 [29]. In CF patients, dysregulated cytokine production was shown in T lymphocytes expressing mutant CFTR [54]. Recently, it has been demonstrated that NF-$_\kappa$B activation and sustained IL-8 expression was consistently greater in primary cultures of CF airway epithelial cells homozygous for the ΔF508 mutation compared to normal airway epithelial cells [46]. The IL-1β-induced $[Ca^{2+}]_i$ response activating NF-$_\kappa$B in CFTR-deficient IB3-1 cells shown in our study, might explain the high susceptibility of airway epithelium from CF patients to express larger amounts of IL-8 in response to inflammatory/infectious agents compared to normal airway epithelium [8,46,55].

Interestingly, we observed a marked release of mitochondrial $[Ca^{2+}]_i$ in CFTR-corrected S9 cells but not in CFTR-deficient IB3-1 in response to IL-1β. On possible explanation could be that various populations of mitochondria sequestered different levels of Ca^{2+} from different sources, as recently demonstrated in two parental and mutated osteosarcoma cell lines [56]. Mitochondria could transiently accumulate an appreciable amount of Ca^{2+} and thereby affect Ca^{2+} homeostasis. Mitochondria at different localizations in the cytosol can be activated by Ca^{2+} to different degrees [57,58]. In the present study, the reason of the drastic difference in the mitochondrial Ca^{2+} mobilization between CFTR-deficient IB3-1 and CFTR-corrected S9 cells remains unexplained and awaits further attention.

We also observed that thapsigargin induced a rapid and greater $[Ca^{2+}]_i$ rise in CFTR-deficient IB3-1cells, indicating that the amount of Ca^{2+} sequestered in ER was higher in CFTR-deficient IB3-1 cells compared to CFTR-corrected S9 cells. This result corroborates with a recent work showing an approximate doubling of ER $[Ca^{2+}]_i$ mobilization in primary CF compared to normal bronchial epithelial cells [22]. The source of Ca^{2+} responsible of this greater ER $[Ca^{2+}]_i$ in CFTR-deficient bronchial epithelial cells remains to be clarified. Unfortunately, we were not able to monitor the YFP-p65NF-$_\kappa$B/I$_\kappa$Bα-CFP dissociation in IL-1β-stimulated CFTR-deficient IB3-1 cells pretreated with thapsigargin. Under these experimental conditions, we observed cell death in more than 85% of CFTR-deficient IB3-1 cells within 1–2 h. Thapsigargin has been reported to rescue the processing defects in the ΔF508-CFTR protein expressed in CFPAC-1 epithelial cells [23] but was not found effective in HEK293 cells [24]. In the latter study, the authors conclude that it was unlikely that simple disruption of ΔF508-CFTR-chaperone interactions in the ER would result in the rescue of the misfolded protein. In fact, it has been previously shown that disruption of CFTR-chaperone interactions accelerated its degradation by the ubiquitin–proteasome pathway [59].

In our study, when CFTR-deficient IB3-1 cells were cultured and tested at low temperature (26 °C), we observed absence of an IL-1β-induced $[Ca^{2+}]_i$ response associated with suppression of NF-$_\kappa$B activation. These results are consistent with the fact that processing of the mutant ΔF508-CFTR protein is temperature sensitive. At reduced temperature, some of the mutant protein could escape from the ER, is fully glycosylated in the Golgi complex, and delivered to the plasma membrane [21,60]. A robust low temperature rescue response has been shown in primary cultures of airway epithelial cells homozygous for the ΔF508 mutation [61,62]. We may interpret our observations obtained with corrected and CFTR-deficient respiratory cell lines with caution for therapy in CF patients. We suggest that the present data will therefore serve as a reference database for analyses on primary normal and CF lung epithelial cells. In futher investigations, we will include primary epithelial cells collected by brushing of nasal mucosa of CF patients to examine both $[Ca^{2+}]_i$ response and NF-$_\kappa$B activation with inflammatory agents to confirm our results.

In conclusion, previous studies have focused on elucidating specific pathways leading from signaling events at the plasma membrane to NF-$_\kappa$B activation. To our knowledge, this study is the first attempt to simultaneously analyze the integration of $[Ca^{2+}]_i$ response and in situ NF-$_\kappa$B activation in individual living airway epithelial cells. We have used state-of-the-art multiparameter non-invasive imaging techniques to follow Ca_i^{2+} mobilization and NF-$_\kappa$B activation occurring in airway epithelial cells in different time domains from seconds to hours. Our study on the dynamics of $[Ca^{2+}]_i$ and NF-$_\kappa$B signaling in single lung epithelial cells allows us to understand the critical role of the $[Ca^{2+}]_i$ response and its later consequence for the NF-$_\kappa$B activation in CF compared to normal airway epithelial cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2005.06.004.

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