ORIGINAL RESEARCH

PLCB3 Loss of Function Reduces *Pseudomonas aeruginosa*–dependent IL-8 Release in Cystic Fibrosis

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Abstract

The lungs of patients with cystic fibrosis (CF) are characterized by an exaggerated inflammation driven by secretion of IL-8 from bronchial epithelial cells and worsened by *Pseudomonas aeruginosa* infection. To identify novel antiinflammatory molecular targets, we previously performed a genetic study of 135 genes of the immune response, which identified the c.2534C>T (p.S845L) variant of phospholipase C-β3 (PLCB3) as being significantly associated with mild progression of pulmonary disease. Silencing PLCB3 revealed that it potentiates the Toll-like receptor's inflammatory signaling cascade originating from CF bronchial epithelial cells. In the present study, we investigated the role of the PLCB3-S845L variant together with two synthetic mutants paradigmatic of impaired catalytic activity or lacking functional activation in CF bronchial epithelial cells. In experiments in which cells were exposed to *P. aeruginosa,* the supernatant of mucopurulent material from the airways of patients with CF or different agonists revealed that PLCB3-S845L has defects of *1*) agonist-induced Ca^{2+} release from endoplasmic reticulum and rise of Ca^{2+} concentration, *2*) activation of conventional protein kinase C isoform β, and *3*) induction of IL-8 release. These results, besides identifying S845L as a loss-of-function variant, strengthen the importance of targeting PLCB3 to mitigate the CF inflammatory response in bronchial epithelial cells without blunting the immune response.

Keywords: phospholipase C-β3; IL-8; calcium signaling; airway inflammation; cystic fibrosis

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Cystic fibrosis (CF), a severe inherited disease evolving toward progressive respiratory insufficiency, is associated with mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (OMIM entry 219700). It is widely known that chronic lung disease is the major complication in these patients, causing the highest morbidity and mortality. This has led in the last two decades to extensive preclinical and clinical investigations on innovative therapies aimed at preventing the onset of or limiting pulmonary deterioration (1, 2).

The bronchial lumen of CF lungs is infiltrated with a huge amount of neutrophils, which release proteases and reactive oxygen species upon continuous activation by bacterial products, a pathway that has a pivotal role in the progression of CF lung tissue damage (3). The secretion of IL-8, which recruits neutrophils in the bronchi, is always present during all stages of the disease and is a hallmark of CF lung pathology (4). Importantly, variant alleles in IL-8 promoter resulting in decreased expression of IL-8 proteins have been linked to reduced severity of progression in CF lung disease (5). This would support the idea that reducing the excess of IL-8–driven neutrophil recruitment could be a significant pharmacological target to ameliorate CF pulmonary damage.

The majority of IL-8 in CF lungs is secreted by the bronchial epithelial cells lining the conductive airways of patients with CF (4, 6). Pattern recognition receptors expressed on the epithelial cell surface, such as Toll-like receptors (TLRs) 2, 4, and 5, activate a series of kinases and adapters upon binding of bacterial components, ultimately triggering nuclear translocation of transcription factors and expression of proinflammatory genes (7). Infection by *Pseudomonas aeruginosa* leads to interaction of the bacteria with different surface receptors, such as asialo ganglio-N-tetraosylceramide receptor (ASGM1R) and TLR5, releasing a large amount of danger signals such as nucleotides (8). In particular, the signaling cascade involves the binding of extracellular ATP to P2Y2 purinergic receptor through an autocrine mechanism. This activates intracellular calcium (Ca^{2+}) release, which, together with TLRs, contributes to IL-8 expression and secretion (9, 10).

In a genotype–phenotype association study previously performed by our group, variants of genes involved in innate immune response were linked to the severity of pulmonary damage in patients with CF. We found a nonsynonymous polymorphism (rs35169799) encoding a serine-to-leucine change at position 845 (p.S845L) in the phospholipase C-β3 (PLCB3) gene on the top (11). Moreover, we also observed that the increase in intracellular Ca^{2+} upon exposure of human bronchial epithelial cells to *P. aeruginosa* is regulated by PLCB3. Thus, PLCB3 plays a pivotal role in IL-8 secretion and neutrophil recruitment in CF airway and lungs.

Because reduced expression of the IL-8 gene has been associated with a mild progression of lung disease in patients with CF (5), we hypothesized that changing the Ser845 to Leu of PLCB3 could cause a reduction of PLCB3 function and consequently affect IL-8 release. In this paper, we report that PLCB3-S845L is a loss-of-function variant that reduces the IL-8 release in human CF bronchial epithelial cells exposed to *P. aeruginosa* and strengthens the potential role of PLCB3 in the proinflammatory signaling in CF.

Methods

Materials

All the reagents used in this study were obtained from Sigma-Aldrich, unless otherwise indicated.

In Silico Analyses of Variants in PLCB3 Structure

The *in silico* analysis was performed as described in the data supplement.

Human Bronchial Epithelial Cell Culture

IB3-1, CuFi-1, and Calu-3 cells were kindly donated by collaborators. Primary cultures derived from a CFTR mutant (genotype F508 del/F508 del) and CFTR wild-type (CFTR-wt) patient were obtained from the Italian Cystic Fibrosis Foundation Primary Culture Core Facility (Genova, Italy). These cells were cultured using standard procedures (*see* data supplement).

Infection with *P. aeruginosa* and Mucopurulent Material from CF Airways

Stimulation was performed using both the PAO1 laboratory strain of *P. aeruginosa* (which is motile nonmucoid) and supernatant from mucopurulent material (SMM). For protocols, *see* the data supplement.

Cell Transfection

Transient transfection of PLCB3-wt, the S845L variant, and the synthetic mutants encoding H332A and L859E cloned in pcDNA3 vector was performed with Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol (*see* data supplement).

Expression of Phospholipase C Isoforms, Mucin, and Cytokine Transcripts

PLC, MUC5, and cytokine (IL-8 and IL-1β) transcripts were quantified by real-time qRT-PCR as previously described (11).

Western Blot Analysis

PLCB3 and secreted IL-8 protein were detected by Western blot analysis according to the procedure reported in the data supplement. Membranes were probed with primary antibodies against PLCB3 (sc-13958; Santa Cruz Biotechnology), anti-IL-8 (sc-7922; Santa Cruz Biotechnology), antiactin (A3853; Sigma-Aldrich), and anti-GAPDH (14C10) (2118; Cell Signaling Technology), as well as with the appropriate horseradish peroxidase–conjugated secondary antibodies.

Detection of IL-8 Protein Release by ELISA

Airway epithelial cells were grown and infected as previously described. Supernatants were then collected from each well, and human IL-8 protein was quantified. The Human IL-8 Instant ELISA Kit (Bender MedSystems) was used for quantitative detection per the manufacturer's instructions.

Fura-2 Acetoxymethyl Ester Experiments

The concentration of Ca^{2+} in the cytosolic fraction was measured using the fluorescent Ca^{2+} indicator Fura-2 acetoxymethyl ester (Life Technologies) using the standardized method of 340/380-nm excitation ratio recorded with a dynamic image analysis system (*see* data supplement).

Aequorin Experiments

Cytosolic aequorin (cytAEQ), endoplasmic reticulum targeted aequorin (erAEQ), and

mitochondrial aequorin (mtAEQ) are the chimeric aequorin probes used to target to the cytosol, endoplasmic reticulum (ER) and mitochondria, respectively (12). All aequorin measurements were performed in Krebs-Ringer bicarbonate buffer supplemented with either 1 mM $CaCl_2$ (cytAEQ and mtAEQ) or 100 μM EGTA (erAEQ). The output of the discriminator was captured by using a Thorn-EMI photon counting board for analyses. The aequorin luminescence data were calibrated off-line into Ca^{2+} concentration ($[Ca^{2+}]$) values, using a computer algorithm based on the Ca^{2+} response curve of wt and mutant aequorins. The rate of ER Ca^{2+} release was calculated as the second derivative of released $[Ca^{2+}]_{er}$ from the ER during agonist stimulation.

PKC Activation Assay

A digital imaging system based on an Axiovert 200 fluorescence microscope (Carl Zeiss Microscopy) was used to record images of PKC translocation as previously described (11).

Statistical Methods

An unpaired, two-tailed Student's *t* test was used with $P < 0.05$ and $P < 0.01$ levels of statistical significance.

Results

In Silico Analyses Suggest that S845 Can Influence the Regulation of PLCB3 Function

A function-to-structure investigation of PLCB3 already characterized the major role the different domains of this enzyme in the transduction signaling leading to modulation of intracellular Ca^{2+} homeostasis (for review, *see* [13]). Other groups have previously characterized the role of different amino acids of PLCB3 by extensive site-directed mutagenesis studies. In particular, Harden's group used two synthetic mutants of residue L859 that were found to be involved in the $G_{\alpha q}$ activation subunit of heterotrimeric GTP-binding proteins (14), as well as a synthetic subunit of residue H332 that was found to be implicated in PLCB3 catalytic activity (15). To date, the role of S845 has not been characterized. At the C-terminal part of PLCB3, the helix–turn–helix Hα1/Hα2 forms the center of the binding interface by making extensive contacts with multiple residues of $G_{\alpha q}$ (Figure 1). It was previously reported that single substitutions of Hα1/Hα2 residues resulted in partial or complete loss of $G_{\alpha q}$-mediated PCLB3 enzymatic activity, as shown by L859, which is known to be critical for $G_{\alpha q}$-mediated activation of PLCB3 (14). Interestingly, S845 is found at the base of the Hα1/Hα2 segment in a region that could influence Hα1/Hα2 mobility and by extension PCLB3 activation by $G_{\alpha q}$ (Figure 1). After these original observations, we decided to compare the functional behavior related to intracellular Ca^{2+} homeostasis of S845 in comparison with that of H332 and L859, which can be considered two paradigms of PLCB3 loss-of-function mutations.

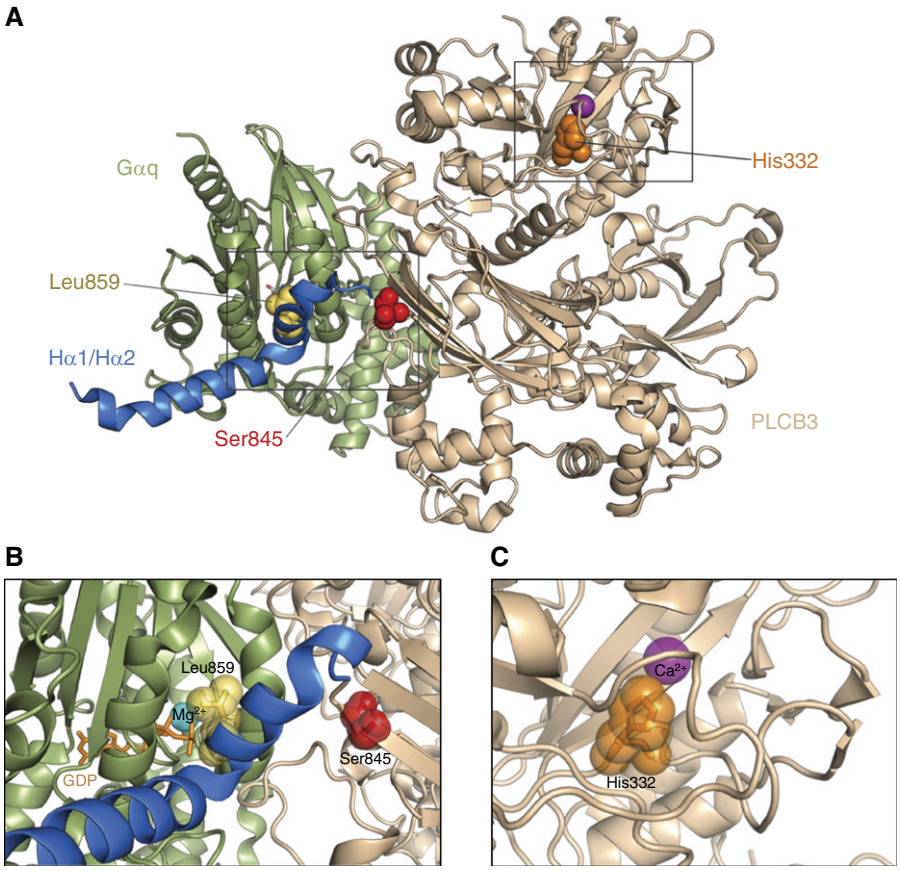

Figure 1. Serine 845 may influence the regulation of phospholipase C-β3 (PLCB3) function. (*A*) Overall structure of the complex $G_{\alpha q}$ (green) and PLCB3 (brown). The single residues Ser845, Leu859, and His332 in PLCB3 are depicted in red, yellow, and orange, respectively. The Hα1/Hα2 segment at the end of the C2 domain of PLCB3 is in blue. (*B* and *C*) Expanded regions highlight the residues Leu859 and His332 in PLCB3 involved in regulating binding with the activated $G_{\alpha q}$ subunit and catalytic activity of PLC. Ser845 in PLCB3 is found at the base of the Hα1/Hα2 segment in a region that could influence Hα1/Hα2 mobility and, by extension, PCLB3 activation by $G_{\alpha q}$.

Overexpression of PLCB3-S845L Does Not Modify Intracellular Ca^{2+} Signaling in Human Bronchial Cells

The hydrolysis of phosphatidylinositol 4,5-biphosphate is catalyzed by PLCB3, generating two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), which in turn activate Ca^{2+} signaling similarly to other phospholipase C isoforms (16, 17). To understand whether the PLCB3-S845L variant is able to modulate intracellular Ca^{2+} homeostasis, we overexpressed wild-type and key variants of PLCB3 in CF human bronchial epithelial cells. Considering that PLCB3 is the major PLCB isoform expressed in bronchial epithelial cells and that the endogenous PLCB3 is the wild type for the SNP S845L in these cells (*see* Figure E1A in the data supplement), we generated a stable PLCB3-knockout (PLCB3-KO) cell clone in CF bronchial cell line IB3-1 to test more clearly the effect of expression of the exogenous PLCB3 constructs. Cell cloning led to isolation of an IB3-B4A7 cell clone representing a

complete knockout of endogenous PLCB3 (Figure E1B). [Ca^{2+}] was detected at different intracellular sites, such as the ER, mitochondria, and cytosol, by expressing organelle-targeted aequorin Ca^{2+} probes (12). Cells were stimulated with the G protein–coupled receptor (GPCR) ligand histamine, inducing Ca^{2+} release from the ER through IP_3 receptor opening. The rate of histamine-induced Ca^{2+} release from the ER in PLCB3-KO IB3-B4A7 cells was significantly reduced in comparison with parental IB3-1 cells (Figures 2A and 2B). A similar difference was observed for Ca^{2+} redistribution in the mitochondria and cytosol (Figures 2C and 2D), confirming that knockout of PLCB3 has a sharp functional effect, although it could be partly compensated by residual PLC isoforms. Reintroduction of PLCB3-wt in the PLCB3-KO IB3-B4A7 cell clone rescued the functional defect obtained by the PLCB3-KO procedure (Figures 2E–2H), resulting in a very pronounced and significant release of Ca^{2+} from the ER with respect to empty pcDNA3 vector (mock) (Figure 2F), with consistent redistribution on mitochondrial and cytosolic Ca^{2+} response after histamine stimulation (Figures 2G and 2H). Interestingly, the overexpression of the PLCB3-H332A variant, which did not alter the luminal ER Ca^{2+} concentration at rest (Figure 3A), resulted in a sharp decrease of the ER Ca^{2+} release rates compared with mock (Figure 3B), affecting the agonist-induced mitochondrial and cytosolic Ca^{2+} response (Figures 3C and 3D). Considering that the Ca^{2+} transients of the mock cells are a result of the activation of endogenously expressed $G_{\alpha q}$ and PLC isoforms, in particular PLCγ2, PLCδ3, and PLCε1 in the case of bronchial epithelial cells (11), this indicates that the transfection of the exogenous catalytic defective PLCB3-H332A variant sequesters a remarkable fraction of $G_{\alpha q}$ protein, acting in dominant-negative fashion, which reduces in part the overall activation of endogenous PLC enzymes (15). Finally, the PLCB3-L859E variant does not increase or reduce the Ca^{2+} transients with respect to the mock cells. Similar behavior has been observed for the PLCB3-S845L variant (Figures 3A–3D).

These results provide evidence that the PLCB3-S845L variant behaves similarly to PLCB3-L859E, a variant lacking the functional activation of the $G_{\alpha q}$-activatory protein but maintaining a functional catalytic site. Similar effects on Ca^{2+} signaling have been observed in parental IB3-1 and human CFTRΔF508 patient-derived primary airway cells stimulated with histamine (Figures E2A–E2D and E3) and the GPCR P2Y2 ligand ATP (18). Challenging human

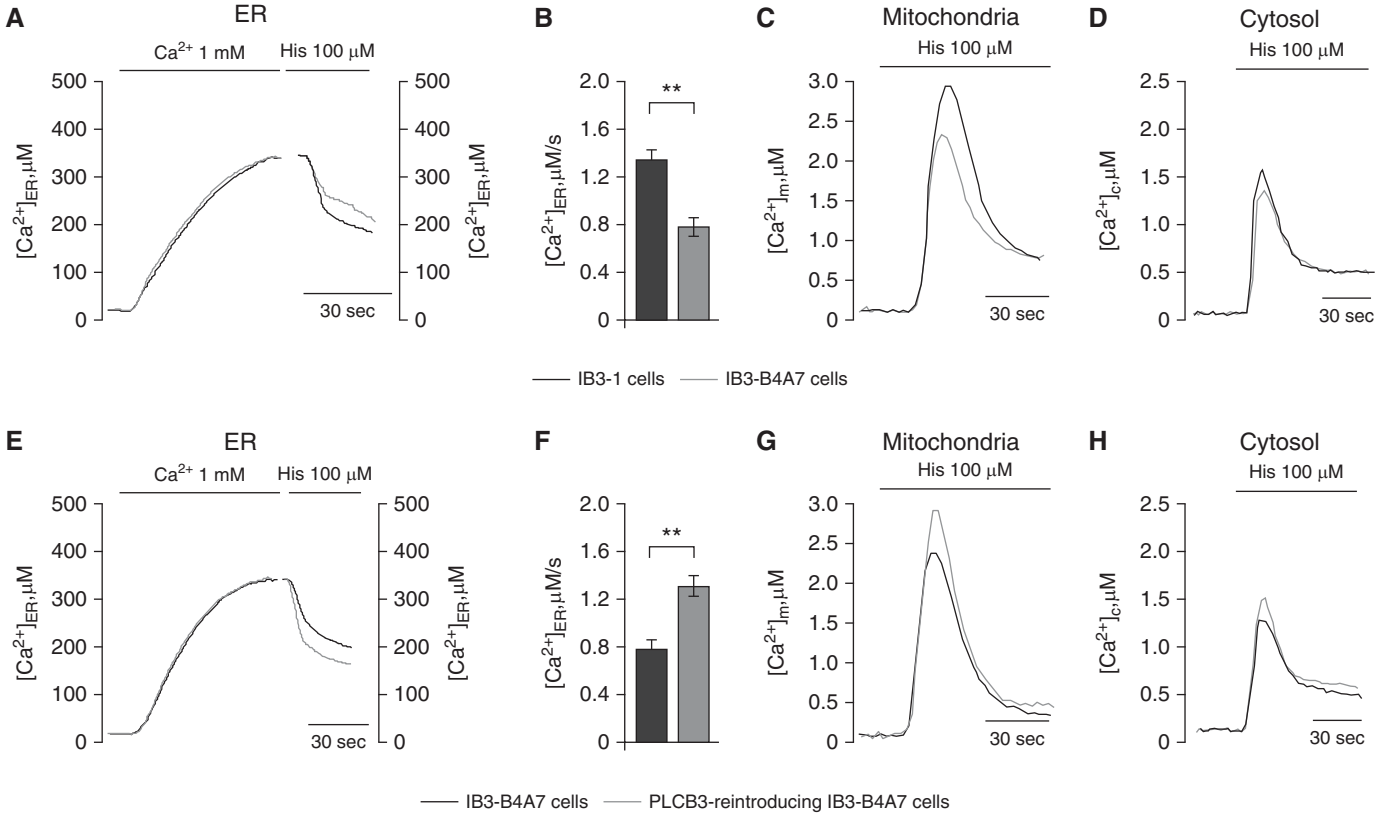

Figure 2. Implication of stable PLCB3 silencing in intracellular Ca^{2+} signaling. The stable silencing of PLCB3 affects the release of Ca^{2+} from the endoplasmic reticulum (ER). (*A*) Representative traces of intraluminal Ca^{2+} accumulation ($[Ca^{2+}]_{er}$) in IB3-1 and IB3-B4A7 cells, by ER-targeted aequorin, in the presence of 1 mM extracellular Ca^{2+} for the time indicated. The ER Ca^{2+} release was induced with the inositol 1,4,5-trisphosphate–dependent agonist histamine (His) 100 μM, as indicated. (*B*) Comparison of ER Ca^{2+} release rate between IB3-1 and IB3-B4A7 cells upon histamine addition, expressed as micromoles per second. All data are expressed as mean ± SEM. Representative histamine-dependent (*C*) mitochondrial and (*D*) cytosolic Ca^{2+} responses in IB3-1 and IB3-B4A7 cells. (*E–H*) The same experiments were performed in IB3-B4A7 cells after reintroducing PLCB3. Traces and bars depict the changes in (*E*) intraluminal ER Ca^{2+} accumulation, (*F*) inositol 1,4,5-trisphosphate–dependent ER Ca^{2+} release, and (*G*) mitochondrial and (*H*) cytosolic Ca^{2+} responses after PLCB3 reintroduction.

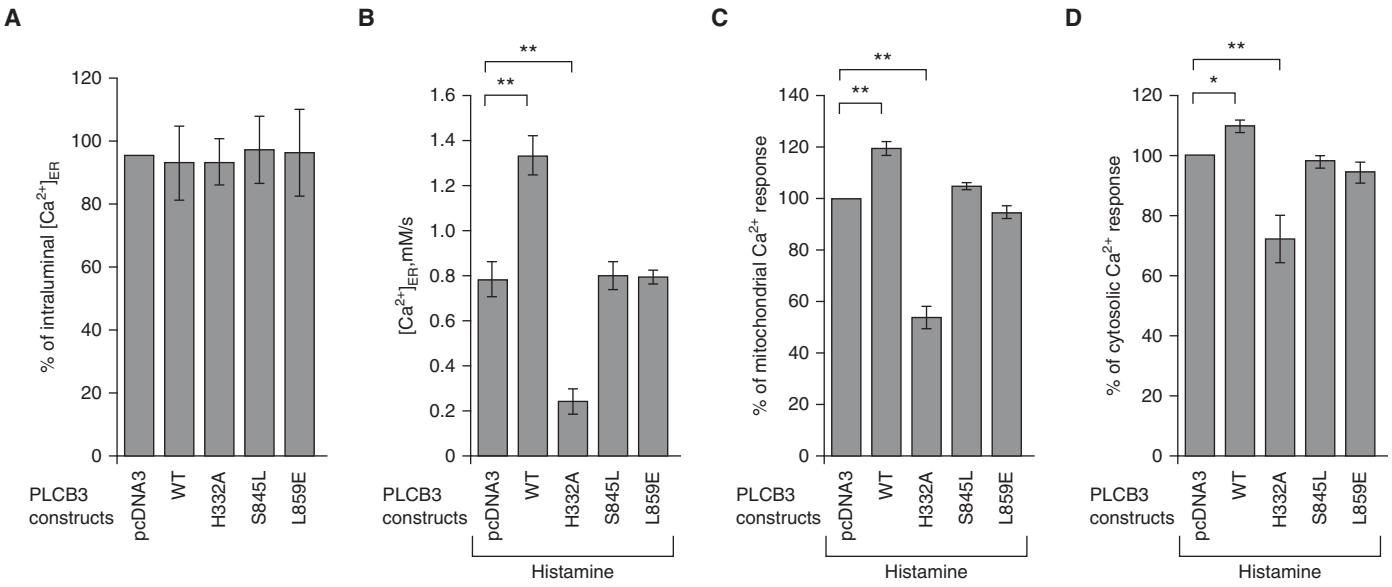

Figure 3. Implication of PLCB3 mutants in intracellular Ca^{2+} signaling mediating G protein–coupled receptor ligand. IB3-B4A7 cells were cotransfected with PLCB3 wild type (WT) or its mutants and the appropriate targeted aequorin probe. After 36 hours, Ca^{2+} measurements were performed as described in experimental procedures (*see* Aequorin Experiments). In detail, (*A*) ER Ca^{2+} accumulation and (*C*) mitochondrial and (*D*) cytosolic Ca^{2+} responses are reported in histograms and expressed as percentage changes of Ca^{2+} responses in PLCB3-reintroducing IB3-B4A7 cells compared with mock cells. (*B*) The bars represent the means of the change in the histamine-dependent ER Ca^{2+} release rates (μM/s) in PLCB3-reintroducing IB3-B4A7 cells. The data are pooled from five or more independent experiments and are expressed as the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

bronchial epithelial cells with ATP produced results similar to those obtained with histamine: *1*) PLCB3-wt produced an increase in Ca^{2+} redistribution from the ER to the mitochondria; and *2*) L859E and S845L variants did not alter the ER Ca^{2+} release rate, whereas the H332A variant produced a reduction, as shown in Figures E2E–E2H.

Overexpression of PLCB3 Wild Type, but Not S845L Variant, Increases IL-8 Expression in Human Bronchial Cells

PLCB3 has been shown to be one of the key players in the IL-8 signaling network of human bronchial epithelial cells stimulated with *P. aeruginosa* (11), a transduction component potentiating the signaling of TLRs mediated by the release of ATP induced by the pathogen–host cell interaction (8, 19). We previously observed that the stimulation of IB3-1 cells with *P. aeruginosa* resulted in a sustained increment of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) (11). Reintroduction of exogenous PLCB3-wt in the PLCB3-KO IB3-B4A7 cell clone exposed to PAO1 reproduced the sustained rise of $[Ca^{2+}]_c$ (Figure 4A), as previously observed in the parental cell line (11). In PLCB3-S845L- and L859E-expressing IB3-B4A7 cells, the pathogen-induced increase of $[Ca^{2+}]_c$ was similar to that obtained in mock cells, whereas reintroduction of PLCB3-H332A produced almost no increase in $[Ca^{2+}]_c$ (Figures 4A and 4B). It is known that PKC isoforms are activated by signals that increase intracellular Ca^{2+} and diacylglycerol, such as those induced by PLCs (20). Moreover, we previously observed that silencing PLCB3 inhibits the translocation of PKCβ to the plasma membrane (11). After transfection of the PLCB3-KO IB3-B4A7 cell clone with recombinant GFP-tagged, Ca^{2+}-dependent conventional PKCβ and infection with *P. aeruginosa*, we observed an activation of PKC shown by the translocation of PKCβ to the plasma membrane. Exposure of the PLCB3-KO IB3-B4A7 cell clone to *P. aeruginosa* for 30 minutes did not induce translocation of recombinant GFP-tagged PKCβ (mock cells), whereas transfection with PLCB3-wt almost doubled the amount of PKCβ on the plasma membrane, as shown by the representative micrographs in Figures 4C and 4D. None of the PLCB3 variants under comparison induced a significant translocation of PKCβ to the plasma membrane, confirming that H332A, L859E, and S845L are loss-of-function mutants of PLCB3 (Figure 4D).

Because it is known that IL-8 expression in bronchial epithelial cells is potentiated by ATP-driven PLCB3 induction together with other ligands activating TLRs (11, 21), we studied the expression and secretion of IL-8 using different PLCB3 variants in PLCB3-KO cells upon stimulation with *P. aeruginosa*. Transfection of the PLCB3-KO IB3-B4A7 cell clone with expression vector encoding PLCB3-wt significantly increased IL-8 mRNA concentration above that of the clone transfected with empty vector (Figure 4E). This was confirmed by the detection of released IL-8 protein in cell culture supernatants by ELISA and Western blotting (Figure 4F and Figure E1B). On the contrary, transfection of the PLCB3-KO IB3-B4A7 cell clone with PLCB3 variants H332A, S845L, and L859E did not significantly increase the concentration of IL-8 mRNA and protein above that observed in the cell clone transfected with the empty vector (Figures 4E, 4F, and E1B). The role of PLCB3 in the model of CF infection/inflammation has been extended by investigating the effects of different PLCB3 mutants on the expression of a panel of cytokines and mucin. On the basis of results shown in Figure 4E, the pathogen also significantly induced the

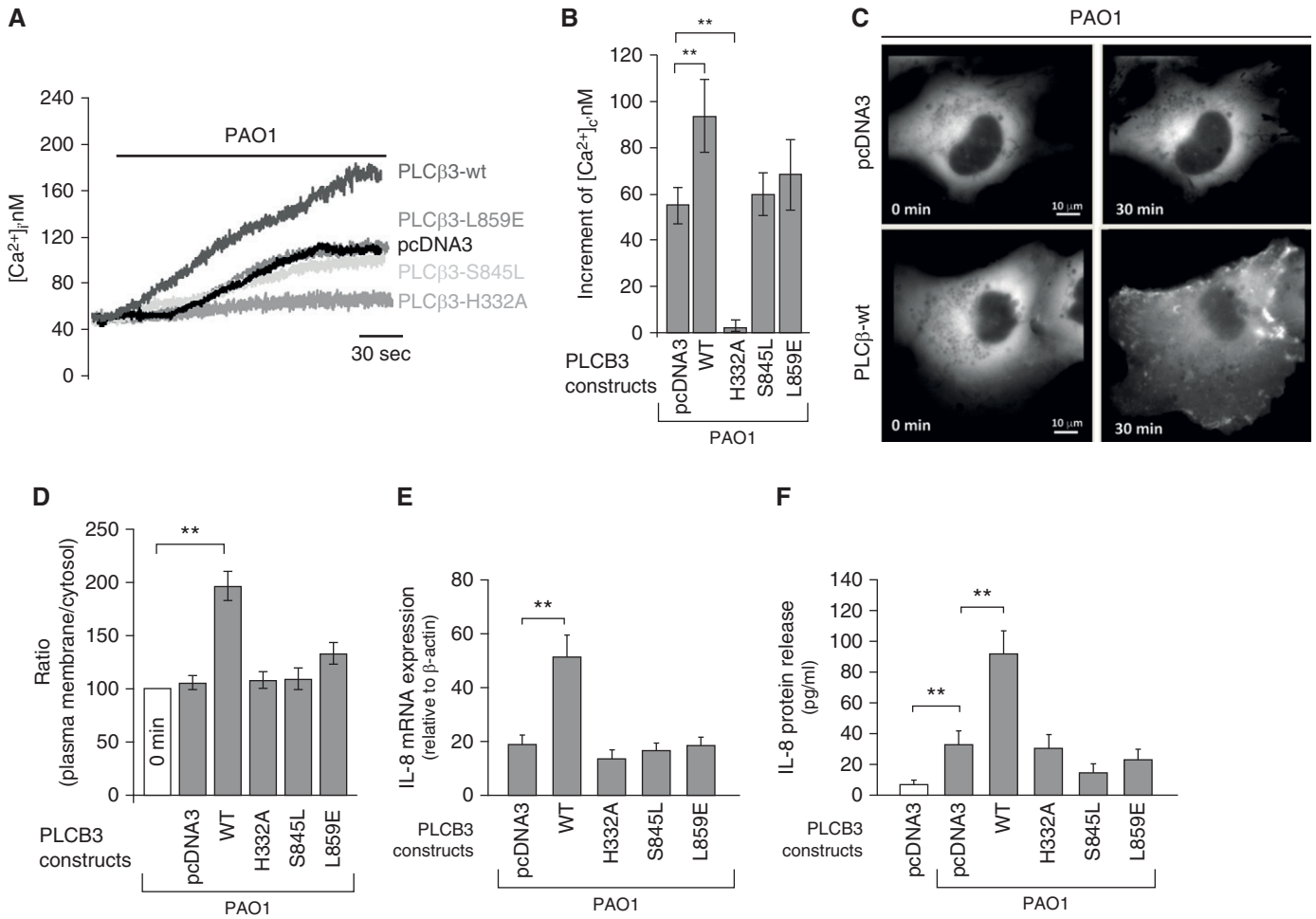

Figure 4. Effects of different PLCB3 mutants on *Pseudomonas aeruginosa*–induced intracellular Ca^{2+} increase, PKCβ activation, and IL-8 secretion in bronchial PLCB3-knockout cells. (*A* and *B*) IB3-B4A7 cells (PLCB3-knockout clone) were transfected with PLCB3 WT or its mutants (H332A, S845L, and L859E) for 36 hours before the addition of PAO1 (100 cfu/ml) and loaded with Fura-2 acetoxymethyl ester dye to measure cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) during acute PAO1 infection. Traces are representative of at least three independent experiments. Fluorescence was recorded every 100 ms, and the $[Ca^{2+}]_c$ (expressed in nM) was calculated by the ratio method using the equation: $[Ca^{2+}]_c = K_d \times (R - R_{min})/(R - R_{max}) \times Sf2/Sf1$ (*A*). (*B*) Increments in $[Ca^{2+}]_c$ after PAO1 addition, expressed as nanomoles of $[Ca^{2+}]_c$. (*C* and *D*) Fluorescence signal of GFP-tagged PKCβ before addition and after 30 minutes from the addition of PAO1 (100 cfu/cell) to IB3-B4A7 cells. The cells were cotransfected with PKCβ-GFP and PLCB3-wt or its mutants (H332A, S845L, and L859E). After 36 hours of expression, cells were infected with PAO1, and the intracellular distribution of PKCβ-GFP was assessed. (*C*) Representative images acquired with a Zeiss Axiovert 200 fluorescence microscope. Upon application of pathogen, clear membrane staining of PKCβ was detected only in PLCB-wt–reintroduced IB3-B4A7 cells. Scale bars: 10 μm. (*D*) Intracellular localization of PKCβ isoform in different experimental conditions, expressed as increase in fluorescence ratio with respect to time 0 (ratio of translocation from cytosol to membrane) as mean ± SEM of at least 12 single cells. (*E*) IB3-B4A7 cells were transfected with different constructs encoding for PLCB3-wt or with PLCB3 mutants (H332A, S845L, and L859E) or with empty vector (pcDNA3). After transfection, the cells were infected with *P. aeruginosa* (PAO1 strain) for a further 4 hours. Expression of IL-8 mRNA was measured by qRT-PCR. (*F*) IL-8 protein release (pg/ml) was measured by ELISA in cells treated as in *E*. Data are expressed as mean ± SEM of five experiments performed in duplicate. Statistical comparisons were made using a paired Student's *t* test. $^{**}P < 0.01$.

expression in PLCB3-wt–expressing cells of the proinflammatory cytokine IL-1β and MUC5 (Figure E4A). Consistent with the data acquired using the PLCB3-KO IB3-B4A7 cell clone under bacterial exposure, the increase in $[Ca^{2+}]_c$ was markedly higher in patient-derived CFTR-ΔF508 and CFTR-wt primary airway cells expressing PLCB3-wt than in airway cells expressing S845L and L859E variants or the empty vector (Figure 5A). The overexpression of the PLCB3-H332A variant in patient-derived primary airway cells resulted in a lower increment of $[Ca^{2+}]_c$ than under other experimental conditions (Figure 5A). These results have also been confirmed by challenging patient-derived primary airway cells with SMM of patients with CF (Figures 5B and 5C), which closely mimics the intracellular Ca^{2+} increment profile previously observed by treating cells directly with pathogen or IP_3-dependent agonists. Bacterial and SMM challenge of transfected patient-derived primary airway cells with PLCB3-wt or variants reproduced the pattern of released IL-8 protein described above. The overexpression of PLCB3-wt significantly increased the IL-8 mRNA expression and protein secretion, whereas the overexpression of three PLCB3

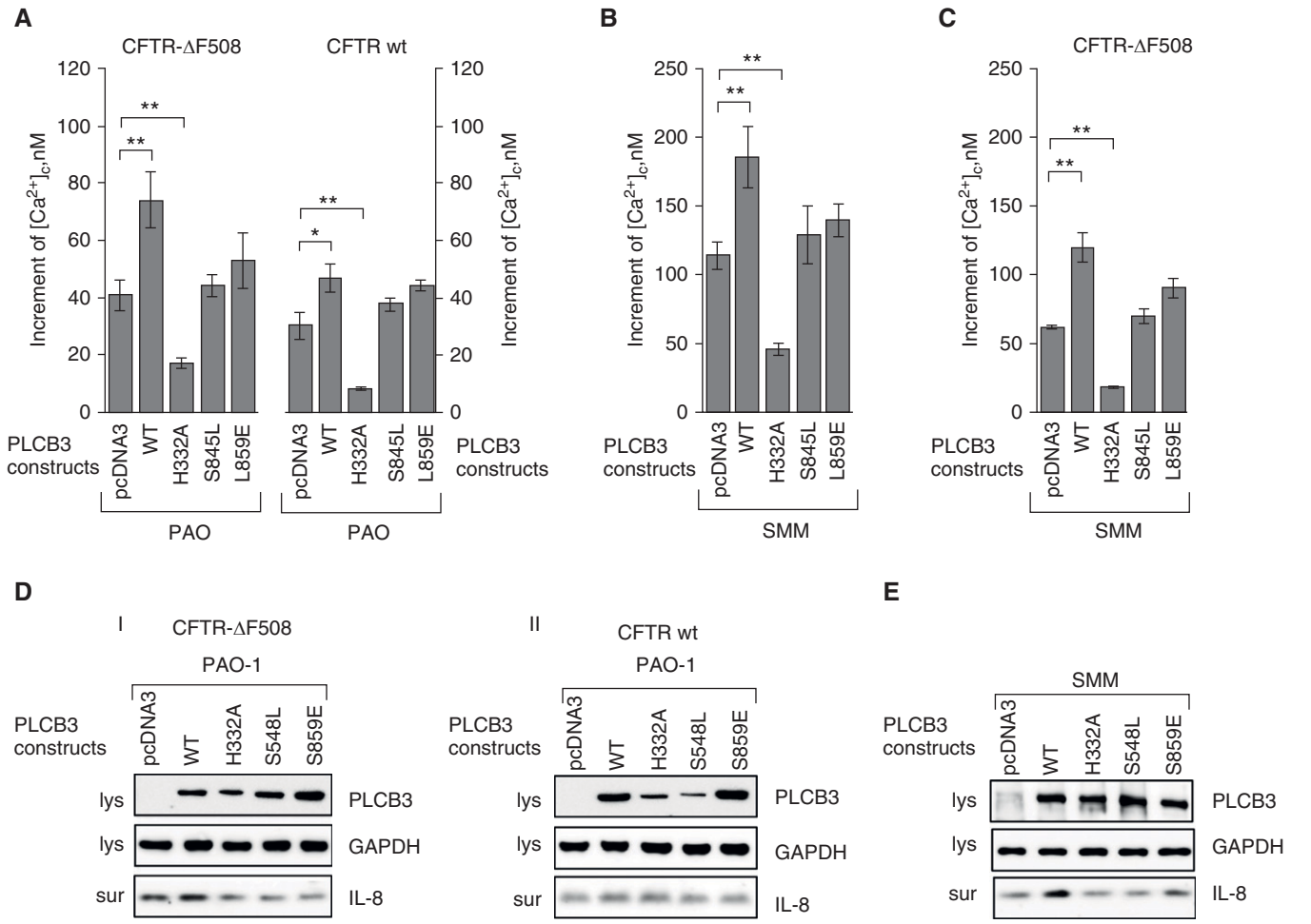

Figure 5. Effects of bacterial and supernatant from mucopurulent material (SMM) challenges on intracellular Ca^{2+} increment and IL-8 secretion in patient-derived primary cystic fibrosis transmembrane conductance regulator (CFTR)-mutant and CFTR-wt airway cells overexpressing PLCB3-wt and its mutants. (*A*) CFTRΔF508 and CFTR-wt patient-derived primary airway cells were transfected with PLCB3-wt or its mutants (H332A, S845L, and L859E) for 36 hours before the addition of PAO1 (100 cfu/ml) for acute infection, as described in Figure 4. The figures depict the increments in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) after PAO1 addition, expressed as nanomoles of $[Ca^{2+}]_c$. Ten microliters of SMM (1:100) were applied to the PLCB-wt and mutants expressing (*B*) IB3-B4A7 and (*C*) CFTRΔF508 patient-derived primary airway cells to induce intracellular Ca^{2+} transient. The data are pooled from five independent experiments and are expressed as mean ± SEM. Statistical comparisons were made using a paired Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$. (*D*) PLCB3, GAPDH, and IL-8 from CFTRΔF508 and CFTR-wt patient-derived primary airway cells transfected with PLCB3-wt or its mutants (H332A, S845L, and L859E) during PAO1 infection. (*E*) PLCB3, GAPDH, and IL-8 were detected in cell lysates (lys) or supernatants (sur) using Western blotting of IB3-B4A7 cells exposed to SMM for 4 hours.

variants did not induce significant increment of IL-8 mRNA and protein compared with mock cells exposed to *P. aeruginosa* and SMM (Figures 5D, 5E, and E4). Similar results have been obtained also for the proinflammatory mediators IL-1β and MUC5 (Figure E4), indicating an extended regulatory role of PLCB3 in inflammation in this scenario.

Discussion

The main cause of bronchial epithelial cell destruction in the lungs of patients affected by CF is excessive lung inflammation. Currently, the discovery of novel drugs and new target molecules involved in proinflammatory pathways is an unmet need (2). To focus on this issue, we previously performed an association study between 721 SNPs (from 135 genes) with either severe or mild phenotypes regarding the progression of lung disease in a population of patients with CF homozygous for the F508del mutation. We found the most significant association with a nonsynonymous polymorphism of the PLCB3 gene, with the minor allele PLCB3-S845L being associated with a mild progression of lung tissue injury (11). The pathophysiology of the exorbitant inflammation in CF is largely related to the huge amount of the neutrophil chemokine IL-8 produced by the bronchial epithelial cells (2). We hence hypothesized that the PLCB3-L845 minor allele could reduce IL-8 release, thus contributing to a mild progression of lung disease in patients affected by CF. In the present study, we investigated the role of PLCB3-S845L and found that the variant has several defective signaling functions regarding *1*) the release of Ca^{2+} from the ER, *2*) the activation of the conventional Ca^{2+}-dependent PKCβ

isoform, and *3*) the *P. aeruginosa*-dependent expression and secretion of IL-8 protein. The comparison of PLCB3-S845L with synthetic variants representative of either defective catalytic activity (H332A) or defective functional activation (L859E) indicates that the PLCB3-S845L variant is a loss-of-function enzyme that reduces both the level of expression and the secretion of IL-8 from bronchial epithelial cells exposed directly to *P. aeruginosa* or SMM.

We previously found that the effect of PLCB3 silencing on the expression of IL-8 driven by *P. aeruginosa* is only partial (11). In fact, *P. aeruginosa* switches on the inflammatory response by interacting with different receptors, including TLRs and ASGM1Rs (22). Thus, the ATP–P2Y2R autocrine loop that increases intracellular Ca^{2+} waves should be regarded as only one of the IL-8–regulating pathways, in parallel with those induced by TLRs via the MyD88 signaling network (23). Moreover, PLCB3 is indeed the most abundantly expressed member of the β-family, but it is not the only isoform of PLC expressed in bronchial epithelial cells (Figure E1) (11), so that residual expression of other PLC isoforms provides a functional substitute to intracellular Ca^{2+} signaling evoked by stimulation of GPCRs. In the present study, we compared the behavior of exogenously expressed S845L with that of variants affecting either the catalytic active site or the interaction with the activatory $G_{\alpha q}$ subunit in different human bronchial epithelial cell models. The activation of PLCB3 was obtained by activating GPCRs either directly with specific ligands such as histamine and ATP or indirectly by bacterial challenge with *P. aeruginosa* and SMM. The activated $G_{\alpha q}$ subunit generated by the ligands, which bind to the GPCRs of the bronchial cells, is likely intervening on both the endogenous and exogenous PLCB moieties. Interestingly, in these models, the H332A variant with defective catalytic function shows consistent reductions of release of Ca^{2+} from the ER (Figures 1 and 2), of cytosolic Ca^{2+} transients (Figure 4), and of cytokine expression and secretion (Figures 4 and 5), indicating its role as a functional dominant-negative variant, in agreement with data previously reported by Harden and collaborators (15). This suggests that the exogenous expression of mutants favors the sequestering of a large fraction of activated G_q subunits, reducing the activation of endogenous PLC isoforms during GPCR stimulation. Although GPCRs lack intrinsic phospho-tyrosine kinase activity, tyrosine phosphorylation of PLC-γ occurs in response to ligation of several such receptors, including those for acetylcholine (muscarinic), angiotensin II, thrombin, platelet-activating factor, and ATP (24–27).

On the contrary, the PLCB3-L859E variant, which has a conserved catalytic site but impaired binding with the activated G_q subunit, does not seem to subtract activated G_q protein from the endogenous PLC isoforms. In parallel experiments, we observed that PLCB3-S845L is a loss-of-function variant at different signal transduction levels, resulting in defective intracellular Ca^{2+} redistribution, PKC activation, and finally IL-8 secretion, confirming the initial working hypothesis of this investigation (Figures 1–5). Considering that PLCB3-S845L behaves in a strictly similar way to that of the PLCB3-L859E variant in our human bronchial epithelial cell models, and considering that its catalytic activity is conserved and that by structural analyses positions S845 close to the interaction site of PLCB3 with activated $G_{\alpha q}$ subunit (14), it is likely that the mechanism of loss of function of PLCB3-S845L may be related to defective activation by the $G_{\alpha q}$ subunit.

Release of nucleotides from bronchial epithelial cells targeting P2Y2 purinergic receptors has been proposed to intervene on different aspects of CF lung pathophysiology (28–33). The chronic infection of CF airways by *P. aeruginosa* amplifies the altered intracellular Ca^{2+} homeostasis of CF epithelial cells, which takes into account different features (10, 33, 34), with direct mitochondrial involvement in proinflammatory signaling, which favors NLRP3 inflammasome activation and IL-1β processing (35), and in turn with proinflammatory cytokines, such as IL-1β, which uses the NF-κB pathway, an inducer of MUC5 expression (36–38). Together, these results support the concept that the modulation of intracellular Ca^{2+} response, also by PLCB3, is a key regulator of amplitude of inflammatory response and lung pathogenesis in CF.

Regarding the design of novel CF tailored antiinflammatory therapies, PLCB3 and Ca^{2+}-dependent proinflammatory signaling seems particularly interesting in CF pathology, because inhibition of PLCB3 could reduce excessive inflammation without blunting completely the antibacterial defenses activated through TLRs (11, 23). Because the expression of PLCB isoforms is implicated in relevant human diseases, such as cancer (39, 40) and cardiovascular defects (41), the identification of small, organic inhibitory molecules is in progress (42), leading the way to development of inhibitory drugs and to a potential drug repositioning in favor of different diseases, including the inflammatory component of CF lung disease. ■

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