Cigarette Smoke-induced Ca\(^{2+}\) Release Leads to Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Dysfunction*

Julia E. Rasmussen\(^1\), John T. Sheridan\(^1\), William Polk\(^1\), Catrin M. Davies\(^6\), and Robert Tarran\(^{1,5,8}\)

From the \(^4\)Cystic Fibrosis/Pulmonary Research and Treatment Center, \(^5\)Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, North Carolina 27599

Background: COPD is the 4th leading cause of death worldwide, yet little is known about its pathogenesis.

Results: Exposure to cigarette smoke elevated intracellular Ca\(^{2+}\) levels, which triggered CFTR internalization/inhibition.

Conclusion: Cigarette smoke-induced Ca\(^{2+}\) release and CFTR inhibition may be involved in COPD pathogenesis.

Significance: Our data provide novel information regarding the underlying mechanism of cigarette smoke-induced lung injury.

Chronic obstructive pulmonary disease affects 64 million people and is currently the fourth leading cause of death worldwide. Chronic obstructive pulmonary disease includes both emphysema and chronic bronchitis, and in the case of chronic bronchitis represents an inflammatory response of the airways that is associated with mucus hypersecretion and obstruction of small airways. Recently, it has emerged that exposure to cigarette smoke (CS) leads to an inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^{-}\) channel, causing airway surface liquid dehydration, which may play a role in the development of chronic bronchitis. CS rapidly clears CFTR from the plasma membrane and causes it to be deposited into aggresome-like compartments. However, little is known about the mechanism(s) responsible for the internalization of CFTR following CS exposure. Our studies revealed that CS triggered a rise in cytoplasmic Ca\(^{2+}\) that may have emanated from lysosomes. Furthermore, chelation of cytoplasmic Ca\(^{2+}\), but not inhibition of protein kinases/phosphatases, prevented CS-induced CFTR internalization. The macrolide antibiotic bafilomycin A1 inhibited CS-induced Ca\(^{2+}\) release and prevented CFTR clearance from the plasma membrane, further linking cytoplasmic Ca\(^{2+}\) and CFTR internalization. We hypothesize that CS-induced Ca\(^{2+}\) release prevents normal sorting/degradation of CFTR and causes internalized CFTR to reroute to aggresomes. Our data provide mechanistic insight into the potentially deleterious effects of CS on airway epithelia and outline a hitherto unrecognized signaling event triggered by CS that may affect the long term transition of the lung into a hyper-inflammatory/dehydrated environment.

Innate lung defense is crucial for removing inhaled pathogens and toxins from the lung (1, 2). Innate lung defense includes mucus clearance, a process that continuously cleans the respiratory tract (3). To maintain mucus clearance, salt and water must be secreted into the lung to balance secretion of "dry" mucins, and together, this mucus/airway surface liquid (ASL)\(^2\) is cleared either by ciliary beat or by coughing (3). When lung inflammation is triggered, normally both salt/water and mucin secretion are enhanced, which serves to increase rates of mucus clearance, thus washing the lung more frequently, until the cause of the infection is resolved (3). One of the major proteins that drives salt/water secretion is CFTR, a cAMP-regulated Cl\(^{-}\) channel that is expressed in airway epithelia (4). Genetic defects in CFTR causes cystic fibrosis, a severe obstructive lung disease characterized by reduced salt secretion and too much dehydrated mucus (5).

Despite clear evidence that tobacco smoking harms lungs and other organs, about 1 billion people worldwide continue to smoke regularly (56). Chronic obstructive pulmonary disease (COPD), the fourth leading cause of death worldwide, is a syndrome associated with chronic airflow obstruction that is most often caused by cigarette smoke (CS) (6–8). Multiple lines of evidence suggest that tobacco exposure negatively affects the lung’s innate defense system (9–11). This sets the stage for chronic inflammation and cellular remodeling (mucus cell metaplasia) as cells adapt to the chronic inflammatory environment (12). More recently, data have emerged that indicate that tobacco exposure decreases CFTR gene expression and CFTR protein levels (13–15). CS is highly oxidizing, which decreases CFTR gene expression, and CS exposure also causes CFTR clearance from the plasma membrane into aggresome-like compartments (14, 16). Both of these effects result in a functional decrease in CFTR activity in the plasma membrane, which reduces salt and water secretion into the ASL in the face of mucin hypersecretion, causing ASL/mucus dehydration and reduced mucus clearance, thus leading to impaired innate responses to inhaled pathogens.

The abbreviations used are: ASL, airway surface liquid; BHK, baby hamster kidney; COPD, chronic obstructive pulmonary disease; CFTR, cystic fibrosis transmembrane conductance regulator; CS, cigarette smoke; HBEC, human bronchial epithelial cells; LAMP1, lysosome-associated membrane protein 1; STIM1, stromal interacting molecule 1; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N’N’,N’-tetraacetic acid tetrakis(acetoxymethyl ester); Ano1, anoctamin 1; PFA, paraformaldehyde; CFP, cyan fluorescent protein; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase; IP\(_3\), inositol 1,4,5-trisphosphate; CCCP, carbonyl cyanide p-chlorophenylhydrazone.
Cigarette Smoke, Ca\(^{2+}\) Release, and CFTR

defense (14, 17). However, as with many aspects of tobacco exposure, the cell biology underlying CFTR’s inhibition following tobacco exposure is likely to be complex, and the underlying mechanisms are currently unknown.

Intracellular Ca\(^{2+}\) is an important second messenger that regulates critical functions ranging from cell growth to differentiation to cell death (18, 19). In the airways, Ca\(^{2+}\) also regulates ciliary beating, Cl\(^{-}\)/ASL secretion, and mucin secretion (20–22). When agonists stimulate increases in cytoplasmic Ca\(^{2+}\), it typically emanates from the endoplasmic reticulum, and excess cytoplasmic Ca\(^{2+}\) is actively recycled into this store soon after release (18). This is desirable because Ca\(^{2+}\) is typically transiently elevated as a cytoplasmic second messenger. Prolonged elevation of Ca\(^{2+}\) is associated with stress responses and transition to an apoptotic state, regardless of whether the cells actually undergo cell death or not (23). Lysosomes are membrane-bound organelles that are responsible for the degradation of macromolecules (24, 25). Lysosomes may contain up to 600 μM Ca\(^{2+}\), suggesting that they may be a considerable source of intracellular Ca\(^{2+}\) (26, 27). In this paper, we looked for the pathway that initiates CFTR internalization following CS exposure. Surprisingly, we found that CS induced prolonged Ca\(^{2+}\) release into the cytoplasm that triggered removal of CFTR from the plasma membrane. Our data suggested that this Ca\(^{2+}\) may emanate from lysosomes and that blockade of this Ca\(^{2+}\) release event protects against CS-induced CFTR internalization.

EXPERIMENTAL PROCEDURES

Cell Culture—BHK\(^{CFTR}\) and HEK293T cells were cultured on glass-bottomed Petri dishes (MatTek) for imaging or standard plastic dishes for biochemical experiments (14). Primary human bronchial epithelial cultures (HBECs) and CALU3 cells were plated on 12-mm Transwell permeable supports (Corning-Costar) under air-liquid interface conditions (14). HBECs and CALU3s were cultured in 7672 human bronchial epithelial cultures (HBECs) and CALU3s were exposed to the vapor phase of whole CS. CS was removed from Ringer’s solution, and 2 mM EGTA was added to provide a nominally Ca\(^{2+}\)-free solution. HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. After ~6 h of transfection, fresh media were added, and cultures were used 1–2 days later.

Cigarette Smoke Exposure—BHK\(^{CFTR}\), HEK293T, HBECs, and CALU3s were exposed to the vapor phase of whole CS. CS was generated by inserting a Cambridge filter pad in the output line of an LMI smoke engine (Borgwaldt); then Kentucky research cigarettes (3R4F) were smoked according to standards from the International Organization for Standardization (2-s/35-ml puff duration/volume). The system was calibrated to deliver a volume of CS that approximated in vivo exposure (28). Acute smoke exposure was 1 puff of cigarette per min for 10 min. Chronic smoke exposure was 1 cigarette (10 puffs) every 2 h for 8 h, as described (13, 14).

Western Blots—BHK\(^{CFTR}\) cultures were exposed to either 10 min of CS or room air puffed through the smoke engine before being lysed with Nonidet P-40 buffer, and Western blot was performed as described (14).

CFTR Surface Labeling—BHK\(^{CFTR}\) cells were cultured in 96-well plates (30,000 cells/well) and studied 24 h later. Cultures were pretreated with compounds or vehicle as needed in BHK media. The media were then replaced with standard Ringer’s solution (10 μL/well, containing compounds if appropriate), and cultures were exposed to CS using a specially designed smoker chamber adapted to fit 96-well plates. As an internal control, half of each plate was sealed with PCR film (Genesee Scientific), so that it was not exposed to CS. After exposure to the volatile phase from two cigarettes over ~15 min, BHK media cultures were added back to the cultures, and they were returned to the 37 °C incubator for 30 min. To halt the experiment, cultures were placed in ice-cold media at 4 °C, followed by a 1-h incubation with a mouse anti-HA antibody (1:2000; Abcam). Cultures were then fixed in 4% PFA, exposed to blocking solution (1% BSA and 1% goat serum), and incubated with secondary antibody (goat anti-mouse Dylight-488; Pierce). Fluorescence was then recorded using a Tecan Infinite multiplate reader. Cultures were then restained with DAPI nuclear dye to give an indicator of cell number. Based on our studies, CS exposure did not affect cell number. However, to account for any discrepancies in total cell number between wells, the 488 nm emission was normalized to DAPI emission. For each plate, data were then normalized to vehicle control to account for variations in gain/fluorescent intensity between experiments.

Intracellular Ca\(^{2+}\) Measurements—Epifluorescence measurements were performed using a Nikon Ti-S microscope with Hamamatsu Orca or Flash Cameras and Ludl Filter wheels and either a ×40 plan fluor oil immersion lens (Fura2 imaging) or a ×60 plan apo-water immersion lens (Rhod-2 imaging). HBECs were bilaterally loaded with 5 μM Fura2-AM and 1 mM probenecid at 37 °C for 30 min. BHK\(^{CFTR}\), HEK293T, and CALU3 cells were loaded with 1 μM Fura2-AM alone at 37 °C for 20 min. The Fura2 ratio (excitation 340/380, emission >450 nm) was collected as described (29). HEK293T cells were labeled with 3 μM Rhod-2 for 1 h at 37 °C followed by a 24-h incubation period as described (30), and measurements of Ca\(^{2+}\) were made by epifluorescence (excitation 540 nm and emission >580 nm). All cells were washed in PBS to remove excess dye before imaging.

Cyclic ADP-ribose and Inositol Phosphate—[\(^{3}H\)]Inositol phosphate accumulation was measured using Dowex columns followed by scintillation counting (29). Cyclic ADP-ribose was measuring using the cycling assay (31).

Quantitation of NAADP\(^{+}\) by LC-MS/MS—Quantitation of NAADP\(^{+}\) was performed with an Acquity liquid chromatography system (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (Thermo Fisher Scientific, Waltham, MA) using electrospray ionization in positive mode. Separation was performed on a 2.0 × 150-mm Hydrosphere C18, 3-μm column (YMC America, Allentown, PA) with gradient elution at a flow rate of 200 μL/min using 5 mM ammonium acetate in water and methanol. Mobile phase composition was held at 1% methanol from 0 to 4 min, changed linearly to 6% methanol over 5 min, held at 6% methanol for 7 min, decreased to 1% methanol over 0.1 min, and held at 1%...
methanol for 3.9 min for a total run time of 20 min. Solvent flow was diverted away from the mass spectrometer to waste for the first 3.5 min of the analysis. The retention time of NAADP was 7.3 min. Infusion of pure standard solutions in water showed a protonated precursor ion at 745 m/z for NAADP. Product ions selected for quantitation and their associated collision energies were 508 m/z (29 eV) for NAADP. Mass spectrometer parameters were optimized for sensitivity and set to the following values: spray voltage of 3000 V, heated vaporizer temperature 300 °C, sheath gas flow rate 35 (arbitrary units), auxiliary gas argon collision gas pressure 1.5 millitorr.

A calibration curve was constructed using NAADP standard solutions diluted in 100 mM oxalic acid in water. After placing 40 µl of standard solution in an autosampler vial, 12 µl of 1 M ammonium acetate was added. Injection of 10 µl of standard solutions gave 100, 250, 1000, and 2500 fmol of NAADP on the column. The limit of quantitation was 100 fmol per injection with an estimated limit of detection of 50 fmol.

Confocal Imaging and ASL Measurements—ASL height and all fluoroescently labeled proteins were measured by confocal microscopy using a Leica SP5 with a ×63 glycerol objective lens (29). CFTR was N-terminally labeled with GFP (32), and Ano1 was C-terminally labeled with mCherry (33). For imaging gfp-CFTR ± CS, cells were fixed for 10 min in 4% PFA 1 h after exposure to air or CS. During imaging, fixed cultures from each group (e.g. air, smoke, and BAPTA) were imaged in the same session and laser power/gain settings remained constant for all image acquisitions.

LAMPI-YCaM3.6 FRET Measurements—Acceptor-photobleaching FRET was performed using a Leica SP5 confocal microscope with a ×63 glycerol immersion objective. The donor (CFP) was excited at 457 nm, and the acceptor (enhanced YFP) was excited at 514 nm. FRET was measured by the acceptor photobleaching method and analyzed using Imager (National Institutes of Health freeware) as described (33). The FRET efficiency (%) was calculated as follows: (donorprebleach − donorpostbleach)/donorprebleach) × 100. In all cases, the acceptor was bleached to ~99% of original intensity without affecting acceptor intensity. Cells were fixed by a 10-min exposure to 4% PFA and imaged in PBS.

Statistical Methods—All data are presented as means ± S.E. Data were inspected to determine whether or not the data were normally distributed. For normally distributed data, paired or unpaired Student’s t tests were used as appropriate. If data were not normally distributed, then the Mann-Whitney U test or Wilcoxon matched pairs test were used. Statistical significance between groups was assessed using paired or unpaired t tests as appropriate. For comparisons of multiple groups, analysis of variance tests were used. Values of n refer to the number of subjects or the number of cultures used in each group. For HBECs, a minimum of four different donors supplied cultures for each experiment. For experiments utilizing BHK-CFTR, CALU3, and HEK293T cells, all experiments were performed on at least three separate occasions.

RESULTS

Cigarette Smoke-induced CFTR Internalization Is Abolished by Ca2+ Chelation—We have previously shown that CS rapidly internalizes CFTR to a detergent-resistant compartment through an unknown mechanism, which manifests as an apparent diminution of CFTR in standard Nonidet P-40 lysis buffer in either BHK-CFTR cells, immortalized CALU3 airway cells, or primary HBECs (14). Regardless of whether we exposed cells to whole cigarette smoke or placed a Cambridge filter pad in the output line to remove the particulate phase and exposed cells to the volatile phase, diminution of CFTR was identical (both n = 3). The volatile phase accounts for 95% of cigarette smoke, and because cigarette smoke particulates are highly fluorescent, we performed all additional experiments using the freshly produced volatile phase of CS. In an attempt to better understand this process, we used CFTR diminution as a marker and performed an initial screen on BHK-CFTR cells with a series of inhibitors including H89 (0.5 µM; PKA), staurosporine (100 nM; PKC/PKA), and okadaic acid (10 nM; serine/threonine protein phosphatases) (Fig. 1A) (34,35). Because CS exposure has been shown to activate PI3K, we also exposed cells to the PI3K inhibitors LY294002 and wortmannin (30 and 0.1 µM, respectively; Fig. 1A) (36). Surprisingly, the only maneuver that blunted the diminution in CFTR was pretreatment with the Ca2+ chelator BAPTA-AM (Fig. 1, A and B).

![Confocal Imaging and ASL Measurements](image_url)

FIGURE 1. Screening for antagonists of CS-induced CFTR diminution by Western blot. BHK-CFTR cultures were exposed to 10 air or CS puffs. A, representative Western blot for mature CFTR following CS exposure ± pretreatment with 5 µM BAPTA-AM. B, mean densitometry of CFTR following pretreatment with either BAPTA-AM or a variety of inhibitors followed by CS exposure. All n = 3. Open bars, air exposure. Closed bars, CS exposure. * denotes p < 0.05 different from control/air. † denotes p < 0.05 different from control/CS.
To confirm the protective effects of BAPTA-AM on CFTR, we transfected gfpCFTR into HEK293T cells. As a control, we also transfected cells with anoctamin 1 (Ano1) tagged with mCherry (Ano1mCherry), a Ca\(^{2+}\)/H\(\text{11001}\)-sensitive plasma membrane Cl\(^{-}\)/H\(\text{11002}\) channel that is also expressed in airway epithelia (37). Under control conditions, both proteins could be seen in the plasma membrane. However, after CS exposure, gfpCFTR, but not Ano1mCherry, clearly internalized (Fig. 2A). A 15-min BAPTA-AM pretreatment had no effect on basal gfpCFTR or Ano1mCherry localization. Importantly, in agreement with the Western blot data (Fig. 1), BAPTA-AM pretreatment prevented gfpCFTR from being internalized post-CS exposure (Fig. 2A).

To quantify CFTR surface densities, we exposed BHK\(^{CFTR}\) cells to CS ± BAPTA-AM and immunofluorescently labeled the extracellular HA tag on CFTR (38). CS and BAPTA-AM exposure had no effect on BHK\(^{CFTR}\) cell number (Fig. 2B). Consistent with the Western blot and immunocytochemistry, CFTR surface levels were diminished by CS, and this effect was prevented by BAPTA-AM pretreatment. As an additional control, we exposed BHK\(^{CFTR}\) cells to the Ca\(^{2+}\) ionophore ionomycin, which serves to increase cytoplasmic Ca\(^{2+}\) levels. Ionomycin caused a decrease in surface CFTR levels, further suggesting that Ca\(^{2+}\) regulates CFTR levels (Fig. 2C).

Cigarette Smoke Exposure Raises Cytosolic Ca\(^{2+}\) through an IP\(_3\)–Independent Mechanism—Because CS-induced CFTR diminution/internalization was dependent on intracellular Ca\(^{2+}\), we next tested the hypothesis that CS exposure triggered an increase in cytoplasmic Ca\(^{2+}\) levels. In polarized HBECs, UTP triggered a rapid spike in cytosolic Ca\(^{2+}\) that returned to baseline within 5 min (Fig. 3A). In contrast, CS caused a sustained increase in cytosolic Ca\(^{2+}\) that was not diminished upon repeated/chronic CS exposure (Fig. 3, A and B). This CS-induced increase in cytosolic Ca\(^{2+}\) had very different kinetics to UTP-induced...
Cigarette Smoke, Ca^{2+} Release, and CFTR

**FIGURE 3. Cigarette smoke exposure increases cytosolic Ca^{2+} levels.** HBECs were preloaded with Fura2 and then exposed to 10 puffs of CS or air in situ under thin film conditions in an environmental chamber on an epifluorescent microscope. **A**, mean change in Fura2 ratio (F_{340/380}) as an indicator of cytosolic Ca^{2+} with time before/after 10 puffs of air (C) or smoke (C). Exposure occurred during the period indicated by the horizontal bar, and afterward, CS was flushed out of the chamber and exchanged for room air. Both n = 12. As an additional control, UTP was added to increase Ca^{2+} (Δ; n = 6). **B**, cytosolic Ca^{2+} was elevated following CS exposure in a variety of cell types. Mean change in Fura2 ratio (F_{340/380}) is shown over time as an indicator of cytosolic Ca^{2+}. ●, HBECs; ■, BHK^CFTR^ cells; ▲, CALU3 cells; ▽, HEK293T cells. **C**, bar graph showing mean change in F_{340/380} after 10 min of air (open bars), CS, removal/chelation of extracellular Ca^{2+} followed by CS, chronic CS exposure (closed bars), and UTP + extracellular Ca^{2+} (gray bars). All n = 6. **D**, [3H]inositol phosphate accumulation in HBECs after air, CS, or UTP exposure. All n = 4. ●, cyclic ADP-ribose (cADPR) levels in CALU3 cells before/after CS exposure. All n = 4. †p < 0.05 different from air control. Fura2 release, and although the UTP response occurred almost immediately, the CS response took ~90 s to begin (Fig. 3A). Identical increases in cytoplasmic Ca^{2+} were elicited in HBECs, BHK^CFTR^, HEK293T, and CALU3 cells (Fig. 3B), suggesting that multiple cell types can be used to study this phenomenon. Furthermore, although UTP-mediated Ca^{2+} release induces store-operated Ca^{2+} entry, which is diminished following removal/chelation of extracellular Ca^{2+}, CS-induced Ca^{2+} release was unaffected by extracellular Ca^{2+} chelation (Fig. 3C) (18). Together, these data suggested that the CS-induced Ca^{2+} was released from a separate compartment than UTP-mediated Ca^{2+}.

UTP binding to its G-protein-coupled receptor (e.g. purinergic receptors) initiates an increase in cellular inositol phosphates (Fig. 3D), which acts as the trigger for endoplasmic reticulum Ca^{2+} release (39). CS exposure did not increase cellular inositol levels (Fig. 3D). After IP_3, cyclic ADP-ribose and NAADP + are the two most potent Ca^{2+}-mobilizing agonists (26). However, cyclic ADP-ribose levels did not change (Fig. 3E), and NAADP + was undetectable by mass spectrometry in CALU3 cells, either before or after CS exposure (n = 3).

**Neither the Endoplasmic Reticulum nor Mitochondria Release Ca^{2+} Following Cigarette Smoke Exposure.** The endoplasmic reticulum is a major source of cellular Ca^{2+} that can be transiently released into the cytoplasm following IP_3 formation, before being pumped back into the endoplasmic reticulum by SERCA pumps (40). STIM1 is an endoplasmic reticulum protein that aggregates and forms puncta upon depletion of endoplasmic reticulum Ca^{2+} (41). Experimentally, the endoplasmic reticulum can be depleted of Ca^{2+} by exposing cells to the SERCA pump inhibitor thapsigargin, causing STIM1 puncta formation (Fig. 4A) (42). In contrast, CS did not induce STIM1 puncta formation, although puncta could still be formed post-CS by adding thapsigargin, suggesting that CS neither activates nor inhibits endoplasmic reticulum Ca^{2+} release (Fig. 4A, B). Pretreatment with thapsigargin abolished UTP-induced Ca^{2+} release but failed to abolish CS-induced increases in cytoplasmic Ca^{2+} (Fig. 4, C and D), further indicating that the endoplasmic reticulum was not the source of Ca^{2+} for the CS response.

Because Ca^{2+} was elevated with time post-CS, we wondered whether the endoplasmic reticulum served as a Ca^{2+} sink that absorbed excess cytoplasmic Ca^{2+}. To test this, we inhibited the SERCA pump with thapsigargin and examined the impact on Ca^{2+} changes induced by CS. When added separately, both CS and thapsigargin significantly increased cytoplasmic Ca^{2+} levels over vehicle (Fig. 4E). Interestingly, cells that were exposed to CS after thapsigargin displayed a significantly larger
increase in cytoplasmic Ca\textsuperscript{2+} over CS alone (Fig. 4E), suggesting that the endoplasmic reticulum accepts some cytoplasmic Ca\textsuperscript{2+} following CS exposure.

To test the specificity of increases in cytoplasmic Ca\textsuperscript{2+} on CFTR, we quantified CFTR surface density after exposure to thapsigargin. Thapsigargin had no effect on cell number (Fig. 4F), but significantly reduced surface expression of CFTR to a similar level as ionomycin (Fig. 4G).

Mitochondria also possess significant Ca\textsuperscript{2+} stores, which are depleted by the mitochondrial uncoupler CCCP (43). To test whether mitochondria were the source of CS-induced Ca\textsuperscript{2+} release, we first measured cytoplasmic Ca\textsuperscript{2+} with Fura2 in HEK293T cells. CCCP alone elicited a small but significant increase in cytoplasmic Ca\textsuperscript{2+} that peaked after \(~5\) min and waned over time (Fig. 5A). CCCP exposure did not prevent the CS-induced increase in Ca\textsuperscript{2+}, suggesting that mitochondria were not the source of Ca\textsuperscript{2+} (Fig. 5A). However, pretreatment with CCCP caused a small but statistically significant increase in CS-induced Ca\textsuperscript{2+} release, suggesting that mitochondria were acting in a minor capacity to buffer some of the CS-induced increase in cytoplasmic Ca\textsuperscript{2+} (Fig. 5A), although to a much lesser extent than the endoplasmic reticulum (Fig. 4E). As a control, we also found that ATP-induced Ca\textsuperscript{2+} release was increased in the presence of CCCP (Fig. 5A). However, this enhancement was much greater, indicating a further difference between CS and ATP-mediated Ca\textsuperscript{2+} signaling. Furthermore, CCCP alone had no impact on cell number or CFTR surface density (Fig. 5, B and C), indicating that the CCCP-mediated release of mitochondrial Ca\textsuperscript{2+} alone was not responsible for CFTR internalization.

To directly visualize mitochondrial Ca\textsuperscript{2+}, we loaded HEK293T cells with Rhod-2-AM. After loading, mitochondria could be directly visualized as distinct puncta (Fig. 5D). Note, only fluorescence (\(I_{540}\)) in distinct puncta was measured for this study, and all other regions of the cell were excluded. After CS exposure, there was a significant increase in Rhod-2 fluores-
cence in the puncta, again suggesting that a small amount of cytoplasmic Ca$^{2+}$ had been taken up into the mitochondria (Fig. 5E). Pretreatment with CCCP reduced the number of puncta that were still detectable and distinct from the cytoplasm, but CS still elicited a significant increase in mitochondrial Ca$^{2+}$, although this increase was significantly reduced compared with CS/non-CCCP-exposed cells (Fig. 5E).

**Cigarette Smoke Induced Ca$^{2+}$ Release and CFTR Internalization Involves Lysosomes**—Because neither the endoplasmic reticulum nor mitochondria were involved in CS-induced Ca$^{2+}$ release, we next assayed the peri-lysosomal environment using a CFP/YFP FRET sensor derived from calmodulin (YCαM3.6) that was fused to the lysosomal membrane protein LAMP1 (44). When expressed for 48 h in HEK293T cells, this construct localized to distinct puncta suggesting that it was lysosome-associated (Fig. 6A). The baseline FRET efficiency (%E) of LAMP1-YCαM3.6 was 44% (Fig. 6, A–C). The maximum %E that can be measured with the CFP/YFP FRET pair is 50% (33); thus, the dynamic range that can be measured is 44–50%. After CS exposure, %E significantly increased to 49%, which is close to the maximum of the measurable range (Fig. 6, A–C). In contrast, CS exposure had no effect on FRET from the positive control (CFP linked to YFP by five glycines) or the negative control (untagged CFP and YFP co-transfected into the same cells).

H$^+$ is accumulated in lysosomes by a bafilomycin A1-sensitive, vacuolar-type H$^+$-ATPase, and Ca$^{2+}$ enters lysosomes by a secondary active process that is linked to H$^+$ uptake (45). To test whether bafilomycin affected CS-induced Ca$^{2+}$ release and CFTR levels, we pretreated cells with bafilomycin A1 and exposed them to CS. The CS-induced increase in cytosolic Ca$^{2+}$ was significantly attenuated by 400 nM bafilomycin pretreatment (Fig. 7A). Similarly, the diminution of CFTR by CS was also prevented by bafilomycin (Fig. 7, B and C). However, bafilomycin had no effect on CFTR levels following air exposure (Fig. 7B). To see if the effect of bafilomycin on whole cell CFTR protein extended to CFTR surface levels, we first looked...
DISCUSSION

Our results demonstrate that CFTR localization and surface density are Ca^{2+}-sensitive (Figs. 1 and 2). Following CS-induced Ca^{2+} increases, CFTR is internalized, whereas Ano1 is unaffected, suggesting specificity for CFTR. To the best of our knowledge, there are no existing reports of CFTR trafficking being Ca^{2+}-sensitive. However, because CFTR is a cAMP/PKA-sensitive channel, the majority of work has focused on constitutive and cAMP-sensitive trafficking, and the effects of Ca^{2+} on CFTR have not been studied (46). We do not know why CFTR but not Ano1 is endocytosed following CS exposure. However, CFTR trafficking is subject to multiple steps of quality control (47). Thus, we speculate that CFTR is damaged by components in CS leading to either adduct formation and/or protein misfolding, causing CFTR to foul the peripheral quality control network (48), leading to its rapid internalization. Further investigation into the effects of CS on CFTR will be required to better understand this phenomenon and its relevance to COPD pathogenesis.

CS exposure consistently elevated cytosolic Ca^{2+} levels (Fig. 3, A–C). This response was identical in all cell types tested (HBECs, CALU3s, BHK/CFTRs, and HEK293Ts). Because the gene expression profile is likely to be different in airway epithelia (which are highly specialized) versus BHK/HEK cells, the as yet unidentified pathway activated by CS exposure is likely very basic in nature. The increase in cytosolic Ca^{2+} seen with CS was similar in magnitude to the UTP-induced Ca^{2+} release but differed greatly in terms of duration (Fig. 3C). Because Ca^{2+} signals are encoded not only by their magnitude, but also by their duration (49), our data suggest that the prolonged CS-induced elevation in cytoplasmic Ca^{2+} may provide drastically different signals than the relatively brief UTP-stimulated response. Although the effect of Ca^{2+} appears to be specific for CFTR, the source of Ca^{2+} appears to be less specific, because prolonged exposure to Ca^{2+} elicited by ionomycin and thapsigargin decreased CFTR surface density to the same extent as CS (Figs. 2 and 4). Furthermore, CS did not induce IP_{3} formation, STIM1 aggregation, nor the influx of extracellular Ca^{2+} (Figs. 3D and 4, A–C), which are all typically observed after UTP exposure (18). Mitochondria are also a significant Ca^{2+} store (43). However, mitochondria played little role in CS-activated Ca^{2+} release and certainly were not the source of Ca^{2+} (Fig. 5). Taken together, these data suggest that “canonical Ca^{2+}-mobilizing pathways” were not being activated by CS exposure. Surprisingly, inhibition of either endoplasmic reticulum SERCA pumps or uncoupling of mitochondrial gradients actually enhanced CS-induced Ca^{2+} release, suggesting that these
FIGURE 7. Bafilomycin-A1 pretreatment abolishes CS-induced increases in cytosolic Ca\textsuperscript{2+} and reduces diminution/internalization of CFTR. A, bar graph showing mean changes in cytosolic Ca\textsuperscript{2+} (F\textsubscript{340/380}) in HBECs following air (open bars; n = 12), CS (closed bars; n = 12), or bafilomycin pretreatment (400 nM) followed by CS (gray bars; n = 12). B, Western blot showing CFTR in the presence/absence of 400 nM bafilomycin in BHK\textsubscript{CFTR} cells. C, mean densitometry taken from B. All n = 3. D, confocal micrographs showing gfpCFTR (green) following air and CS exposure in the presence of 400 nM bafilomycin. E, CS and bafilomycin exposure do not affect DAPI fluorescence, an indicator of total BHK\textsubscript{CFTR} cell number. AU, arbitrary units. F, surface CFTR fluorescence from BHK\textsubscript{CFTR} cells (normalized to DAPI and vehicle control) following air or CS exposure in the presence and absence of 300 nM bafilomycin. n = 12–24 cultures from three separate experiments. G, typical confocal micrographs showing ASL height after air or CS exposure in the presence of 400 nM bafilomycin. H, graph taken from G showing mean changes in ASL height with time before and after air or CS exposure. ■, air, vehicle; ▲, air, BAF; ●, CS, vehicle; ◆, CS, BAF. All n = 8. * denotes p < 0.05 different from control (air). † denotes p < 0.05 different from CS.
organelles act as sinks to accept small amounts of excess Ca\(^{2+}\) released during CS exposure (Figs. 4 and 5). Importantly, the increase in cytosolic Ca\(^{2+}\) did not desensitize with chronic smoke exposure (Fig. 3C), indicating that it may be a relevant signaling event in chronic smokers.

In addition to their known roles in degrading proteins and the induction of autophagy, lysosomes sequester a large amount of Ca\(^{2+}\) and are thought to be an additional source of Ca\(^{2+}\) that can be released as needed (26, 27). Lysosomal Ca\(^{2+}\) release can also be stimulated by the second messengers cyclic ADP-ribose and NAADP\(^{+}\) (26). However, we found no increase in either of these molecules following CS exposure (Fig. 3D). We did find that cytoplasmic Ca\(^{2+}\) became elevated in the vicinity of lysosomes (Fig. 6), potentially implicating lysosomes in this response. Lysosomes can sequester Ca\(^{2+}\) through a variety of processing, including direct Ca\(^{2+}\) uptake via Ca\(^{2+}\)-ATPase, Na\(^{+}\)-Ca\(^{2+}\) exchange, and secondary (bafilomycin-sensitive) uptake of Ca\(^{2+}\) linked to H\(^{+}\)-ATPase activity (26).

However, because bafilomycin did not fully abolish the CS-induced rise in cytoplasmic Ca\(^{2+}\) (Fig. 7A), additional pathways may exist to sequester Ca\(^{2+}\) prior to CS exposure. Bafilomycin also repressed the effects of Ca\(^{2+}\) chelation with BAPTA on CFTR protein and surface densities (Figs. 1, 2, and 7, B–D), suggesting that the bafilomycin-dependent inhibition of Ca\(^{2+}\) release accounted for the protection of surface CFTR following CS exposure (Fig. 7, B–F). Bafilomycin is known to affect receptor trafficking and recycling (50); thus, we cannot rule out the possibility that CS-damaged CFTR molecules are internalized through an alternative mechanism. Bafilomycin pretreatment also prevented CS-induced ASL volume depletion (Fig. 7, G and H), indicating that CS/Ca\(^{2+}\)-induced CFTR inhibition is relevant in airway epithelia. Despite being approved for human usage, bafilomycin may not be a suitable drug to protect against or reverse the effects of chronic CS on CFTR because it inhibits CFTR maturation (51).

Altered lysosomal function has previously been described in COPD airways and is thought to trigger induction of autophagy (11). Whether the increase in Ca\(^{2+}\) with CS is associated with this event remains to be determined, although lysosomes appear to be involved in both processes. Why CFTR is sequestered into an insoluble aggregate after CS exposure is also unknown (14). CFTR that is targeted for degradation normally traffics to lysosomes (52). However, we hypothesize that CS-induced lysosomal dysfunction causes CFTR to be re-routed into aggresome-like compartments leaving it detergent-insoluble. Elevated cytoplasmic Ca\(^{2+}\) is usually associated with apoptosis and cell death (53). However, we have tracked CS-exposed BHCFCTR cells and HBECs for >24 h post-CS exposure; and the cultures remain intact and, in the case of the HBECs, can still maintain a functional ASL beyond this period. Elevated Ca\(^{2+}\) levels are also known to activate transcription factors such as NFkB and nrf2, which can trigger changes in gene expression (54, 55). Thus, the CS-induced Ca\(^{2+}\) response may be an adaptive/survival response of the epithelia that prepare the lung for survival in a chemically toxic environment (i.e. chronic CS exposure) by facilitating altered gene expression. In summary, we have provided evidence that CS exposure induces drastic changes in cellular Ca\(^{2+}\) levels that lead to CFTR inter-

Acknowledgments—We thank the University of North Carolina, Chapel Hill, Cystic Fibrosis Center Tissue Core for isolating and providing cells from donor lungs and the University of North Carolina, Chapel Hill, Biomarker Mass Spectrometry Facility for analyzing NAADP\(^{+}\). BHCFCTR cells, Ano1mCherry, gfpCFTR, yfpSTIM, and LAMPI-YCaM3.6 were kindly provided by Drs. Martina Gentzsch (University of North Carolina, Chapel Hill), Criss Hartzell (Emory University), Peter Haggie (University of California at San Francisco), Tobias Meyer (Stanford University), and Lee Haynes (University of Liverpool) respectively.

REFERENCES

1. Vareille, M., Kieninger, E., Edwards, M. R., and Regamey, N. (2011) The airway epithelium: soldier in the fight against respiratory viruses. Clin. Microbiol. Rev. 24, 210–229
2. Bartlett, J. A., Fischer, A. J., and McCray, P. B., Jr. (2008) Innate immune functions of the airway epithelium. Contrib. Microbiol. 15, 147–163
3. Schmid, A., Clunes, L. A., Salathe, M., Verdugo, P., Dietl, P., Davis, C. W., and Tarran, R. (2011) Nucleotide-mediated airway clearance. Subcell. Biochem. 55, 95–138
4. Com, G., and Clancy, J. P. (2009) Adenosine receptors, cystic fibrosis, and airway hydration. Handb. Exp. Pharmacol. 363–381
5. Chmiel, J. F., and Davis, P. B. (2003) State of the art: why do the lungs of patients with cystic fibrosis become infected and why can’t they clear the infection? Respir. Res. 4, 8
6. Mannino, D. M., Homa, D. M., Akinbami, L. J., Ford, E. S., and Redd, S. C. (2002) Chronic obstructive pulmonary disease surveillance—United States, 1971–2000. MMWR Surveill. Summ. 51, 1–16
7. Hogg, J. C. (2005) Infection and COPD. Exp. Lung Res. 31, Suppl. 1, 72–73
8. Petty, T. L. (2002) COPD in perspective. Chest 121, 1165–1208
9. Cosio, M. G., Saetta, M., and Agusti, A. (2009) Immunologic aspects of chronic obstructive pulmonary disease. N. Engl. J. Med. 360, 2445–2454
10. Donnelly, L. E., and Barnes, P. J. (2012) Defective phagocytosis in airways disease. Chest 141, 1055–1062
11. Ryter, S. W., Nakahira, K., Haspel, J. A., and Choi, A. M. (2012) Autophagy in pulmonary diseases. Annu. Rev. Physiol. 74, 377–401
12. Kim, V., Rogers, T. J., and Criner, G. J. (2008) New concepts in the pathobiology of chronic obstructive pulmonary disease. Proc. Am. Thorac. Soc. 5, 478–485
13. Cantin, A. M., Hanrahan, J. W., Bilodeau, G., Ellis, L., Dupuis, A., Liao, J., Zielenski, J., and Durie, P. (2006) Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. Am. J. Respir. Crit. Care Med. 173, 1139–1144
14. Clunes, L. A., Davies, C. M., Coakley, R. D., Aleksandrov, A. A., Henderson, A. G., Zeman, K. L., Worthington, E. N., Gentzsch, M., Kredo, S. M., Cholon, D., Bennett, W. D., Riordan, J. R., Boucher, R. C., and Tarran, R. (2012) Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration. FASEB J. 26, 533–545
15. Sloane, P. A., Shastry, S., Wilhelm, A., Courville, C., Tang, L. P., Backer, K., Levin, E., Raju, S. V., Li, Y., Mazur, M., Byan-Parker, S., Grizzle, W., Sorsch, E. J., Dransfield, M. T., and Rowe, S. M. (2012) A pharmacologic approach to acquired cystic fibrosis transmembrane conductance regulator dysfunction in smoking related lung disease. PLoS One 7, e93809
16. Cantin, A. M., Bilodeau, G., Ouellet, C., Liao, J., and Hanrahan, J. W. (2006) Oxidant stress suppresses CFTR expression. Am. J. Physiol. Cell Physiol. 290, C262–C270
17. Rubin, B. K., Ramirez, O., Zayas, J. G., Finegan, B., and King, M. (1992) Respiratory mucus from asymptomatic smokers is better hydrated and more easily cleared by mucociliary action. Am. Rev. Respir. Dis. 145, 545–547
18. Homolya, L., Watt, W. C., Lazarowski, E. R., Koller, B. H., and Boucher,
R. C. (1999) Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y(2) receptor (−/−) mice. J. Biol. Chem. 274, 26456–26460

19. Feske, S. (2009) ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca2+ entry in the immune system and beyond. Immunity 31, 1017–1018

20. Korngreen, A., and Priel, Z. (1996) Purinergic stimulation of rabbit ciliated airway epithelia: control by multiple calcium sources. J. Physiol. 497, 53–66

21. Lorenzo, I. M., Liedtke, W., Sanderson, M. J., and Valverde, M. A. (2008) TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 105, 12611–12616

22. Davis, C. W., and Lazarowski, E. (2008) Coupling of airway ciliary activity and mucin secretion to mechanical stresses by purinergic signaling. Respir. Physiol. Neurobiol. 163, 208–213

23. Agopyan, N., Head, J., Yu, S., and Simon, S. A. (2004) TRPV1 receptors mediate particulate matter-induced apoptosis. Am. J. Physiol. Lung Cell Mol. Physiol. 286, L563–L572

24. Sun-Wada, G. H., Wada, Y., and Futai, M. (2003) Lysosome and lysosome-related organelles responsible for specialized functions in higher organisms, with special emphasis on vacuolar-type proton ATPase. Cell Struct. Funct. 28, 455–463

25. Eskelinen, E. L., Tanaka, Y., and Saitg, P. (2003) At the acidic edge: emerging functions for lysosomal membrane proteins. Trends Cell Biol. 13, 137–145

26. Morgan, A. J., Platt, F. M., Lloyd-Evans, E., and Galioune, A. (2011) Molecular mechanisms of endolysosomal Ca2+ signalling in health and disease. Biochem. J. 439, 349–374

27. Kiselyov, K., Yamaguchi, S., Lyons, C. W., and Muallem, S. (2010) Aberrant Ca2+ handling in lysosomal storage disorders. Cell Calcium 47, 103–111

28. Clunes, L. A., Bridges, A., Alexis, N., and Tarran, R. (2008) In vivo versus in vitro airway surface liquid nicotine levels following cigarette smoke exposure. J. Appl. Toxicol. 32, 201–207

29. Coakley, R. D., Sun, H., Clunes, L. A., Rasmussen, J. E., Stackhouse, J. R., Okada, S. F., Fricks, I., Young, S. L., and Tarran, R. (2008) 17β-Estradiol inhibits Ca2+-dependent homeostasis of airway surface liquid volume in human cystic fibrosis airway epithelia. J. Clin. Investig. 118, 4025–4035

30. Fukushima, R., Takakura, T., Nakamichi, N., Kambe, Y., Kawagoe, H., Nakazato, R., and Yoneda, Y. (2010) Requirement of both NR3A and NR3B subunits for dominant negative properties on Ca2+ mobilization mediated by acquired N-methyl-D-aspartate receptor channels into mitochondria. Neurochem. Int. 57, 730–737

31. Young, G. S., and Kirkland, J. B. (2006) Modifications to increase the lifetime and epithelial cell number of human cystic fibrosis airway epithelia. J. Cell Sci. 119, 281–286

32. Haggie, P. M., Stanton, B. A., and Verkman, A. S. (2002) Diffusional mobility of the cystic fibrosis transmembrane conductance regulator mutant, delta F508-CFTR, in the endoplasmic reticulum measured by photo bleaching of GFP-CFTR chimeras. J. Biol. Chem. 277, 16419–16425

33. Sheridan, J. T., Worthington, E. N., Yu, K., Gabriel, S. E., Hartzell, H. C., and Tarran, R. (2011) Characterization of the oligomeric structure of the Ca2+-activated Cl− channel Ano1/TMEM16A. J. Biol. Chem. 286, 1381–1388

34. Jia, Y., Mathews, C. J., and Hannah, J. W. (1997) Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. J. Biol. Chem. 272, 4978–4984

35. Thelin, W. R., Kesimer, M., Tarran, R., Kreda, S. M., Grubb, B. R., Sheehan, J. K., Stutts, M. J., and Milgram, S. L. (2005) The cystic fibrosis transmembrane conductance regulator is regulated by a direct interaction with the protein phosphatase 2A. J. Biol. Chem. 280, 41152–41152