Chronic Airway Infection/Inflammation Induces a Ca^{2+}_i-dependent Hyperinflammatory Response in Human Cystic Fibrosis Airway Epithelia*

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Hyperinflammatory responses to infection have been postulated as a component of cystic fibrosis (CF) lung disease. Studies have linked intracellular calcium (Ca^{2+}_i) mobilization with inflammatory responses in several systems. We have reported that the pro-inflammatory mediator bradykinin (BK) promotes larger Ca^{2+}_i signals in CF compared with normal bronchial epithelia, a response that reflects endoplasmic reticulum (ER)/Ca^{2+} store expansion induced by chronic luminal airway infection/inflammation. The present study investigated whether CF airway epithelia were hyperinflammatory and, if so, whether the hyperinflammatory CF phenotype was linked to larger Ca^{2+} stores in the ER. We found that ΔF508 CF bronchial epithelia were hyperinflammatory as defined by an increased basal and mucosal BK-induced interleukin (IL)-8 secretion. However, the CF hyperinflammation expressed in short-term (6–11-day-old) primary cultures of ΔF508 bronchial epithelia was lost in long-term (30–40-day-old) primary cultures of ΔF508 bronchial epithelia, indicating this response was independent of mutant cystic fibrosis transmembrane conductance regulator. Exposure of 30–40-day-old cultures of normal airway epithelia to supernatant from mucopurulent material (SMM) from CF airways reproduced the increased basal and mucosal BK-stimulated IL-8 secretion of short-term CF cultures. The BK-triggered increased IL-8 secretion in SMM-treated cultures was mediated by an increased Ca^{2+}_i mobilization consequent to an ER expansion associated with increases in protein synthesis (total, cytokines, and antimicrobial factors). The increased ER-dependent, Ca^{2+}_i-mediated hyperinflammatory epithelial response may represent a general beneficial airway epithelial adaptation to transient luminal infection. However, in CF airways, the Ca^{2+}_i-mediated hyperinflammation may be ineffective in promoting the eradication of infection in thickened mucus and, consequently, may have adverse effects in the lung.

In CF[1] airway epithelia, the absence of the cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl^- secretion, coupled with increased Na^+ absorption, results in a reduced periciliary liquid layer depth (1), adherence of thickened mucus to airway surfaces, and persistent airway infections (2–5). It remains controversial whether the inflammatory response of CF airways to infection is intrinsically excessive and, thus, accelerates lung disease. As evidence for excessive inflammation, cytokines are elevated in sputa from CF compared with disease control (asthmatic) patients (6), and bronchoalveolar lavage studies have revealed higher levels of cytokines and inflammatory cells referenced to bacterial number or endotoxin levels in CF *versus* non-CF patients with acute lung infection (4, 7).

Many studies have addressed the magnitude of airway epithelial inflammatory responses to infection in CF by focusing on the regulation of cytokine production by the transcription factor nuclear factor (NF)-κB (8, 9), including its regulation by toll receptors (10). In addition, intracellular calcium (Ca^{2+}_i) signals resulting from heterotrimeric G protein-coupled receptor (GPCR) activation by inflammatory mediators or infectious agents also modulate NF-κB activation by a Ca^{2+}_i-dependent mechanism (11–17). For example, the pro-inflammatory mediator bradykinin (BK) triggers Ca^{2+}_i mobilization (18) and induces interleukin (IL)-8 secretion in non-CF and CF human airway epithelia (19). Furthermore, the CF pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* promote IL-8 secretion by a Ca^{2+}_i mobilization-dependent mechanism in airway epithelial cells (20).

We have shown that apical purinoceptor ($P2Y_2$ receptors) or BK receptor activation induces larger Ca^{2+}_i signals in CF compared with normal human airway epithelia (21, 22), and this exaggerated Ca^{2+}_i response results from luminal infection/inflammation-induced up-regulation of the apically confined endoplasmic reticulum (ER) Ca^{2+} stores (22). The possibility that the amplified ER-derived Ca^{2+}_i responses provide a clue to the pathogenesis of CF hyperinflammation was raised by studies of other diseases. For example, in a model for Gaucher disease, hippocampal cells expressed a greater glutamate-dependent ER Ca^{2+} release, consequent to an increased ER size, which

appeared linked to the neuronal toxicity characteristic of this disease (23). Moreover, presenillin mutations in Alzheimer disease have been associated with increases in ER size, Ca^{2+} stores, and cytokine production (24, 25). In the case of CF, the possible relationships among ER expansion, enhanced cytokine release, and CF airways inflammation are unknown.

In the present study, we tested the hypothesis that CF airway epithelia express a hyperinflammatory phenotype resulting from luminal infection/inflammation-induced up-regulation of ER Ca^{2+} stores. Accordingly, we investigated the following: 1) whether CF airway epithelia have increased IL-8 release in response to the inflammatory peptide BK as compared with normal airway epithelia; 2) whether this response is an epithelial adaptation to luminal infection/inflammation or depends on mutant ΔF508 CFTR; and 3) whether greater ER-derived Ca$^{2+}_i$ responses contribute to the increased cytokine release in an *in vitro* model for CF airway epithelial inflammation.

MATERIALS AND METHODS

Cell Culture and Freshly Excised Tissue—Tissues and cells were obtained under the auspices of protocols approved by the Institutional Committee on the Protection of the Rights of Human Subjects. Excess tissues from human donor lungs and excised recipient lungs were obtained at the time of lung transplantation from main stem or lobar bronchi. Tissues were either fixed in 4% paraformaldehyde and embedded in paraffin or used for cell isolation. Bronchial epithelial cells were provided by the University of North Carolina Cystic Fibrosis Center Tissue Core. Normal and CF (ΔF508 homozygous) cells were harvested and cultured as previously described (22). Cultures were maintained at an air-liquid surface interface, and polarized primary cultures were studied at 6–11 days (short-term cultures) or at 30–40 days (long-term cultures).

IL-8 Secretion—For basal, apical BK-stimulated, UTP-stimulated, or SMM from CF airways (see "Studies with Infectious/Inflammatory Material from CF Airways")-stimulated IL-8 secretion, the serosal media were collected at selected times from short-term or long-term cultures of normal and CF bronchial epithelia. IL-8 was measured by enzyme-linked immunosorbent assay (R&D Systems) in duplicate.

Studies with Infectious/Inflammatory Material from CF Airways—Mucopurulent material was harvested from the airway lumens of excised human CF lungs infected with *P. aeruginosa* and *S. aureus* at the time of transplant and provided by the Tissue Core of the University of North Carolina Cystic Fibrosis Center as recently described (22). This material was centrifuged at 100,000 rpm (60 min, 4 °C), and the supernatant was filtered through a 0.2-μm filter and frozen at -80 °C. Preliminary studies revealed that treatment of airway epithelia with SMM from individual CF lungs infected with *P. aeruginosa* and *S. aureus* induced epithelial hyperinflammation. Because of the small volume of SMM per patient and the large number of experiments, SMMs from nine CF lungs (five males and four females; age, 17–48 years; four ΔF508 homozygous and five unknown genotypes) were pooled to assure homogeneous stimulus throughout experiments.

PBS or SMM (40 μl) was applied to the mucosal surfaces of normal or CF bronchial epithelia, and the following protocols were utilized. First, to compare the inflammatory response of normal *versus* CF cultures to luminal infectious/inflammatory stimuli, cultures were treated with PBS or SMM, and a time course was performed for IL-8 secretion. Second, to test whether SMM-treated cultures were sensitized to a subsequent inflammatory stimulus, cultures were treated with PBS or SMM for 24–36 h, and the serosal media were replaced by fresh media, followed by mucosal BK (5 μM) or UTP (100 μM) and serial measurements of IL-8 secretion. Third, to address the role of Ca$^{2+}_i$ mobilization on SMM-potentiated BK-induced IL-8 secretion, cultures were treated with PBS or SMM for 36 h, followed by removal of the medium and BAPTA/acetoxymethyl ester loading as previously described (21). BK (5 μM) was subsequently mucosally added to cultures in a nominally Ca^{2+}-free solution (21), and Ca$^{2+}_i$ mobilization was assessed as previously reported (22); in parallel experiments, IL-8 release into the serosal compartment was measured 2 h later. Fourth, to assess the effect of SMM on IL-8, IL-6, and IL-1β secretion, cultures were treated with PBS or SMM for up to 120 h, and the serosal media were collected and replaced every 24 h for cytokine measurements. Fifth, to address the effect of SMM on total protein synthesis, the incorporation of ^{35}S-labeled amino acids into proteins synthesized by cultures metabolically labeled with [^{35}S]cysteine/

methionine (26) was measured after 48 h of PBS or SMM treatment.

Immunostaining of ER Protein Synthetic Markers—The immunostaining of ER chaperone proteins in cultures of bronchial airway epithelia or deparaffinized native bronchial epithelial sections was performed according to a modification of our previous method (27). Cultures or epithelial sections were incubated with the primary antibodies, a rabbit (1:100 dilution) and a mouse (5 μg/ml; Stressgen) antibody, for BIP and PDI, respectively, followed by a fluorescein-labeled goat anti-rabbit antibody and a Texas Red-labeled goat anti-mouse antibody (1:200 dilution; Jackson Immunoresearch Laboratories). As controls, the primary antibodies were omitted. The fluorescent signals were studied by laser confocal microscopy (Leica model TCS 4D; PL APO 63x/1.20 mm water lens) in the XZ or XY scanning mode.

The quantification of the fluorescence intensity of labeled BIP and PDI was performed according to a previous method (22). Multiple scans were obtained from each sample, and regions of interest were designated in the apical domains with the MetaMorph software. The same acquisition parameters (*e.g.* laser power, contrast, brightness, and pinhole value) for each channel were employed to acquire the images from paired normal bronchial epithelial cultures treated in different ways or native normal *versus* CF bronchial epithelia in experiments performed on the same day. The fluorescence intensity values from the regions of interest were averaged from paired cultures and native bronchial epithelial tissues.

XBP-1 mRNA Splicing—The assessment of XBP-1 mRNA splicing was an adaptation of a previous method (28, 29). Long-term bronchial epithelial cultures were mucosally treated with 40 μl of PBS or SMM for 48 h or 1 μM thapsigargin (TG) for 24 h, followed by three washes with PBS. 1 ml of RNAlater^{TM} (Qiagen) was added to the mucosal and serosal surfaces, and cultures were frozen at -20 °C. Total RNA was isolated using RNeasy (Qiagen), and 200 ng of RNA was reverse transcribed by SuperScript II RNase H+ reverse transcriptase (Invitrogen) using 250 ng of random primers. PCR primers were designed to amplify a 270-bp region of XBP-1 (sequence number NM_005080). The PCR product spans a 26-bp intron that, when spliced by IRE1, results in a translated XBP-1 mRNA that codes for an active transcription factor. The unspliced PCR product contains a PstI site, which is destroyed after splicing. The PCR protocol was 1 cycle at 94 °C for 7 min; 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; followed by 1 cycle of 72 °C for 7 min and holding at 4 °C. The PCR products were run on a 2% agarose gel, column-purified using a QIAquick PCR Purification Kit (Qiagen), and digested with PstI to visualize the 270-bp product (spliced product) and the two ~140-bp products (unspliced product). To quantify the amount of unspliced product, a Southern blot was performed using the PstI-digested PCR product from PBS- and SMM-treated cultures. DNA was run on a 2% agarose gel, capillary blot transferred, cross-linked to the membrane, and probed (Megaprime DNA Labeling Kit; Amersham Biosciences) with the 270-bp PCR product from PBS-treated samples. Data were quantified with a Storm phosphorimaging system.

Quantitation of BIP mRNA—Quantitative real-time PCR analysis was used to evaluate changes in BIP mRNA from PBS- and SMM-treated cultures utilizing the LightCycler (Roche Applied Science). RNA was isolated according to standard RNA isolation procedures involving phenol/choloroform extraction and purification/DNase treatment using the Qiagen RNeasy purification kit. cDNA was generated from RNA samples (200 ng) using SuperScript II reverse transcriptase. PCR amplification utilized the following primer sequences: 5′ forward, TCCTATGTCGCCTTCACTCC; and 3′ reverse, TTTCCCAATAACCTCAGC.

Primers were initially tested for specificity by PCR and then by melting curve analysis in the LightCycler instrument. A standard curve was run utilizing a positive control cDNA to determine PCR reaction efficiency. PBS- or SMM-treated samples were run in duplicate, and cross-points were calculated using the LightCycler internal software. As a control gene, glyceraldehyde-3-phosphate dehydrogenase was also run in duplicate on each of the samples. Ratios of gene expression were calculated as previously reported (30).

Statistics—Data represent the mean $\pm$ S.E. from at least three experiments from three individual donors and were analyzed by unpaired t test or two-way analysis of variance. Statistical significance was defined as $p < 0.05$.

RESULTS

Short-term Primary Cultures of CF Airway Epithelia Exhibit Increased Basal and BK-stimulated IL-8 Secretion—To investigate whether CF airway epithelia exhibit hyperinflammation,

FIG. 1. **Short-term primary cultures of CF bronchial airway epithelia exhibit increased basal and BK-stimulated IL-8 secretion, but their hyperinflammatory phenotype reverts with time in the prolonged absence of luminal infection/inflammation, independently of defective ΔF508 CFTR.** *A*, time course for baseline IL-8 secretion in short-term (6–11-day-old) cultures of normal and CF epithelia. *B*, time course for mucosal BK (5 μM)-stimulated IL-8 secretion in 6–11-day-old cultures of normal and CF epithelia. *C*, time course for baseline IL-8 secretion in long-term (30–40-day-old) cultures of normal and CF epithelia. *D*, time course for mucosal BK (5 μM)-stimulated IL-8 secretion in 30–40-day-old cultures of normal and CF epithelia. *White* and *black bars* correspond to normal and ΔF508 CF epithelia, respectively. Data are expressed as mean ± S.E.; n = 3–4. *, $p < 0.05$, CF *versus* normal cultures.
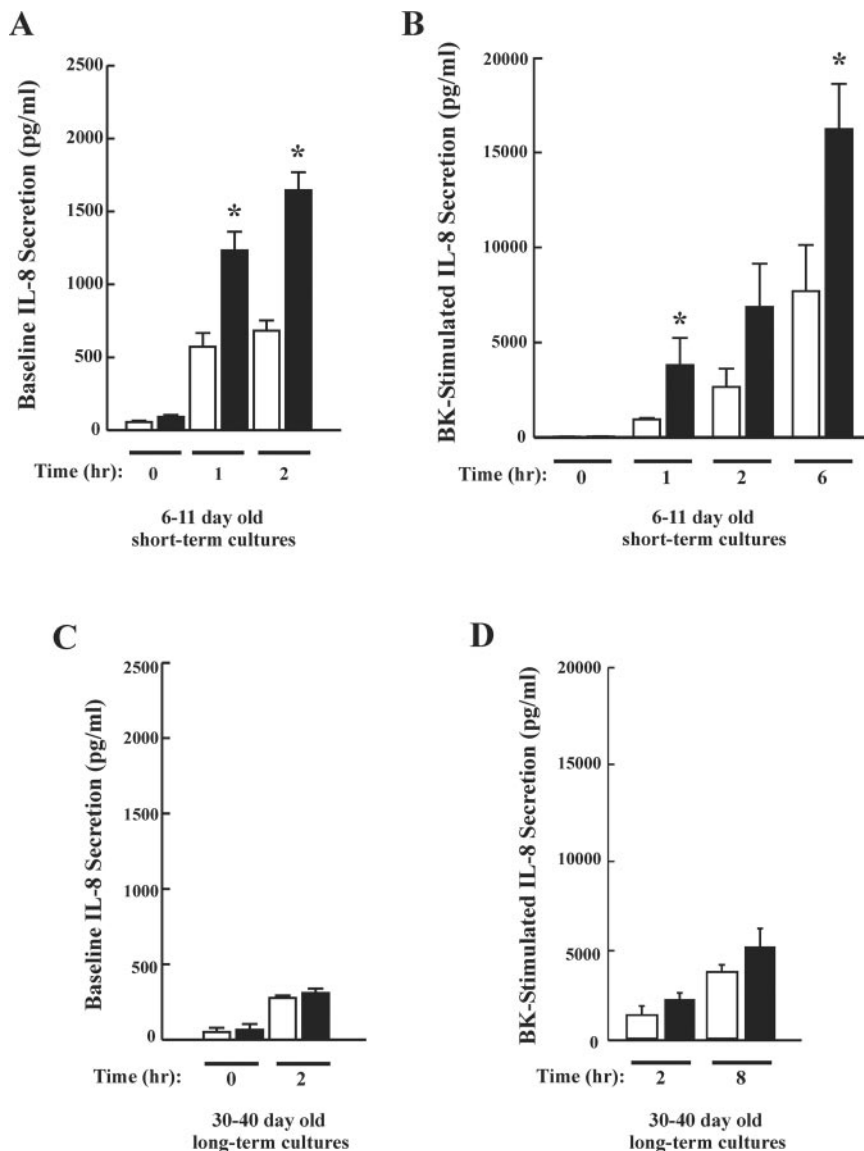
we first compared the basal IL-8 secretion in 6–11-day-old normal *versus* ΔF508 homozygous CF primary bronchial epithelial cultures. Fig. 1*A* illustrates that the baseline IL-8 secretion was increased in short-term CF compared with normal cultures. The effect of the pro-inflammatory agonist BK on IL-8 secretion was measured in parallel 6–11-day-old primary cultures of normal and ΔF508 homozygous CF bronchial epithelia. Mucosal treatment with a maximal dose of BK (5 μM) (18) promoted greater IL-8 secretion in CF compared with normal cultures (Fig. 1*B*). These data demonstrate that short-term primary cultures of CF bronchial airway epithelia express a hyperinflammatory phenotype.

Is the CF Hyperinflammatory Phenotype the Result of an Intrinsic ΔF508 CFTR Defect or an Acquired Epithelial Response to Chronic Airway Infection/Inflammation?—To address this issue, IL-8 secretion under basal and BK-stimulated conditions was studied in long-term normal *versus* ΔF508 homozygous CF primary bronchial epithelial cultures. Fig. 1*C* demonstrates that the increased baseline IL-8 secretion in short-term CF cultures compared with normal cultures (Fig. 1*A*) was lost after 30–40 days in culture. Furthermore, 30–40-day-old CF cultures responded in the same manner to 5 μM mucosal BK-dependent IL-8 secretion as 30–40-day-old normal cultures (Fig. 1*D*). These data strongly suggest that the

hyperinflammatory state observed in short-term CF cultures does not represent a defect intrinsic to the ΔF508 CFTR genotype but, rather, is a response to the persistent infectious/inflammatory milieu found in CF airways *in vivo* that wanes with prolonged periods in the absence of the stimulus.

Can the CF Hyperinflammatory Phenotype Be Transferred to Normal Airway Epithelia by Mucosal Exposure to CF Airway Mucopurulent Material?—To test more directly for the role of persistent infection and inflammation on hyperinflammatory responsiveness, we prepared a SMM from CF airway lumens, exposed the lumens of airway epithelial cultures to it for prolonged periods, and measured basal and BK-induced IL-8 release.

SMM-dependent IL-8 secretion was first investigated in long-term 30–40-day-old primary cultures of normal and CF bronchial epithelia acutely exposed to mucosal SMM for up to 6 h. Fig. 2*A* illustrates that the acute SMM treatment triggered IL-8 secretion, but this response was similar in long-term normal and CF cultures for each interval.

We next assessed whether prolonged SMM pretreatment (36 h) conferred a "CF-like hyperinflammatory phenotype," *i.e.* an increased IL-8 secretory response to mucosal BK, in normal airway epithelia and whether this response was different in long-term cultures of CF epithelia. SMM-pretreated 30–40-day-old normal epithelia exhibited an increased IL-8 secretion
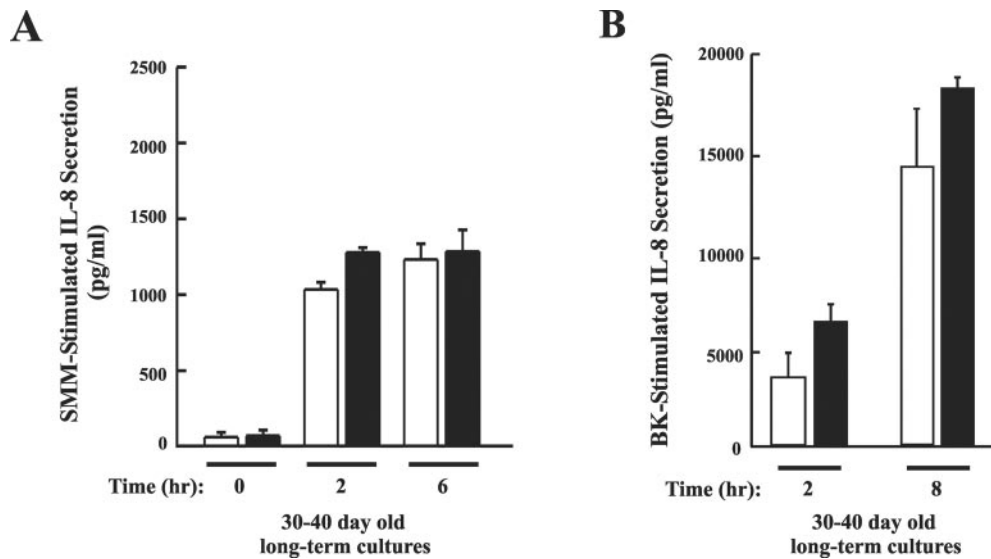
FIG. 2. **Long-term primary cultures of normal and CF epithelia respond in the same manner to luminal infectious/inflammatory stimuli-induced IL-8 secretion.** *A*, time course for IL-8 secretion following acute mucosal exposure of long-term (30–40-day-old) cultures of normal and CF epithelia to SMM. SMM also induces IL-8 secretion in short-term cultures (data not shown). *B*, time course for mucosal BK (5 μM)-stimulated IL-8 secretion in 30–40-day-old cultures of normal and CF epithelia pretreated for 36 h with mucosal SMM. The *y* axis scales are the same as those from Fig. 1. *White* and *black bars* correspond to normal and ΔF508 CF epithelia, respectively. Data are expressed as mean $\pm$ S.E.; *n* = 3.

to BK (Fig. 2*B*) compared with 30–40-day-old normal epithelia exposed to BK in the absence of SMM pretreatment (Fig. 1*D*). Moreover, an increased BK-dependent IL-8 secretion was induced in 30–40-day-old CF cultures pretreated for 36 h with SMM (Fig. 2*B*), as compared with non-SMM treated cultures (Fig. 1*D*). Notably, the IL-8 response of SMM-pretreated CF cultures in the absence (Fig. 2*A*) or presence (Fig. 2*B*) of mucosal BK was similar to that of normal cultures. Thus, these data again argue against an intrinsic hyperinflammatory defect associated with the ΔF508 genotype but suggest that chronic infection/inflammation (SMM) itself can induce the hyperinflammatory phenotype.

Can the CF-like Hyperinflammatory Phenotype, e.g., the Amplified BK-dependent IL-8 Secretion in SMM-pretreated Normal Cultures, Be Reproduced with the Activation of Another Receptor Coupled to Ca$^{2+}_i$ Mobilization?—Like BK receptors, P2Y$_2$ receptors are coupled to Ca$^{2+}_i$ mobilization (22). Therefore, we investigated whether activation of this class of receptors with UTP stimulated IL-8 secretion in airway epithelia and, if so, whether SMM potentiated this response. Following a 24-h PBS pretreatment, 100 μM mucosal UTP induced a modest increase in IL-8 secretion in 30–40-day-old normal bronchial epithelia compared with vehicle-treated cultures (Fig. 3). In contrast, cultures pretreated with SMM secreted much higher levels of IL-8 in response to 100 μM mucosal UTP compared with PBS-treated cultures (Fig. 3). These data suggest that the increased IL-8 secretion triggered by apical activation of BK receptors in SMM-pretreated cultures (Fig. 2*B*) may be a general finding associated with the activation of GPCRs that trigger increased Ca$^{2+}_i$ mobilization in chronically infected/inflamed airway epithelia (22).

Is SMM-potentiated BK-dependent IL-8 Secretion Dependent on SMM-induced Expansion of the Releasable ER Ca^{2+} Stores?—To address the role of Ca$^{2+}_i$ signals on the amplified SMM-induced BK-dependent inflammatory response, we investigated BK-induced Ca$^{2+}_i$ mobilization in 30–40-day-old normal bronchial airway epithelial cultures treated with PBS *versus* SMM for 36 h. Mucosal BK (5 μM) elicited Ca$^{2+}_i$ mobilization in both PBS- and SMM-treated cultures, but the BK-dependent Ca$^{2+}_i$ signal was amplified in SMM-treated epithelia (Fig. 4, *A* and *B*, respectively). The compiled data for
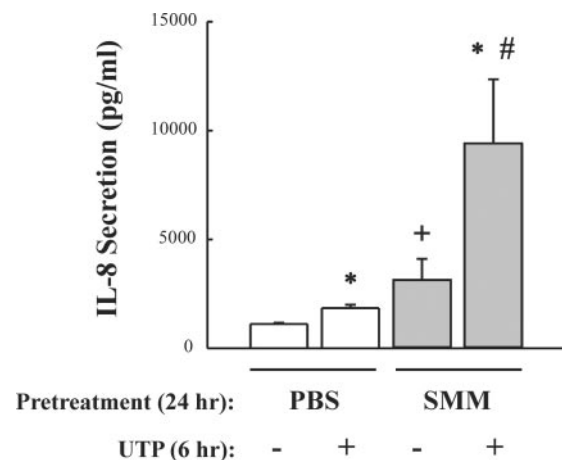

FIG. 3. **Prolonged mucosal exposure of long-term primary cultures of normal bronchial airway epithelia to the Ca$^{2+}_i$-mobilizing agent UTP promotes IL-8 secretion, and this response is potentiated by SMM pretreatment.** 30–40-day-old primary cultures of normal bronchial airway epithelia were mucosally exposed for 24 h to PBS or SMM, followed by apical addition of vehicle or 100 μM UTP, and assessment of IL-8 secretion 6 h later. Data are expressed as mean $\pm$ S.E.; *n* = 3. *, $p < 0.05$, UTP- *versus* vehicle-treated cultures; +, $p < 0.05$, SMM- *versus* PBS-treated cultures; #, $p < 0.05$, UTP + SMM- *versus* UTP + PBS-treated cultures.

BK-dependent Δ340/380 Fura-2 fluorescence (peak − baseline values, an index of ER Ca^{2+} store capacity in airway epithelia) (22) illustrate that SMM pretreatment increased the ER Ca^{2+} stores that can be mobilized following apical BK receptor activation (Fig. 4*E*). Utilizing a protocol previously shown to effectively buffer Ca$^{2+}_i$ signals in airway epithelia (21), the Ca$^{2+}_i$-mobilizing effect of BK was completely blocked in BAPTA-loaded PBS- or SMM-pretreated cultures (Fig. 4, *C* and *D*).

We next tested the effect of Ca$^{2+}_i$ clamping on BK-dependent IL-8 secretion in epithelia treated with PBS *versus* SMM for 24 h. Fig. 5 illustrates that BAPTA was ineffective in blocking BK-dependent IL-8 secretion in PBS-treated cultures. In contrast, the "hyperinflammatory" component of BK-dependent IL-8 secretion in SMM-treated cultures was inhibited by BAPTA. These data suggest that BK induces IL-8 secretion by
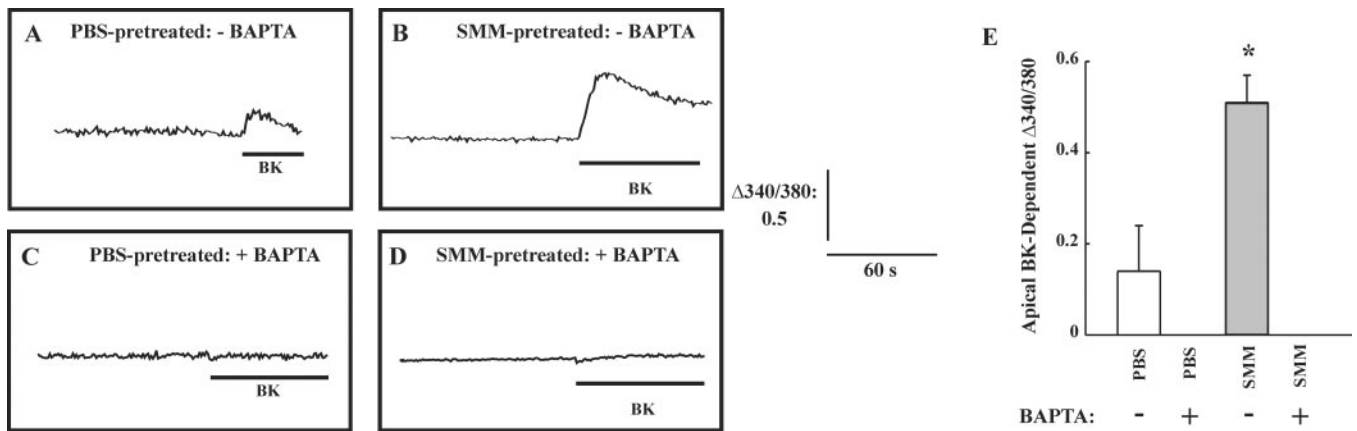
FIG. 4. **Prolonged mucosal exposure of long-term primary cultures of normal bronchial airway epithelia to SMM increases apical BK-sensitive ER Ca²⁺ stores.** *A* and *B*, representative Ca^{2+}_i tracings depicting the effect of 5 μM apical BK-mobilized Ca^{2+}_i in Fura-2-loaded 30–40-day-old primary cultures of normal bronchial airway epithelia mucosally exposed for 36 h to PBS or SMM, respectively. *C* and *D*, representative Ca^{2+}_i tracings from 30–40-day-old primary cultures of normal bronchial airway epithelia mucosally exposed for 36 h to PBS or SMM, respectively, illustrating that the effect of 5 μM apical BK-mobilized Ca^{2+}_i is abolished by Ca^{2+}_i buffering with BAPTA. *E*, compiled data for apical BK-mobilized Ca^{2+}_i (Δ340/380 signal from Fura-2) from PBS- and SMM-pretreated cultures in the absence or presence of Ca^{2+}_i chelation with BAPTA. Data are expressed as mean ± S.E.; $n = 3$–4. *, $p < 0.05$, SMM- *versus* PBS-treated cultures in the absence of BAPTA.
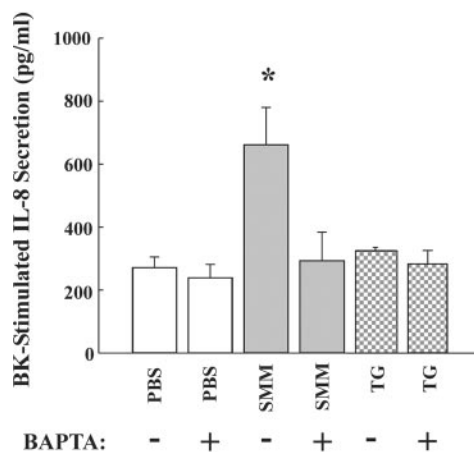

FIG. 5. **Prolonged mucosal exposure of long-term primary cultures of normal bronchial airway epithelia to SMM reproduces a CF-like BK-dependent amplified IL-8 secretion that is mediated by Ca^{2+}_i mobilization.** 30–40-day-old primary cultures of normal bronchial airway epithelia were mucosally exposed to PBS, SMM, or TG (1 μM) for 24 h, followed by vehicle or BAPTA addition in nominally Ca^{2+} free buffer as described under "Materials and Methods." Cultures were subsequently exposed to 5 μM mucosal BK, and IL-8 secretion was assessed 2 h later. Data are expressed as mean ± S.E.; $n = 3$ for all groups. *, $p < 0.05$, SMM- *versus* PBS-treated cultures in the absence of BAPTA.

Ca^{2+}_i-independent and -dependent mechanisms. We speculate that the absence of an effect of Ca^{2+}_i buffering on BK-induced IL-8 secretory responses in PBS-treated cultures reflects the fact that the small BK-induced Ca^{2+}_i signals in cells with normal ER Ca^{2+} stores did not reach the critical threshold necessary to induce the Ca^{2+}_i-mediated component of IL-8 secretion (Fig. 4*A*). In contrast, the larger BK-dependent Ca^{2+}_i signals resulting from the up-regulation of ER Ca^{2+} stores by SMM (Fig. 4*B*) were sufficient to produce a significant Ca^{2+}_i-dependent activation of signal transduction pathway(s) involved in amplified IL-8 secretion.

To further investigate the Ca^{2+}_i-independent component of BK-induced IL-8 secretion in normal airway epithelia, cultures were pretreated for 24 h with the ER Ca^{2+}-ATPase inhibitor TG to deplete the ER Ca^{2+} stores (31). As shown in Fig. 5, the IL-8 response to BK in cultures whose ER Ca^{2+} stores were depleted with TG was the same as that seen in PBS-treated cultures. Furthermore, BAPTA had no additional effect on

BK-dependent IL-8 secretion in TG-treated cultures (Fig. 5).

Collectively, these data suggest that apical BK receptor activation triggers a Ca^{2+}_i-independent component of IL-8 secretion in PBS-, SMM-, and TG-pretreated cultures (indexed as BAPTA-insensitive secretion) and a Ca^{2+}_i-dependent, BAPTA-sensitive component that requires SMM-dependent up-regulation of ER Ca^{2+} stores (Figs. 4 and 5).

Does SMM Induce Increased Protein Synthesis That Is Linked to ER Expansion and Increased Ca^{2+} Stores?—We have recently shown that mucosal treatment of 30–40-day-old primary cultures of normal bronchial epithelia with SMM from CF airways induces expansion of the apical ER compartment and increases its Ca^{2+} stores (22). ER expansion can be triggered by conditions that increase protein synthesis, *e.g.* cellular differentiation, antibody production, or infection (32–39). Thus, we tested whether SMM produced an increase in protein synthesis directly by measuring cytokine and total protein synthesis rates in the absence and presence of SMM. SMM induced IL-8 secretion that was sustained for 120 h compared with PBS-treated cultures (Fig. 6*A*). As a control, treatment of long-term normal cultures with supernatant from luminal material isolated from the lungs of two normal subjects elicited an IL-8 secretion that was only slightly higher than that triggered by PBS (data not shown). SMM-treated long-term normal cultures also exhibited increased secretion of IL-1β and IL-6, compared with PBS-treated cultures (Fig. 6, *B* and *C*, respectively). Moreover, the mRNA levels of the antimicrobial β-defensin2 were increased by 6-fold in SMM-treated cultures compared with PBS-treated cultures (data not shown).

The increased secretion of cytokines was a component of a larger increase in total protein synthesis. Treatment of 30–40-day-old normal airway epithelia with SMM for 48 h increased total protein synthesis by ~50% compared with PBS-treated cultures (Fig. 6*D*). Together, these observations suggest that SMM-increased cytokine secretion and antimicrobial factors are components of a general increase in cellular protein synthesis associated with expansion of the ER and ER Ca^{2+} stores (22).

Is SMM-increased Synthesis of Inflammatory Mediators Associated with Increased Expression of ER Proteins Involved in Protein Synthesis?—Increases in cellular protein synthetic rates are detected in the ER by sensors that trigger the expression of proteins that facilitate the folding requirements of newly synthesized normal proteins (35–38). In mammalian cells, increased protein synthesis is detected by two ER stress
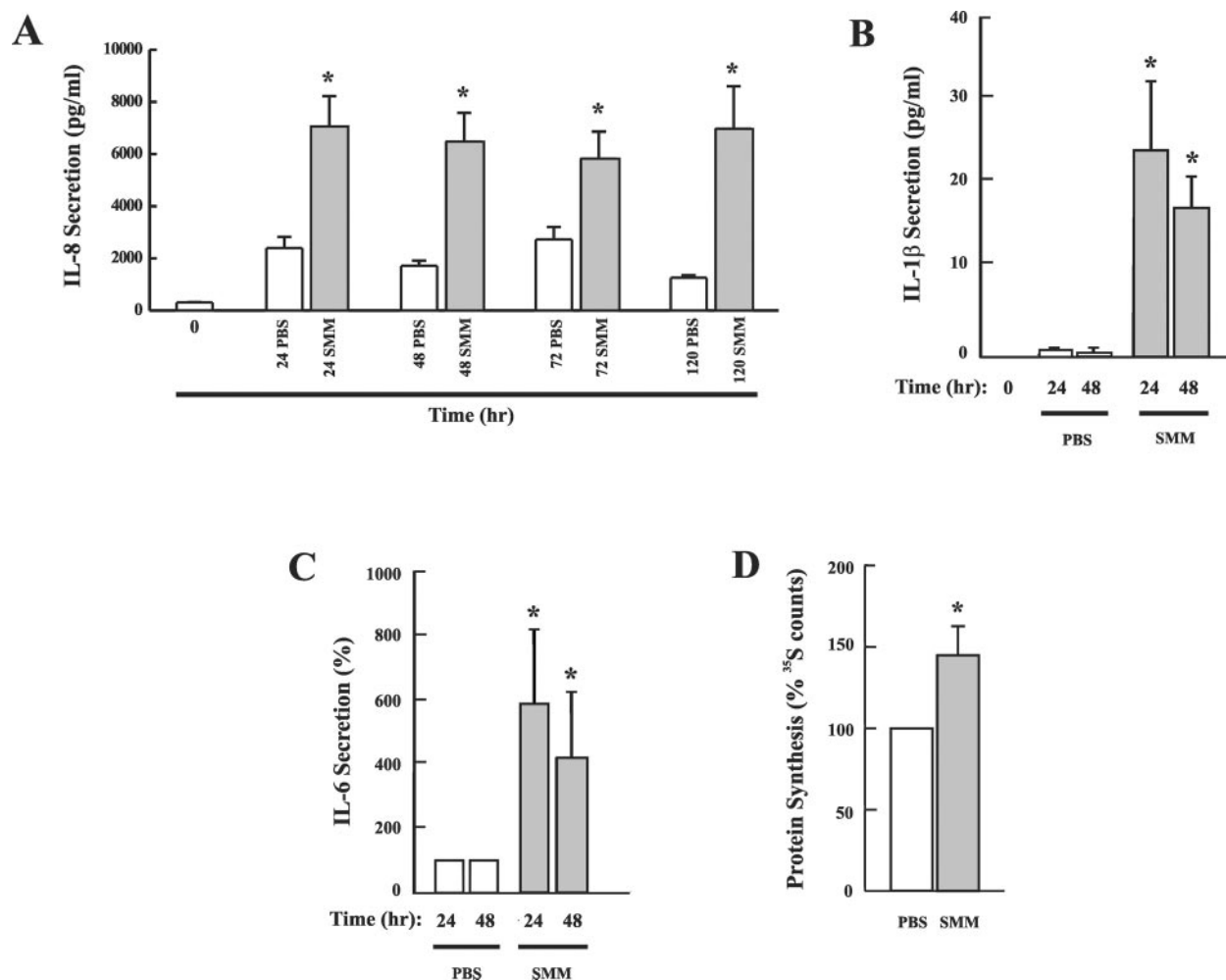
FIG. 6. **Prolonged mucosal exposure of long-term primary cultures of normal bronchial airway epithelia to SMM increases the syntheses of cytokines and total proteins.** *A*, time course for mucosal PBS- and SMM-dependent IL-8 secretion in normal cultures. *B*, time course for mucosal PBS- and SMM-induced IL-1β secretion in normal cultures. *C*, time course for mucosal PBS- and SMM-dependent IL-6 secretion in normal cultures. *D*, total protein synthesis from normal epithelia exposed to mucosal PBS or SMM for 48 h (normalized to [^{35}S]methionine counts from PBS-treated cultures). Data are from 30–40-day-old primary cultures of normal bronchial epithelia and are expressed as mean $\pm$ S.E.; $n =$ 3 for all groups. *, $p < 0.05$, SMM- *versus* PBS-treated cultures.

sensors, IRE1 (40, 41) and the activating transcription factor 6 (ATF6) (35, 38, 42). For example, with increased protein synthesis, the lumenal ER chaperone BIP leaves a BIP·IRE1 complex (43, 44) to facilitate the folding of the newly synthesized proteins. Then, IRE1 dimerizes, *trans*-autophosphorylates, and activates its C-terminal endoribonuclease (36, 38). This activity splices the leucine zipper transcription factor XBP-1 mRNA via removal of a 26-nucleotide intron and induces a frameshift of the mRNA transcript (28, 29). The resulting XBP-1 mRNA is efficiently translated into a potent transcription factor that up-regulates genes such as those encoding ER chaperones involved in protein folding, *e.g.* BIP and PDI (37, 45). ATF6, on the other hand, undergoes proteolysis upon activation to produce a basic leucine zipper transcription factor that is responsible for inducing transcription of ER chaperones as well as XBP-1 itself (46).

We tested whether increases in protein synthesis induced by SMM treatment of 30–40-day-old normal bronchial epithelia triggered XBP-1 mRNA splicing. Fig. 7*A* shows the effect of 48-h mucosal SMM *versus* PBS treatment on XBP-1 mRNA splicing in four different cultures. SMM induced an ~2.5-fold increase of spliced XBP-1 mRNA over PBS (Fig. 7*B*). 6- or 24-h SMM treatment also induced increases of XBP-1 mRNA splicing as compared with PBS-treated cultures (data not shown). Total unspliced XBP-1 mRNA was not increased after SMM

treatment (data not shown), suggesting that ATF6 activation-dependent transcriptional regulation of XBP-1 was not triggered by infection/inflammation. In addition, treatment of 30–40-day-old normal bronchial epithelia with SMM increased BIP mRNA (Fig. 7*C*) and BIP protein (Fig. 7, *D* and *E*) levels. These results suggest that SMM-induced splicing of XBP-1 mRNA is associated with increases in the expression of the ER chaperone BIP.

To investigate whether persistent luminal infection/inflammation was also associated with markers of increased ER protein synthesis in native CF airway epithelia, we studied the expression of the ER chaperones BIP and PDI in CF bronchial epithelia freshly isolated from chronically infected airways. The distribution of both proteins (Fig. 7*F*) matched that of the ER network in native airway epithelia as recently reported (22), and their expression was increased in native CF compared with normal epithelia (Fig. 7, *G* and *H*).

These data suggest that chronic airway infection/inflammation-dependent increases in protein synthesis induce an adaptive ER stress response associated with ER expansion and an increased ER protein folding capacity in CF epithelia *in situ*. A consequence of the ER expansion was an increase in calreticulin mRNA (fold increase after SMM treatment = 1.4, $p < 0.05$) and protein levels in the ER (22) that provide increased ER Ca^{2+} storage, resulting in the amplified Ca$^{2+}_i$ signals in re-
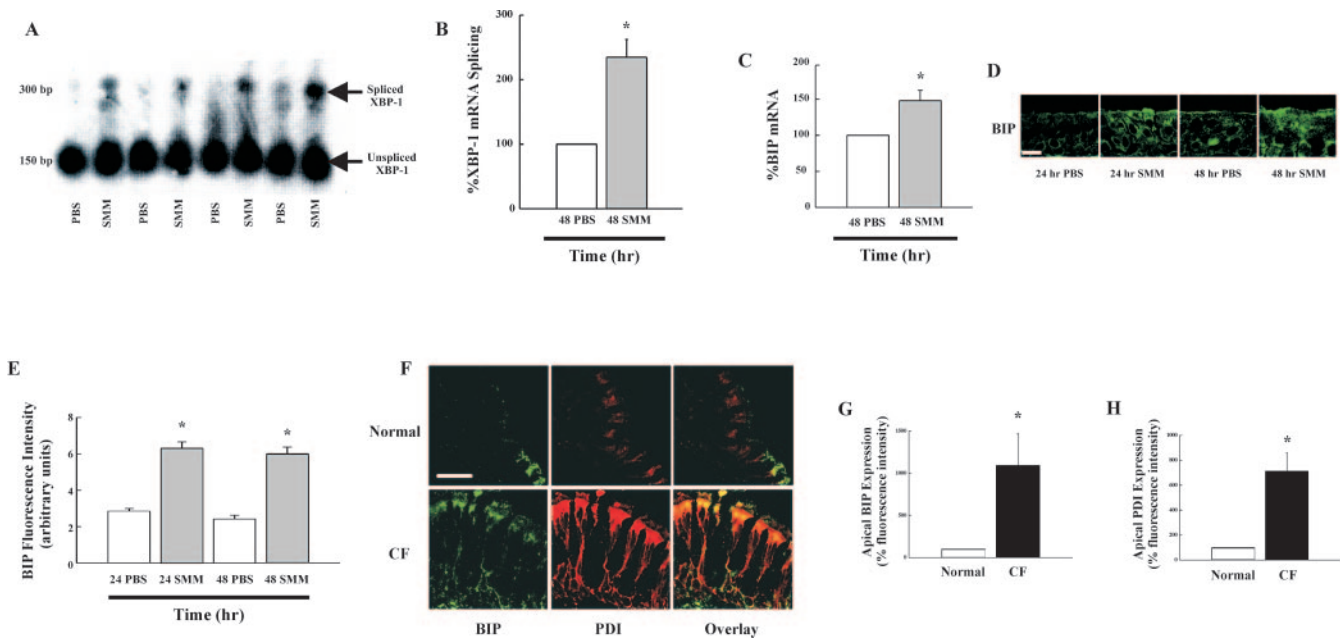
FIG. 7. **Airway infection/inflammation induces an ER stress response associated with increases in XBP-1 mRNA splicing and protein folding ER chaperones in airway epithelia.** *A*, Southern blot illustrating that 48 h mucosal SMM induces IRE1-mediated XBP-1 mRNA splicing in 30–40-day-old cultures of normal bronchial epithelia. *B*, compiled data for XBP-1 mRNA splicing from PBS- and SMM-treated cultures (normalized to XBP-1 mRNA splicing from PBS-treated cultures). *C*, mucosal SMM exposure increases BIP mRNA expression in 30–40-day-old cultures of normal bronchial epithelia (data are normalized to BIP mRNA levels from PBS-treated cultures). *D*, time course illustrating BIP expression by immunostaining in 30–40-day-old normal bronchial epithelia exposed to SMM over 48 h. *Bar*, 10 μm. *E*, compiled data for BIP apical fluorescence intensity from PBS- or SMM-treated cultures. *F*, XY confocal scans from normal and CF native human bronchial epithelia immunostained for BIP and PDI. *Bar*, 10 μm. G and *H*, percentage of apical fluorescence intensity from BIP and PDI, respectively (normalized to fluorescence intensity values from normal epithelia). Data are expressed as mean $\pm$ S.E.; $n = 3$–6 for cultures and $n = 5$ for native epithelia. *, $p < 0.05$, SMM- *versus* PBS-treated cultures or CF *versus* normal epithelia.

sponse to apical GPCR activation that promote amplified IL-8 release, *i.e.* hyperinflammation.

DISCUSSION

The pathogenesis of CF airway disease is complex. Persistent intraluminal infection appears to reflect the failure to clear thickened mucus from the CF lung (47). Airway epithelia play an active role in the response to the bacterial infection of mucus by secreting an array of inflammatory mediators and antimicrobial factors into the airway lumen (48–50). The ineffectiveness (and possibly the magnitude of the inflammatory response) appears to promote airway wall damage and, eventually, respiratory failure in CF.

Previous data suggest that inflammation does not precede infection in CF airways (51, 52). However, the cytokine, *e.g.* IL-8, and cellular responses to a defined bacterial load may be greater in CF than in normal airways, *i.e.* they are "excessive" (4, 7, 53). Several reports suggest that one pathway scaling the magnitude of the cytokine secretion to airways infection is via Ca^{2+}_i, and the pathway connecting ΔCa^{2+}_i responses and cytokine secretion appears to involve activation of transcription factors such as NF-κB. These reports have demonstrated the following: 1) activation of GPCRs induces NF-κB activation and Ca^{2+}_i-dependent secretion of inflammatory mediators (11–14); and 2) infectious agents, or their products, activate NF-κB in a Ca^{2+}_i mobilization-dependent way (15–17). Recent data support the view that alterations in airway epithelial Ca^{2+}_i signaling could, indeed, be implicated in the excessive inflammatory responses of CF airways. These data have revealed that CF airway epithelia exhibit larger ΔCa^{2+}_i responses than normal epithelia following apical purinoceptor or BK receptor activation, which reflect an expansion of the apically confined ER Ca^{2+} stores (22).

In the present study, we tested the hypothesis that CF airway epithelia exhibit excessive inflammatory responses, as

indexed by IL-8 secretion, due to increased ΔCa^{2+}_i responses resulting from ER/Ca^{2+} store expansion. Our findings confirmed that primary cultures of CF airway epithelia are hyperinflammatory when studied as 6–11-day-old short-term cultures (Fig. 1). However, the CF hyperinflammatory phenotype is acquired due to chronic luminal infection/inflammation and is independent of ΔF508 CFTR based on the following observations: 1) long-term primary cultures of CF airway epithelia did not exhibit increased baseline or BK-dependent IL-8 secretion when cultured in the absence of luminal infection for 30–40 days (Fig. 1); 2) the IL-8 secretory responses of long-term primary CF and normal airway epithelial cultures to SMM exposure were not different (Fig. 2); and 3) a CF-like hyperinflammatory phenotype, *e.g.* the amplified BK (or UTP)-induced IL-8 secretion, could be induced in long-term normal airway epithelia by exposing their luminal surfaces to SMM (Figs. 3 and 5). Although it has been controversial whether the ΔF508 CFTR mutation is directly linked with a hyperinflammatory CF airway epithelial response (54), our data suggest that the hyperinflammatory state of CF airway epithelia represents an adaptive epithelial response to the chronic infectious/inflammatory milieu found in CF airway lumens *in vivo* and is independent of mutant CFTR.

This study revealed that a mechanism of CF airway epithelial hyperinflammation reflects an alteration in ER Ca^{2+}_i signaling based on the following observations. First, the hyperinflammatory state observed in short-term primary cultures of CF epithelia (Fig. 1) was associated with larger ER-derived Ca^{2+}_i mobilization than normal cultures (22). Second, exposure of normal cultures to SMM potentiated mucosal BK-mobilized Ca^{2+}_i as a result of ER Ca^{2+} store expansion (Fig. 4), reproducing the larger mucosal BK-dependent Ca^{2+}_i signals from short-term primary cultures of CF bronchial epithelia (22). Third, the CF-like hyperinflammation (*i.e.* increased mucosal
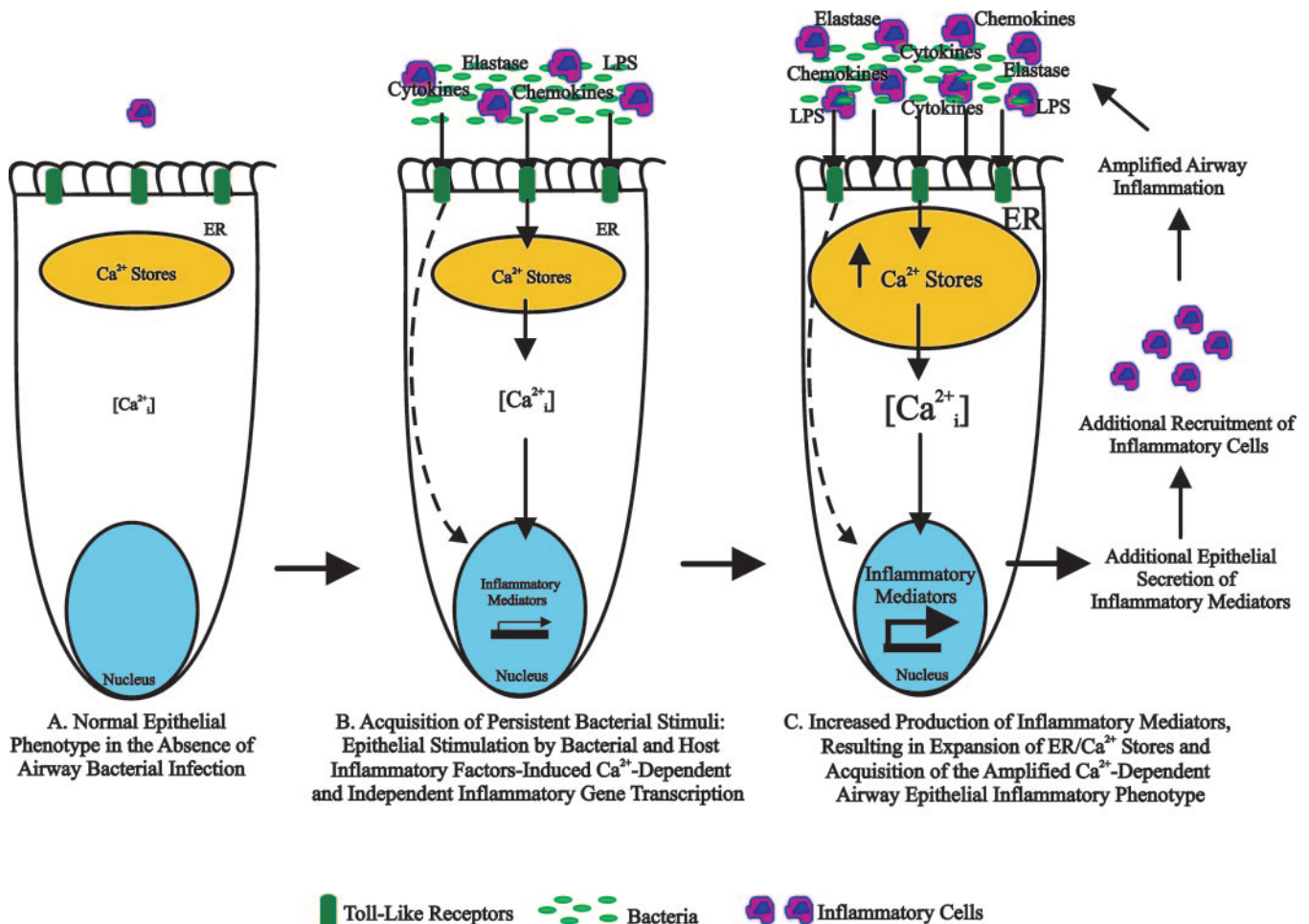
FIG. 8. **Proposed model linking the hyperinflammatory response of CF airway epithelia to ER Ca²⁺ store expansion induced by persistent luminal infection/inflammation.** Illustration of the sequence of events that leads to the Ca^{2+}_i-dependent CF hyperinflammatory phenotype. The initiating stimulus is persistent intraluminal airways infection (*B*). A response to this infection, the increased epithelial synthesis and secretion of antimicrobial factors and cytokines (*B*), triggers ER expansion and increased ER Ca²⁺ stores (*C*). The consequent increase in the magnitude of Ca^{2+}_i signals mediates a component of CF airway hyper-responsiveness, *i.e.* it primes the airways to secrete cytokines via a Ca^{2+}_i-dependent pathway in response to luminal infectious/inflammatory stimuli (*C*). In CF, elements of this amplified response are protective, *e.g.* nucleotide-induced Ca^{2+}_i-enhanced mucociliary clearance (22), whereas other elements may be maladaptive, *e.g.* the increased Ca^{2+}_i-dependent IL-8 secretion recruits inflammatory cells that are ineffective in thick mucus and paradoxically injure airway walls by proteolytic enzyme release. Thus, the increased ER-dependent, Ca^{2+}_i-mediated hyperinflammatory epithelial response is responsible, at least in part, for the acquisition of the amplified CF airways inflammation.

BK-dependent IL-8 secretion resulting from SMM exposure) was abolished by Ca^{2+}_i chelation with BAPTA (Figs. 4 and 5). These data suggest that the larger magnitude of $[Ca^{2+}]_i$ resulting from BK receptor activation in SMM-treated cultures reached the critical threshold for Ca^{2+}_i-dependent activation of IL-8 transcription. The delay between the GPCR-triggered ΔCa^{2+}_i and onset of IL-8 protein secretion reflects the kinetics of activation of Ca^{2+}_i-dependent transcription (55) and protein translation. Based on previous studies, we speculate that following BK-induced $[Ca^{2+}]_i$ elevation, NF-κB is freed from IκB inhibition and translocates to the nucleus, where it can reside for tens of minutes, resulting in persistent transcriptional activation (55), despite the relaxation of Ca^{2+}_i levels toward baseline levels (Fig. 4) (22).

The proposed role of amplified ER-derived Ca^{2+}_i responses in CF pathogenesis in the present study is in agreement with previous findings of increases in ER Ca²⁺ stores in the pathogenesis of Gaucher disease (23) and Alzheimer disease (24, 25), although different mechanisms may participate in the genesis of ER expansion in these diseases. In the case of CF, we speculate that an airway epithelial adaptation to chronic luminal stimulation by bacterial/host products is the increased synthesis and secretion of cytokines and antimicrobial proteins (Fig.

6). The increased protein synthesis requires an increased ER protein folding capacity, triggering an ER response that is characterized by increased XBP-1 mRNA splicing (Fig. 7, *A* and *B*), increased expression of ER chaperones (Fig. 7, *C*−*H*), and expansion of the ER compartment and its Ca²⁺ stores (Fig. 4) (22). A model for describing the linkage between increased protein synthesis and ER expansion has been the B lymphocyte and plasma cell differentiation path. The high rate of immunoglobulin secretion by plasma cells requires ER expansion (39), and this process depends on XBP-1 mRNA splicing (56). The link among chronic airway epithelial infection/inflammation, ER expansion, and an increase in ER Ca²⁺ stores was recently reported in studies that demonstrated SMM-induced increases in the ER mass and the ER Ca²⁺ store marker calreticulin (22). Of note, ER expansion has been previously described in response to other forms of bacterial infection (57).

ER expansion likely impacts on the adaptation by airway epithelia to intraluminal infection in multiple ways. First, it provides a key cellular adaptation to mediate the increased protein synthesis required for infection-stimulated increases in secretion of host defense factors and cellular repair. Second, it may amplify the inflammatory response to persistent airway infection. For example, airway epithelia exposed to intralumi-

nal bacterial infection for prolonged periods (>24 h) become primed via greater ER Ca^{2+} stores to secrete higher levels of cytokines as a result of increased Ca^{2+}_i mobilization in response to stimuli that signal through ΔCa^{2+}_i such as BK (Figs. 1, 4, and 5) (22). We speculate that under normal circumstances, the amplification of the inflammatory response consequent to expansion of ER/Ca^{2+} stores is beneficial. For example, the increased inflammatory mediator-dependent IL-8 secretion that may occur 24–48 h after initiation of infection would result in progressive recruitment of inflammatory cells to rid the lumen of infection (Figs. 3 and 5). In CF, however, amplified cytokine responses following chronic bacterial infection may be maladaptive because the bacteria are protected in the thickened mucus environment in CF airways, and inflammatory cells cannot eradicate these bacteria (47). Thus, the persistent but ineffective "amplified" inflammatory cascade in CF may have an adverse impact on the host, *i.e.* destruction of the airway wall, rather than the bacterial target.

A summary of the sequence of events that leads to the CF hyperinflammatory phenotype is depicted in Fig. 8. The initiating stimulus is persistent intraluminal airway infection (Fig. 8*B*). A response to this infection, the increased epithelial synthesis and secretion of antimicrobial factors and cytokines (Fig. 8*B*), triggers ER expansion and increased ER Ca^{2+} stores (Fig. 8*C*). The consequent increase in the magnitude of Ca^{2+}_i signals mediates a component of CF airway "hyper-responsiveness", *i.e.* it primes the epithelial cells to secrete cytokines via a Ca^{2+}_i-dependent pathway in response to luminal infectious/inflammatory stimuli (Fig. 8*C*). In CF, elements of this amplified response are protective, *e.g.* Ca^{2+}_i-enhanced mucociliary clearance (22), whereas other elements may be maladaptive, *e.g.* the increased Ca^{2+}_i-dependent IL-8 secretion recruits inflammatory cells that are ineffective in thick mucus and paradoxically injure airway walls by proteolytic enzyme release. Thus, the most effective way to treat the inflammation-induced damage to airway walls is to remove the persistent stimulus, *i.e.* infected mucus plaques/plugs, from CF airway surfaces.

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