THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 11, Issue of March 18, pp. 10202–10209, 2005
Printed in U.S.A.

Cystic Fibrosis Airway Epithelial Ca^{2+}_i Signaling

THE MECHANISM FOR THE LARGER AGONIST-MEDIATED Ca^{2+}_i SIGNALS IN HUMAN CYSTIC FIBROSIS AIRWAY EPITHELIA*

Received for publication, September 15, 2004, and in revised form, January 6, 2005
Published, JBC Papers in Press, January 12, 2005, DOI 10.1074/jbc.M410617200

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In cystic fibrosis (CF) airways, abnormal epithelial ion transport likely initiates mucus stasis, resulting in persistent airway infections and chronic inflammation. Mucus clearance is regulated, in part, by activation of apical membrane receptors coupled to intracellular calcium (Ca^{2+}_i) mobilization. We have shown that Ca^{2+}_i signals resulting from apical purinoceptor ($P2Y_2$-R) activation are increased in CF compared with normal human airway epithelia. The present study addressed the mechanism for the larger apical $P2Y_2$-R-dependent Ca^{2+}_i signals in CF human airway epithelia. We show that the increased Ca^{2+}_i mobilization in CF was not specific to $P2Y_2$-Rs because it was mimicked by apical bradykinin receptor activation, and it did not result from a greater number of $P2Y_2$-R or a more efficient coupling between $P2Y_2$-Rs and phospholipase C-generated inositol 1,4,5-trisphosphate. Rather, the larger apical $P2Y_2$-R activation-promoted Ca^{2+}_i signals in CF epithelia resulted from an increased density and Ca^{2+} storage capacity of apically confined endoplasmic reticulum (ER) Ca^{2+} stores. To address whether the ER up-regulation resulted from ER retention of misfolded ΔF508 CFTR or was an acquired response to chronic luminal airway infection/inflammation, three approaches were used. First, ER density was studied in normal and CF sweat duct human epithelia expressing high levels of ΔF508 CFTR, and it was found to be the same in normal and CF epithelia. Second, apical ER density was morphometrically analyzed in airway epithelia from normal subjects, ΔF508 homozygous CF patients, and a disease control, primary ciliary dyskinesia; it was found to be greater in both CF and primary ciliary dyskinesia. Third, apical ER density and $P2Y_2$-R activation-mobilized Ca^{2+}_i, which were investigated in airway epithelia in a long term culture in the absence of luminal infection, were similar in normal and CF epithelia. To directly test whether luminal infection/inflammation triggers an up-regulation of the apically confined ER Ca^{2+} stores, normal airway epithelia were chronically exposed to supernatant from mucopurulent material from CF airways. Supernatant treatment expanded the apically confined ER, resulting in larger apical $P2Y_2$-R activation-dependent Ca^{2+}_i responses, which reproduced the increased Ca^{2+}_i signals observed in CF epithelia. In conclusion, the mechanism for the larger Ca^{2+}_i signals elicited by apical $P2Y_2$-R activation in CF airway epithelia is an expansion of the apical ER Ca^{2+} stores triggered by chronic luminal airway infection/inflammation. Greater ER-derived Ca^{2+}_i signals may provide a compensatory mechanism to restore, at least acutely, mucus clearance in CF airways.

Airway epithelia constitute the major interface between inspired air and the airway wall. These epithelia are highly polarized and exhibit a series of integrated functions that provide mechanical cleansing, *i.e.* mucus clearance, as a primary mode of lung defense. The individual airway epithelial functional components that mediate mucus clearance, including ion transport, mucin secretion, and ciliary beat frequency (1), are regulated in part by intracellular calcium (Ca^{2+}_i) (2, 3). Airway epithelial Ca^{2+}_i mobilization can be elicited by selective autocrine and/or paracrine activation of apical or basolateral membrane heterotrimeric G protein-coupled receptors (GPCRs)[1] linked to phospholipase C (PLC) stimulation, which generates inositol 1,4,5-trisphosphate (IP_3) and induces Ca^{2+} release from endoplasmic reticulum (ER) stores (3, 4). 5′ nucleotides (ATP/UTP), released by airway epithelial cells and sensed by apical membrane purinoceptors ($P2Y_2$-Rs) (5), may be the dominant autocrine regulators of Ca^{2+}_i mobilization in airway epithelia (3).

In airway epithelia of patients with cystic fibrosis (CF), the functional absence of the cystic fibrosis transmembrane conductance regulator (CFTR) results in a diminished periciliary liquid layer depth (6) and a reduction in mucus clearance. Likewise, abnormal mucus clearance is found in patients with primary ciliary dyskinesia (PCD), a syndrome that results from defective airway epithelial ciliary proteins linked to a decreased ciliary activity (7). In both diseases, persistent airways infection and inflammation are the predominant clinical phenotypic characteristics consequent to the impairment of mucus clearance (8).

There are clues that airway epithelia may regulate the Ca^{2+}_i

* This work was supported in part by Cystic Fibrosis Foundation Grants CFF RIBEIR00Z0 and CFF RIBEIR00G0 (to C. M. P. R.) and by National Institutes of Health Grants HL34322 and HL60280 (to R. C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; $P2Y_2$-R, purinoceptor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PCD, primary ciliary dyskinesia; SMM, supernatant from mucopurulent material from CF airways; PBS, phosphate-buffered saline; RPA, ribonuclease protection assay; $DIOC_6(3)$, dihexaoxacarbocyanine; CaCC, Ca^{2+}-activated Cl^- channel; Ins, inositol.

signaling pathway in response to airways disease. For example, *in vivo* studies established that the nasal transepithelial electrical potential difference responses to agents that activate $P2Y_2$-Rs and promote $Ca^{2+}{}_i$ mobilization (*e.g.* ATP/UTP) are greater in CF patients than in normal subjects (9, 10). *In vitro* studies also detected a larger apical $Ca^{2+}{}_i$-dependent Cl^- conductance in CF airway epithelia (9, 11–13). In addition, we recently reported that apical $P2Y_2$-R activation promotes greater $Ca^{2+}{}_i$ mobilization in CF compared with normal human nasal epithelia (4). However, the underlying mechanism(s) responsible for the regulation of the increased $Ca^{2+}{}_i$ responsiveness of CF airway epithelia and its functional consequences are unknown.

Therefore, in the present study we investigated the mechanism for the augmented apical GPCR-derived $Ca^{2+}{}_i$ signals in CF airway epithelia by probing the $P2Y_2$-R/Ca^{2+} signaling system in functional, biochemical, and cell biological studies in normal and CF human airway epithelia. Further, we investigated whether the raised $Ca^{2+}{}_i$ signals in CF are a manifestation of the molecular pathogenesis of mutant CFTR (ΔF508 CFTR) or are an acquired host response to chronic infection/inflammation *in vivo*. We compared ER density in non-infected human sweat duct epithelia from normal and ΔF508 CF individuals and in human airway epithelia from normal, ΔF508 CF, and PCD patients with chronic airway infection and inflammation. In addition, ER density and apical $P2Y_2$-R activation-induced $Ca^{2+}{}_i$ mobilization were studied in short term and long term cultures of normal and CF bronchial epithelia. Finally, to directly address whether chronic airway infection/inflammation, independently of defective CFTR, up-regulated apically confined ER Ca^{2+} stores, ER density and UTP-mobilized ER Ca^{2+} were investigated in normal airway epithelia chronically exposed to supernatant from CF airways mucopurulent material (SMM).

EXPERIMENTAL PROCEDURES

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 CF Center Tissue Core. Normal and CF (ΔF508 homozygous) cells were harvested and cultured as described previously (4). Cultures were maintained at an air-liquid surface interface, and polarized primary cultures were studied at 6–11 days (short term monolayers) or 30–40 days later (long term well differentiated). Normal and CF cultures were apically washed with sterile PBS, and the serosal media were replenished every 2–4 days.

Nasal scrapes obtained from normal (n = 4, ages 33–35), ΔF508 homozygous CF (n = 4, ages 25–46), or PCD (n = 3, ages 34–57) individuals and skin biopsies from normal (n = 4, ages 28–47) and ΔF508 homozygous CF (n = 5, ages 31–47) individuals were used for ER density studies.

$Ca^{2+}{}_i$ Studies—6–11- or 30–40-day-old cultures were loaded with fura-2/AM, and $Ca^{2+}{}_i$ was measured under bilateral perfusion as reported previously (4). $Ca^{2+}{}_i$ signals were calibrated as described previously (14). There were no systematic differences between CF and normal epithelia as a result of differential fura-2 behavior, including dye loading. Baseline $Ca^{2+}{}_i$ levels were the same in CF and normal epithelia, as reported previously (4), and no consistent differences were observed between CF and normal epithelia in the sustained phase of $Ca^{2+}{}_i$ mobilization following activation of $P2Y_2$-Rs.

Ribonuclease Protection Assay (RPA)—A 398-base pair human $P2Y_2$-R fragment was prepared by reverse transcriptase-PCR from human airway RNA and ligated into the pCRII vector (Invitrogen). $P2Y_2$-R primer sequences were as follows: 253F, 5′AGACCTGGGCCCCTGGAATGACACCATC3′; and 651R, 5′AGACGCCCAGACACCGGTGCACGCTGATG3′.

Individual clones were verified by automatic sequencing. Templates for RPAs were prepared by PCR from the $P2Y_2$-R plasmid with the following primers for the PCRII vector: PCRII SP6, 5′ATGATTACGCCAAGCTATTTAGGTGACACT3′ and PCRII T7, 5′GACGGCCAGTGAATTGTAATACGACTCACT3′.

50 ng of purified PCR fragment was transcribed, incorporating the [^{32}P]UTP label with the Maxiscript kit (Ambion), and RPA was performed with the RPA III kit (Ambion). Protected fragments were quantified by phosphorimaging with ImageQuant software (Amersham Biosciences).

Phospholipase C Activity—After 4 days in culture, monolayers of normal and CF bronchial epithelia were radiolabeled with [^{3}H]inositol for 4–5 days (15). For 20 min prior to study, cultures were placed in serum-free media containing 10 mM lithium and [^{3}H]inositol followed by 5 min 100 μM mucosal UTP-induced PLC activity measured as reported previously (15). The levels of ^{3}H-labeled inositol phosphates were normalized to [^{3}H]IP_6 levels because IP_6 levels are not affected by agonist stimulation (16). Assays were performed in duplicate.

Immunostaining of Calreticulin and IP_3 receptors (IP_3Rs) and $DIOC_6$(3) Staining—The immunostaining of calreticulin and IP_3Rs in cultures and deparaffinized native bronchial epithelial or skin biopsy sections was performed according to a modification of our previous method (17). For calreticulin and IP_3Rs staining, samples were incubated with a rabbit anti-calreticulin antibody (1:100 dilution, Affinity Bioreagents) and a mouse anti-IP_3R antibody that recognizes all IP_3R isoforms (1:100 dilution, Calbiochem) for 60 min at 37 °C. This incubation was followed by three PBS washes and a 30-min incubation at 25 °C with a fluorescein-labeled goat anti-rabbit antibody (1:20 dilution for calreticulin, Kirkegaard & Perry Laboratories) and with a Texas Red-labeled goat anti-mouse antibody (1:200 dilution for IP_3Rs, Jackson Immunoresearch Laboratories). As controls, the primary antibodies were omitted or substituted with rabbit and mouse γ-globulins (Jackson Immunoresearch Laboratories). For antibody-independent ER staining, sections of native bronchial epithelia were labeled with the fluorescent dihexaoxacarbocyanine dye $DIOC_6$(3) by incubation with 250 ng/ml $DIOC_6$(3) for 1 min at room temperature in PBS followed by three washes with PBS as we have reported previously (17). The Ca^{2+} store markers and the $DIOC_6$(3) 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.

To quantify the fluorescence intensity of labeled calreticulin and IP_3Rs and $DIOC_6$(3), 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) were employed for each channel to acquire the images from normal and CF cultures or native tissue in experiments performed on the same day. The fluorescence intensity values from the regions of interest were averaged from paired normal and CF cultures, native bronchial epithelia, skin biopsies, or normal cultures treated with PBS or supernatant from CF airways mucopurulent material (SMM, see "Studies with Infectious/Inflammatory Material from CF Airways").

Calreticulin Western Blot Analysis—Monolayers of normal and CF bronchial epithelia were lysed and blotted as reported previously (18). The calreticulin antibody was a rabbit polyclonal (1:5000 dilution, Affinity Bioreagents), and the secondary antibody was an anti-rabbit horseradish peroxidase (1:10,000 dilution, Amersham Biosciences). Blots were developed with enhanced chemiluminescence.

Electron Microscopy—Nasal scrapes from normal, ΔF508 homozygous CF, and PCD individuals were fixed in 2% glutaraldehyde plus 2% paraformaldehyde plus 0.25% tannic acid, post-fixed in 1% OsO_4, and processed for electron microscopy (19). Apical ER morphometric scoring was performed double blind. Only ciliated cells were analyzed, and repairing cells with migrating basal bodies (procentrioles) and micrographs with tangential cuts were excluded. The apical domain was defined as the region 1–3.4 μm (to exclude basal bodies) from the apical plasma membrane. The criteria for identifying ER were: parallel membranes in elongated or oval strands, containing an amorphous center without cristae (to exclude mitochondria), or electron-dense particles (to exclude rough ER, because it is increased in repairing cells), and not stacked (to exclude Golgi). Data are expressed as number of ER strands/μm^2.

Studies with Infectious/Inflammatory Material from CF Airways—Mucopurulent material was harvested from the airway lumens of excised human CF lungs infected with *Pseudomonas aeruginosa* and *S. aureus* at the time of transplant and provided by the University of North Carolina CF Center Tissue Core. 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 *Staphyloccocus aureus* induced epithe-

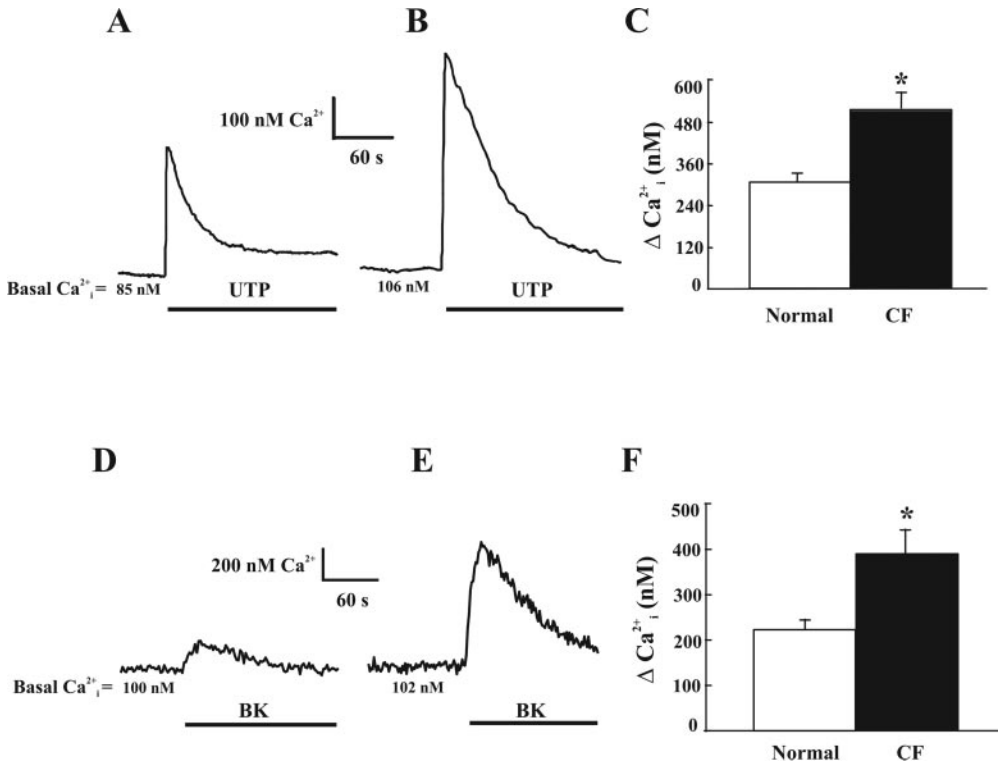

FIG. 1. **The larger apical UTP-mobilized Ca^{2+}_i in CF airway epithelia is not agonist specific.** *A* and *B*, representative Ca^{2+}_i tracings depicting the effect of apical UTP-mobilized Ca^{2+}_i in short term primary culture monolayers of normal and CF human bronchial epithelia, respectively. *C*, summary ΔCa^{2+}_i values (peak-baseline Ca^{2+}_i) from UTP-stimulated cultures. *D* and *E*, representative Ca^{2+}_i tracings depicting the effect of apical bradykinin (*BK*)-mobilized Ca^{2+}_i in short term primary culture monolayers of normal and CF human bronchial epithelia, respectively. *F*, compiled ΔCa^{2+}_i values (peak-baseline Ca^{2+}_i) from BK-stimulated cultures. Data are expressed as mean ± S.E. ($n = 4$ for UTP studies in both groups; $n = 3$ for BK studies in both groups). *, $p < 0.05$.

lial hyperinflammation. Because of the small volume of SMM/patient, 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.

To address the effect of SMM on ER size and Ca^{2+} storage, PBS or SMM (40 μl) was applied to the mucosal surfaces of well differentiated cultures of normal bronchial epithelia for 48 h, and then either ER volume was studied by calreticulin immunostaining as described above or peak Ca^{2+}_i responses to 100 μM mucosal UTP were measured, respectively.

Statistics—Data represent the mean ± 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 with $p < 0.05$.

RESULTS

Larger Apical GPCR-modulated Ca^{2+}_i Responses Are a Generalized Feature of CF Airway Epithelia—We have shown previously that CF human nasal epithelia exhibit larger rises in Ca^{2+}_i in response to apical $P2Y_2$-R activation than normal nasal epithelia (4). To investigate whether this difference was generalized to lower airways, we studied the effect of a maximal dose (100 μM) UTP applied to the apical surface of 6–11-day-old monolayers of normal and CF human bronchial epithelial cultures on Ca^{2+}_i mobilization (4). UTP addition to normal bronchial epithelium induced an initial Ca^{2+}_i peak, resulting from IP_3-dependent ER Ca^{2+} store release, and a sustained rise in Ca^{2+}_i, resulting from activation of capacitative Ca^{2+} entry (3) (Fig. 1*A*). $P2Y_2$-R activation in CF epithelium (Fig. 1*B*) resulted in a markedly higher Ca^{2+}_i mobilization peak than in normal epithelium, in agreement with our findings in nasal epithelia (4). Fig. 1*C* illustrates the summary ΔCa^{2+}_i data (peak-baseline Ca^{2+}_i values) from these studies.

We next addressed whether the larger Ca^{2+}_i mobilization in CF was a specific property of $P2Y_2$-R activation or was generalized to other GPCRs linked to PLC at the apical membrane. Fig. 1, *D* and *E*, shows representative Ca^{2+}_i tracings from 6–11-day-old monolayers of normal and CF bronchial epithelial cultures, respectively, following apical addition of a maximal dose (5 μM) of bradykinin. Fig. 1*F* depicts the summary ΔCa^{2+}_i data from these experiments, illustrating that apical bradykinin receptor activation in CF also resulted in an increased ΔCa^{2+}_i mobilization. Because the greater bradykinin-induced Ca^{2+}_i signal in CF mimicked that elicited by $P2Y_2$-R activation, the larger ΔCa^{2+}_i signal in CF does not depend on a specific class of GPCRs.

$P2Y_2$-R Number and $P2Y_2$-R-dependent PLC Activation Are the Same in Normal and CF Airway Epithelia—We tested whether $P2Y_2$-R expression was increased in CF. Because no antibodies or radioligands were available for $P2Y_2$-R quantification, we used an RPA to assess $P2Y_2$-R steady-state mRNA levels in 6–11-day-old primary culture monolayers of normal and CF bronchial airway epithelia. No difference was found in $P2Y_2$-R mRNA levels from normal compared with CF human airway epithelia (Fig. 2, *A* and *B*).

We next investigated whether the larger Ca^{2+}_i mobilization in CF was driven by an enhanced PLC response to $P2Y_2$-R activation. $P2Y_2$-R-regulated PLC activity was assayed by measuring IP_3 and the accumulation of its downstream metabolites in the presence of lithium to prevent recycling of inositol phosphates back to inositol (20). We found that PLC activation in response to a 5-min treatment with 100 μM apical UTP was not elevated in 6–11-day-old primary culture monolayers of CF (Fig. 2*C*). These findings indicate that the larger ΔCa^{2+}_i in CF does not reflect increased $P2Y_2$-R number or increased efficiency of $P2Y_2$-R coupling to PLC.

Apical ER Ca^{2+} Stores Are Functionally Increased in CF Airway Epithelia—We next addressed whether 6–11-day old monolayers of CF cultures exhibited an increased quantity of

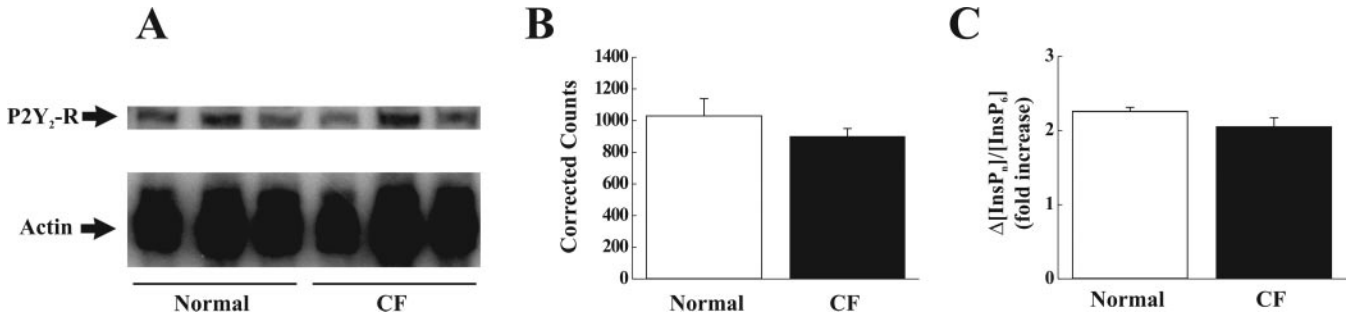

FIG. 2. **$P2Y_2$-R expression and $P2Y_2$-R-PLC coupling efficiency are similar in normal and CF bronchial epithelial cultures.** *A*, $P2Y_2$-R steady-state mRNA levels from three different samples from short term primary culture monolayers of normal and CF human bronchial epithelia. *B*, $P2Y_2$-R mRNA expression levels (corrected for actin mRNA levels) from the data shown in *A* ($n = 3$ for both groups). *C*, UTP-dependent PLC activity (the sum of IP_3 metabolites (Ins-1-P, Ins-2-P, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,3,4,6)P_4) in short term primary culture monolayers of normal and CF human bronchial epithelia. Inositol phosphate levels were normalized to IP_6 and expressed as -fold increase over the inositol phosphate values in the absence of UTP. Data are expressed as mean ± S.E. ($n = 3$ for both groups). CF and normal airway epithelial cells also showed similar levels of IP_3, as well as Ins(3,4,5,6)P_4, an inhibitor of Ca^{2+}-activated Cl^- channels (20) (data not shown).

ER Ca^{2+} that could be mobilized in response to IP_3 generation resulting from GPCR activation. To measure the Ca^{2+} storage capacity of the apical ER, a protocol was developed based on findings that ER Ca^{2+} stores are functionally confined to the plasma membrane domain ipsilateral to $P2Y_2$-R activation (21). 6–11-Day-old normal and CF monolayers, bathed in bilateral nominally Ca^{2+}-free buffer, were exposed to 100 μM basolateral UTP to deplete $P2Y_2$-R-sensitive basolateral Ca^{2+} stores. Basolateral UTP increased $Ca^{2+}{}_i$ to the same extent in both cultures (Fig. 3*A*), suggesting that basolateral ER Ca^{2+} store capacity was the same in normal and CF epithelia. Apical ER Ca^{2+} store capacity was then measured with perfusion with 0.6 mM bilateral La^{3+} to block plasma membrane Ca^{2+}-ATPases and Ca^{2+} influx channels (22) followed by 1 μM apical thapsigargin (an ER Ca^{2+}-ATPase inhibitor) to release Ca^{2+} from apical ER stores (23). $Ca^{2+}{}_i$ rose and was sustained because of inhibition of the plasma membrane and ER Ca^{2+}-ATPases by La^{3+} and thapsigargin, respectively (Fig. 3*A*). The greater rise in $Ca^{2+}{}_i$ in CF (Fig. 3*A*) indicated that the quantity of Ca^{2+} sequestered in the apical ER was functionally greater in CF compared with normal bronchial epithelial cultures. Fig. 3*B* illustrates the summary $\Delta Ca^{2+}{}_i$ data from these experiments. These results led us to speculate that the density of apical ER Ca^{2+} stores is increased in short term 6–11-day-old cultures of CF airway epithelia.

Apical ER Ca^{2+} Stores Are Morphologically Expanded in Short Term CF Airway Epithelial Cultures and Native CF Airway Epithelia—We next measured the expression of two ER Ca^{2+} store markers, IP_3Rs and calreticulin (an intraluminal ER protein involved in Ca^{2+} sequestration), in 6–11-day-old monolayers of normal and CF bronchial epithelia by confocal immunofluorescence microscopy (Fig. 4). The relative cellular distribution of these Ca^{2+} store markers was similar in normal and CF epithelia (*i.e.* they localized predominantly toward the apical pole and the fluorescent signals from calreticulin and IP_3Rs were increased in CF cultures (Fig. 4, *A* and *B*)). No immunostaining of Ca^{2+} store markers was detected when the primary antibodies were omitted or nonspecific IgGs were used (data not shown). These data suggest an increased expression of calreticulin and IP_3Rs in CF cultures; however, because the IP_3R antibody used recognizes all IP_3R isoforms, it is possible that the change in IP_3R signal may reflect a switch in the predominance of one isoform *versus* others rather than a change in the total amount of all IP_3Rs in CF. The increase in CF calreticulin immunofluorescence was confirmed by calreticulin immunoblotting from whole cell lysates of early stage culture monolayers of normal and CF bronchial epithelia (Fig. 4*C*).

The apical ER volume of normal and CF epithelia was also studied by an antibody-independent method, utilizing $DIOC_6(3)$ fluorescence to stain the ER (17). Fig. 4*D* illustrates that $DIOC_6(3)$ staining was greater in native CF compared with normal bronchial airway epithelia. The mean apical fluorescence intensity from the CF group exceeded that of the normal group by a factor proportional to that observed with antibody-dependent methods (Fig. 4*E*).

To test whether the topography and expression levels of the ER Ca^{2+} stores found in CF *versus* normal short term primary culture monolayers mimicked those *in vivo*, confocal immunofluorescence microscopy studies of IP_3Rs and calreticulin were performed in native normal and CF bronchial epithelia (Fig. 4, *F* and *G*). Similar to our findings in short term primary culture monolayers (Fig. 4, *A* and *B*), ER Ca^{2+} stores were distributed toward the apical domain of native epithelia, and their expression was increased in CF (Fig. 4, *F* and *G*). Collectively, these data, coupled with the direct measurement of ER density by electron microscopy (Fig. 5), strongly suggested that the functional increase in apical ER Ca^{2+} storage detected in CF epithelia (Fig. 3) was because of an increase in the ER volume in the apical domain.

Is the ER Expansion in CF a Result of Misfolded ΔF508 CFTR or an Acquired Response to Chronic Airway Infection?—Two approaches were used to address this question. First, we tested the hypothesis that the ER up-regulation could be linked to abnormal CFTR folding in the absence of infection/inflammation. We elected to study sweat ducts because they are a source of non-infected CF epithelium and they exhibit relatively high levels of CFTR expression (24). Fig. 5, *A* and *B*, depict the immunostaining of calreticulin in native sweat ducts from normal and ΔF508 homozygous CF individuals, respectively. Quantification of calreticulin immunofluorescence revealed that its expression was the same in normal and CF sweat duct epithelia (Fig. 5*C*), suggesting that the ER expansion observed in CF airway epithelia was not a consequence of ER retention of ΔF508 CFTR.

Second, we evaluated whether the ER expansion in CF was acquired by studying airway epithelia from patients with an unrelated disease characterized by chronic airways infection, *e.g.* PCD (8). We quantified morphometrically the ER density in freshly isolated nasal scrapes from normal (Fig. 5*D*), ΔF508 homozygous CF (Fig. 5*E*), and PCD (Fig. 5*F*) individuals. ER density, as depicted by *red arrows*, was increased in both CF and PCD compared with normal epithelium (Fig. 5*G*). Apical UTP-mobilized $Ca^{2+}{}_i$ was also increased 2-fold in short term primary culture monolayers of PCD compared with normal bronchial epithelia (data not shown). These data suggest that

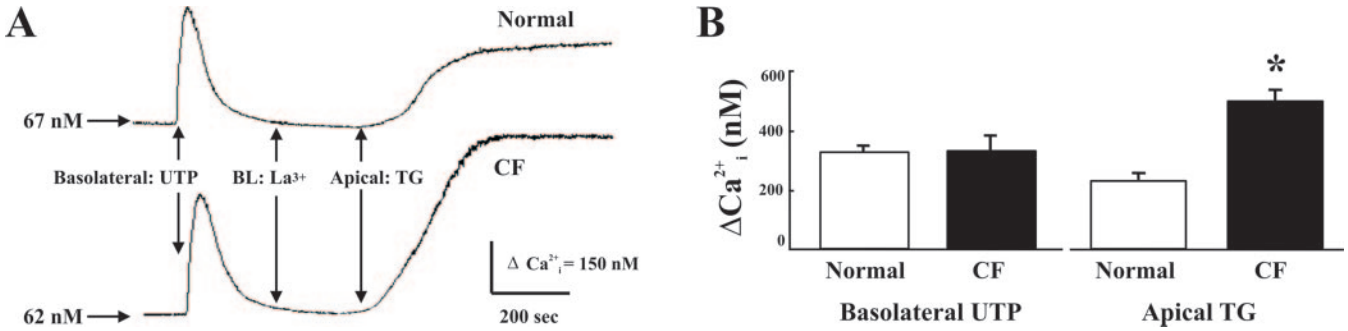

FIG. 3. **The increased $Ca^{2+}{}_i$ mobilization triggered by apical purinergic receptor activation in CF airway epithelia reflects larger apically confined functional ER Ca^{2+} stores.** *A*, representative $Ca^{2+}{}_i$ tracings depicting the $Ca^{2+}{}_i$ responses to sequential exposure of basolateral UTP (to assess the capacity of UTP-releasable basolateral ER Ca^{2+} stores), bilateral (*BL*) La^{3+}, and apical thapsigargin (*TG*) (to assess the capacity of apically confined ER Ca^{2+} stores) in short term primary culture monolayers of normal and CF human bronchial epithelia. Cultures were bilaterally perfused with a nominally Ca^{2+}-free buffer throughout the entire experiment. *B*, compiled $\Delta Ca^{2+}{}_i$ values (steady state-baseline $Ca^{2+}{}_i$) from the experiments shown in *A* ($n = 4$). Data are expressed as mean ± S.E.; *, $p < 0.05$, CF *versus* normal cultures.

the apical ER volume is expanded in CF in response to luminal airway infection and inflammation.

The ER Expansion Observed in Short Term CF Cultures Reverts to Normal in CF Epithelia Cultured for Long Term in the Absence of Luminal Infection—To address directly the possible relationship between luminal infection/inflammation and ER expansion in CF airway epithelia, normal and ΔF508 homozygous CF bronchial epithelia were cultured for long term for 30–40 days, and ER density was investigated by calreticulin immunofluorescence as described in the studies depicted in Fig. 4*A*. Fig. 6, *A* and *B*, depicts the calreticulin expression in a normal culture and a ΔF508 homozygous CF culture, respectively, illustrating that apical calreticulin expression was no longer up-regulated in the long term CF compared with the normal culture. Fig. 6*C* shows the summary data for apical calreticulin staining from long term normal and CF cultures. These findings demonstrate that the ER expansion observed in short term CF cultures is lost upon prolonged culturing under sterile conditions. We next investigated whether the morphological reversal of ER expression to normal correlated with a functional reversal of agonist-sensitive Ca^{2+} stores to normal in long term ΔF508 CF cultures. Fig. 6, *D* and *E*, illustrates that activation of apical $P2Y_2$-Rs with mucosal ATP (100 μM) elicited a similar $Ca^{2+}{}_i$ mobilization in long term normal and CF cultures, respectively. Fig. 6*F* depicts the summary $\Delta Ca^{2+}{}_i$ data (peak-baseline $Ca^{2+}{}_i$ values) from these studies. These data demonstrate that the characteristic phenotype of short term 6–11-day-old ΔF508 CF cultures (*e.g.* apical ER expansion associated with increased agonist-sensitive ER Ca^{2+} stores) reverts to normal after long term culturing in the absence of luminal infection and is independent of mutated CFTR.

Can the CF Phenotype, e.g. Increased Density of Apically Confined ER Ca^{2+} Stores Coupled to Larger Apical $P2Y_2$-R Activation-dependent $Ca^{2+}{}_i$ Signals, Be Transferred to Normal Airway Epithelia by Prolonged Exposure to Mucopurulent Material from CF Airways?—The above findings suggest that the expanded apical ER phenotype does not depend on ER retention of ΔF508 CFTR but results from an epithelial adaptation to the *in situ* CF airway infectious/inflammatory milieu, and it is lost *in vitro* with time in the absence of airway infection. Based on these findings, we hypothesized that the ER expansion and the consequent up-regulation of functional Ca^{2+} stores could be induced in normal airway epithelia exposed to an infectious/inflammatory luminal environment. To test this notion, the luminal surfaces of 30–40-day-old normal bronchial epithelia were exposed for up to 48 h to either PBS or SMM harvested from CF airways, and the following parameters were measured. First, the apical ER compartment, visualized by calreticulin immunofluorescence, was assessed in PBS- *versus* SMM-treated cultures. Fig. 7*A* illustrates that apical ER density was increased in SMM- compared with PBS-treated cultures in a time-dependent manner. The compiled data from these studies are shown in Fig. 7*B*. SMM-treated short term cultures of normal epithelia also expressed an increased ER density (not shown). Second, we tested whether the increased apical ER expression in SMM-treated cultures resulted in larger $Ca^{2+}{}_i$ signals in response to apical $P2Y_2$-R activation with 100 μM mucosal UTP. UTP-mobilized $Ca^{2+}{}_i$ was increased in cultures pretreated with SMM compared with PBS (Fig. 7*C*), mimicking the larger mucosal UTP-dependent $Ca^{2+}{}_i$ signals found in short term primary cultures of CF bronchial airway epithelia (Fig. 1, *A–C*). These findings demonstrate that chronic luminal exposure of normal cultures to SMM reproduces the increased ER density and apical GPCR activation-dependent $Ca^{2+}{}_i$ signals observed in short term cultures of CF airway epithelia.

DISCUSSION

We have hypothesized previously that an increased $Ca^{2+}{}_i$ signal coupled to Cl^- secretion would provide a compensatory mechanism to offset the absent cAMP-mediated Cl^- transport in CF (3). Perhaps the strongest evidence for the role of $Ca^{2+}{}_i$-mediated Cl^- secretion in protecting against CF lung disease came from CF knock-out mice, which express a large endogenous Ca^{2+}-activated Cl^- conductance in the airway epithelia and are devoid of airway disease (13, 25). CF patients do exhibit larger $Ca^{2+}{}_i$-dependent responses triggered by luminal purinoceptor agonists (9, 10), but the mechanism(s) of this response in human airways has remained unclear.

In this study, we have elucidated the mechanism for the increased apical GPCR activation-dependent $Ca^{2+}{}_i$ signals in CF human airway epithelia (4) (Fig. 1) by showing that it resulted from the expansion of the apical ER/Ca^{2+} store compartment (Figs. 3–5 and 7) rather than from a greater number of $P2Y_2$-Rs or coupling efficiency between $P2Y_2$-R activation and PLC activity (Fig. 2). Although it is plausible that the higher $Ca^{2+}{}_i$-dependent Cl^- secretion in human CF airway epithelia may in part be a consequence of an increased Ca^{2+}-activated Cl^- channel (CaCC) number, our data demonstrating higher $Ca^{2+}{}_i$ mobilization in CF compared with normal human nasal (4) or bronchial (Fig. 1) epithelia suggest that higher $Ca^{2+}{}_i$ levels at the vicinity of the apical membrane CaCC likely mediate this response.

A number of observations led to the conclusion that the enlarged ER in CF reflected an acquired epithelial response to luminal airway infection/inflammation rather than an ER stress response to misfolded ΔF508 CFTR (26). First, non-infected native sweat duct epithelia from ΔF508 homozygous

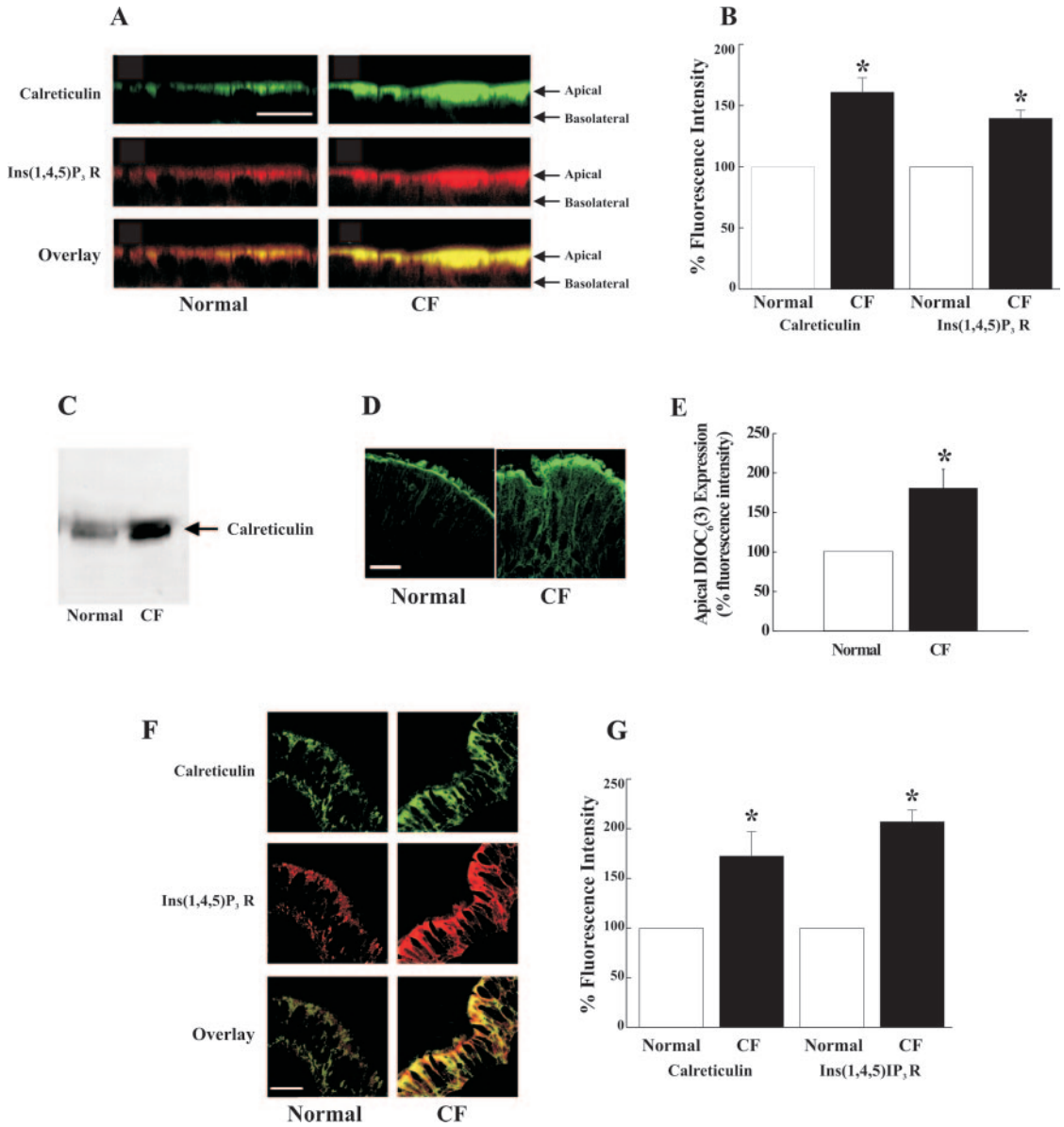

FIG. 4. **Apical ER Ca^{2+} stores are morphologically expanded in CF cultures and native CF tissue.** *A*, XZ confocal scans from short term primary culture monolayers of normal and CF bronchial airway epithelia immunostained for calreticulin or IP_3Rs. *Bar*, 10 μm. *B*, percent of apical fluorescence intensity from calreticulin and IP_3Rs (normalized to fluorescence intensity values from normal cultures; $n = 5$). *C*, Western blot for calreticulin from whole cell lysates (50 μg of protein/lane) from short term primary culture monolayers of normal and CF bronchial airway epithelia (representative from three normal and three CF cultures). *D*, XY confocal scans from normal and CF sections of native bronchial epithelia stained with $DIOC_6(3)$. *E*, percent of apical $DIOC_6(3)$ fluorescence intensity (normalized to fluorescence intensity values from normal sections; $n = 5$). *F*, XY confocal scans from normal and CF sections of native bronchial epithelia immunostained for calreticulin and IP_3Rs. *Bar*, 10 μm. *G*, percent of apical fluorescence intensity from calreticulin and IP_3Rs (normalized to fluorescence intensity values from normal sections; $n = 5$ for calreticulin, and $n = 3$ for IP_3Rs in normal and CF). Data are expressed as mean ± S.E.; *, $p < 0.05$, CF *versus* normal epithelia.

CF patients exhibited normal ER size (Fig. 5). Second, the apical ER density was similarly increased in native CF and PCD airway epithelia (Fig. 5). Third, the expanded ER phenotype observed in short term (6–11-day-old, Fig. 4) primary cultures of ΔF508 CF airway epithelia reverted to normal in long term (30–40-day-old, Fig. 6) ΔF508 CF cultures. Fourth, chronic *in vitro* exposure of normal airway epithelia to bacterial and inflammatory factors (such as SMM) harvested from CF airways increased the ER size and Ca^{2+} storage (Fig. 7), mimicking the phenotype of short term CF cultures. These data suggest that the elaborated apical ER phenotype observed in short term primary cultures of CF airway epithelia, which mimics that of native CF airway epithelia, 1) reflects a "memory" of the *in situ* infectious environment of CF airways, 2) reverts *in vitro* in the absence of luminal airway infection, and 3) is independent of the intrinsic ΔF508 CFTR defect.

Regarding host defense mechanisms that clear airway surfaces, the raised $Ca^{2+}{}_i$ release due to ER expansion, especially confined to the apical domain (4), (27), may provide an adaptive response for both the normal and the CF airways. The higher $Ca^{2+}{}_i$ mobilization may be particularly useful to CF patients, who depend solely on CaCC to compensate for the absent cAMP-mediated Cl^- secretion in CF (3). Thus, the approximate doubling of $Ca^{2+}{}_i$ mobilization in CF is predicted to double the magnitude of Cl^- secretion (4). This relatively larger component of Cl^- secretion may allow CF airways to transiently restore defective mucus clearance. In this scenario, cough (shear stress)-induced release of ATP/UTP (5) into airway surface liquid would produce larger $Ca^{2+}{}_i$ signals via apical $P2Y_2$-R activation and would more effectively activate CaCC

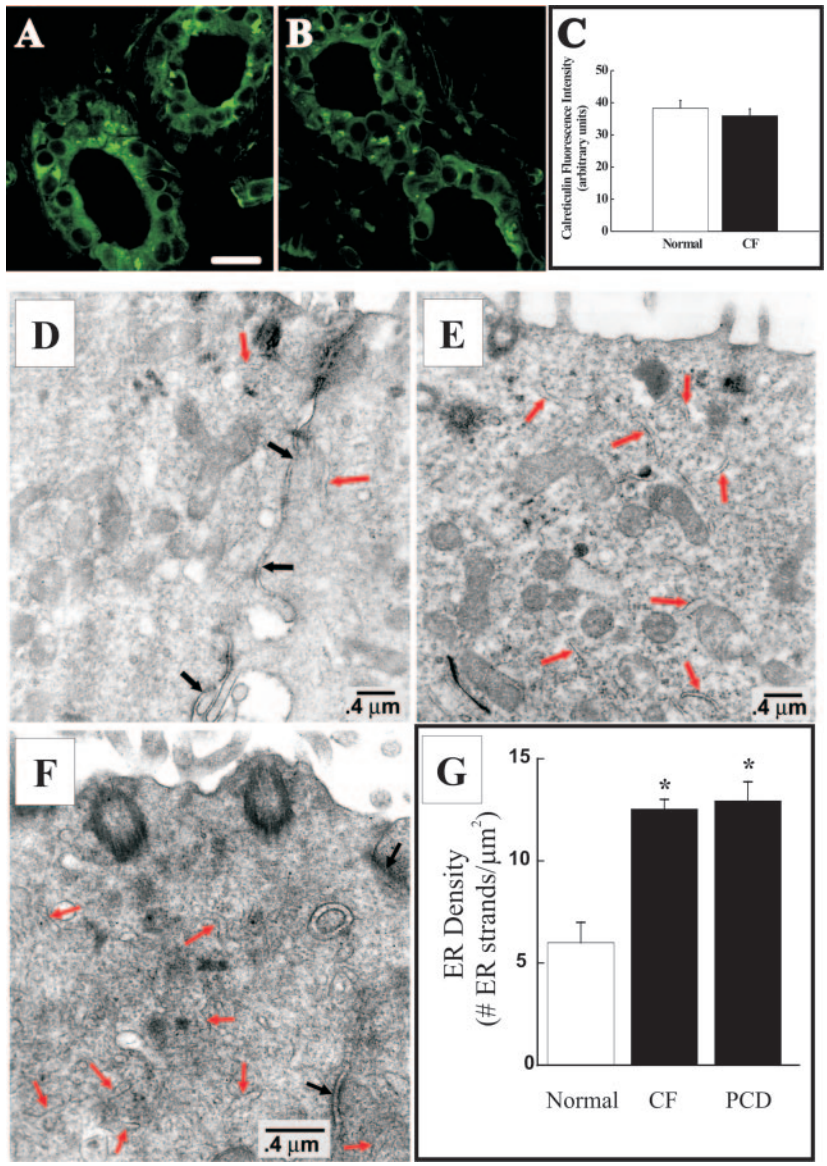

FIG. 5. **ER density is similar in native ΔF508 homozygous CF *versus* normal sweat duct epithelia but greater in airway epithelia from native CF and PCD infected airways.** XY confocal scans of the ER (as depicted by calreticulin staining) in normal (*A*) and ΔF508 homozygous CF (*B*) native sweat duct epithelia are shown. *Bar*, 10 μm. *C*, compiled ER density data from the apical epithelial domain of normal and ΔF508 homozygous CF sweat ducts (n = 4 and 5 individuals for normal and CF, respectively). Micrographs of the apical region of normal (*D*), ΔF508 homozygous CF (*E*), and PCD (*F*) nasal ciliated epithelial cells are shown. *Red* and *black arrows* depict the ER and the intercellular spaces, respectively (see *panels D*, *E*, and *F*). *G*, compiled ER density data from the apical epithelial domain of the three groups (n = 3–4 individuals). Data are expressed as mean ± S.E.; *, $p < 0.05$, CF or PCD *versus* normal epithelia.

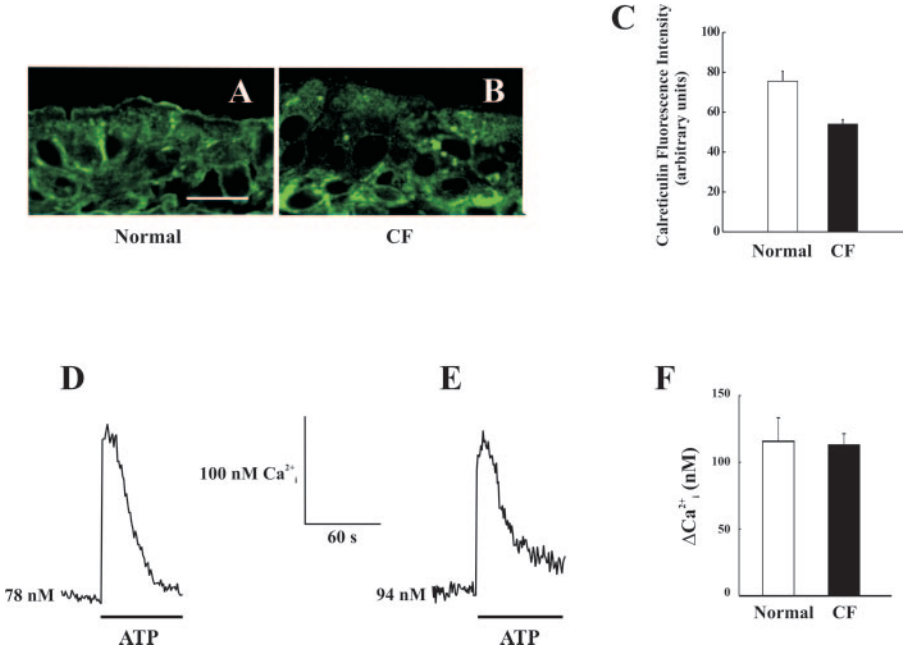

FIG. 6. **Apical ER density and ER Ca^{2+} storage is the same in normal and CF airway epithelia cultured for long term in the absence of luminal infection.** ER density, depicted by calreticulin staining, in 30–40-day-old long term cultures of normal (*A*) and CF (*B*) bronchial airway epithelia. The apical surface corresponds to the upper area in figures. *Bar*, 10 μm. *C*, compiled data for calreticulin fluorescence from the apical domain of normal and CF cultures. *D* and *E*, representative $Ca^{2+}{}_i$ tracings depicting the effect of (100 μM) apical ATP-mobilized $Ca^{2+}{}_i$ in 30–40-day-old long term cultures of normal and CF human bronchial epithelia, respectively. *F*, compiled $\Delta Ca^{2+}{}_i$ values (peak-baseline $Ca^{2+}{}_i$) from ATP-stimulated cultures. Data are expressed as mean ± S.E. (n = 3–5).

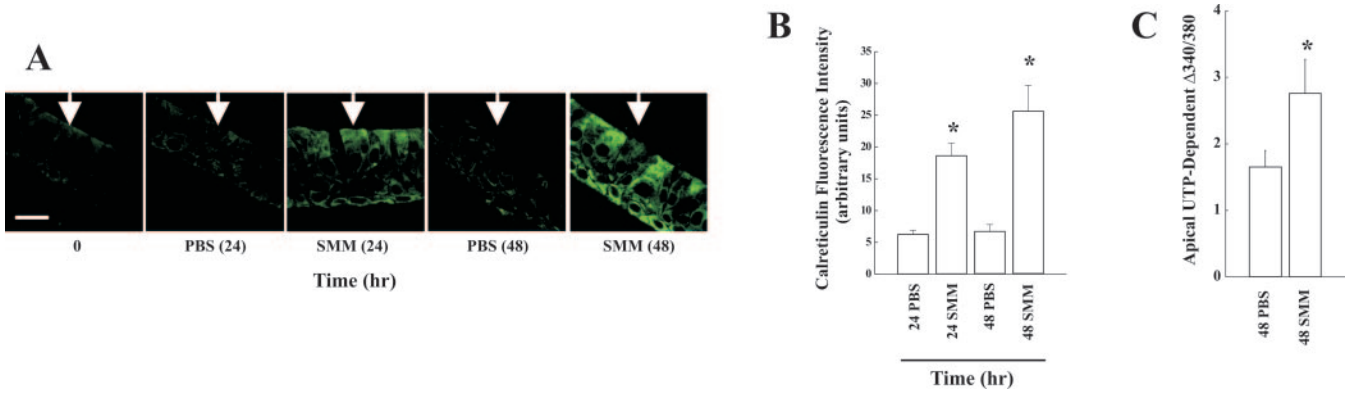

FIG. 7. **The CF phenotype, *e.g.* increased density of apically confined ER Ca^{2+} stores coupled to larger apical $P2Y_2$-R activation-dependent $Ca^{2+}{}_i$ signals, is transferred to normal airway epithelia by prolonged mucosal exposure to CF airways mucopurulent material.** *A*, time course illustrating the ER expansion, depicted by calreticulin staining, in 30–40-day-old normal airway epithelia exposed to mucosal supernatant from CF airways mucopurulent material over 48 h. *Arrows* point to the apical surface. *Bar*, 10 μm. *B*, compiled data for ER density from the apical domain of PBS- or SMM-treated cultures. *C*, effect of a 48-h SMM treatment of 30–40-day-old normal cultures of human bronchial airway epithelia on apical UTP-mobilized $Ca^{2+}{}_i$ (Δ340/380 signal from fura-2). Data are expressed as mean ± S.E. ($n = 3$; *, $p < 0.05$).

and ciliary beat to transiently restore mucus clearance (28).

Conversely, this adaptive airway epithelial response to chronic luminal infection/inflammation involving the up-regulation of apical ER Ca^{2+} stores can have an adverse effect, because the increased agonist-induced $Ca^{2+}{}_i$ mobilization in CF airway epithelia may also play a role in $Ca^{2+}{}_i$-dependent airway inflammatory responses (29). A role for increased ER Ca^{2+} stores has been raised in the pathogenesis of neurological diseases. For example, ER expansion has been described in a model for Gaucher disease, where neuronal cells expressed an increase in ER and ryanodine receptors and a greater ER-dependent Ca^{2+} release in response to glutamate or caffeine stimulation (30). Thus, the increased glutamate-dependent $Ca^{2+}{}_i$ signal may be linked with the neuronal toxicity and cell death characteristic of Gaucher disease. In contrast, most cases of Alzheimer disease are caused by presenilin mutations, and it is thought that ER protein (presenilin) retention (31) leads to increases in ER size and Ca^{2+} storage capacity in this disease (32–34).

In conclusion, our findings add to the understanding of the mechanisms and roles of $Ca^{2+}{}_i$-dependent signal transduction in airway epithelia by elucidating that 1) ER Ca^{2+} stores are predominantly distributed toward the apical domain, 2) the apical ER compartment and its capacity to sequester Ca^{2+} are increased in CF and PCD airway epithelia, and 3) the expansion of the apical ER network is an adaptive innate epithelial response to chronic luminal airway infection/inflammation. We speculate that the up-regulation of the ER/Ca^{2+} stores in CF may reflect a response that can be generalized to other diseases characterized by persistent epithelial exposure to an infectious and inflammatory milieu. These observations may prove important in designing strategies and selecting dosages of therapeutic agents aimed at restoring mucus clearance in patients with chronic infectious lung disease (*e.g.* CF and PCD) via regulation of $Ca^{2+}{}_i$-dependent mechanisms.

Acknowledgments—We are deeply indebted to Margaret Leigh, Johnny Carson, and Jackie Kylander for advice on ER morphometry, James Yankaskas and Scott Randell for supplying primary cells and tissue blocks, Laurenell Burch for performing RPAs, Kim Burns for supplying electron microscopy pictures, Tracy Eldred for providing tissue sections, Hamsa Suchindran for performing Western blots, and Lisa Brown for editorial assistance.

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