*Am J Physiol Lung Cell Mol Physiol* 293: L1250–L1260, 2007.
First published September 7, 2007; doi:10.1152/ajplung.00231.2007.

# Effects of cystic fibrosis transmembrane conductance regulator and ΔF508CFTR on inflammatory response, ER stress, and $Ca^{2+}$ of airway epithelia

**Kevin Hybiske, Zhu Fu, Christian Schwarzer, Jill Tseng, Jiun Do, Natalie Huang, and Terry E. Machen**
*Department of Molecular and Cell Biology, University of California-Berkeley, Berkeley, California*

Submitted 11 June 2007; accepted in final form 3 September 2007

**Hybiske K, Fu Z, Schwarzer C, Tseng J, Do J, Huang N, Machen TE.** Effects of cystic fibrosis transmembrane conductance regulator and ΔF508CFTR on inflammatory response, ER stress, and $Ca^{2+}$ of airway epithelia. *Am J Physiol Lung Cell Mol Physiol* 293: L1250–L1260, 2007. First published September 7, 2007; doi:10.1152/ajplung.00231.2007.—We tested whether cystic fibrosis (CF) airway epithelia have larger innate immune responses than non-CF or cystic fibrosis transmembrane conductance regulator (CFTR)-corrected cells, perhaps resulting from ER stress due to retention of ΔF508CFTR in the endoplasmic reticulum (ER) and activation of cytosolic $Ca^{2+}$ ($Ca_i$) and nuclear factor (NF)-κB signaling. Adenovirus infections of a human CF (ΔF508/ΔF508) nasal cell line (CF15) provided isogenic comparisons of wild-type (wt) CFTR and ΔF508CFTR. In the absence of bacteria, there were no or only small differences among CF15, CF15-lacZ (β-galactosidase-expressing), CF15-wtCFTR (wtCFTR-corrected), and CF15-ΔF508CFTR (to test ER retention of ΔF508CFTR) cells in NF-κB activity, interleukin (IL)-8 secretion, $Ca_i$ responses, and ER stress. Non-CF and CF primary cultures of human bronchial epithelial cells (HBE) secreted IL-8 equivalently. Upon infection with *Pseudomonas aeruginosa* (PA) or flagellin (key activator for airway epithelia), CF15, CF15-lacZ, CF15-wtCFTR, and CF15ΔF508CFTR cells exhibited equal PA binding, NF-κB activity, and IL-8 secretion; cells also responded similarly to flagellin when both CFTR (forskolin) and $Ca_i$ signaling (ATP) were activated. CF and non-CF HBE responded similarly to flagellin + ATP. Thapsigargin (Tg, releases ER $Ca^{2+}$) increased flagellin-stimulated NF-κB and ER stress similarly in all cells. We conclude that ER stress, $Ca_i$, and NF-κB signaling and IL-8 secretion were unaffected by wt- or ΔF508CFTR in control and during exposure to PA, flagellin, flagellin + ATP, or flagellin + ATP + forskolin. Tg, but not wt- or ΔF508CFTR, triggered ER stress. Previous measurements showing hyperinflammatory responses in CF airway epithelia may have resulted from cell-specific, rather than CFTR- or ΔF508CFTR-specific effects.

nuclear factor-κB; interleukin-8; adenovirus; inflammation; endoplasmic reticulum stress; ire1α

ALTHOUGH IT IS CLEAR THAT cystic fibrosis (CF) airways exhibit exaggerated secretion of cytokines and recruitment of neutrophils to the airways, it remains questionable whether this inflammatory response is determined by excessive accumulation of bacteria because of reduced clearance in sticky mucus or whether CF epithelial cells are also different in how they bind and interact with bacteria. Some in vivo measurements have indicated that inflammation precedes or occurs in the absence of bacterial colonization in CF humans (5, 33, 51, but see Ref. 10) and mice (55, but see Ref. 6). However, it may be difficult to control for bacteria that may elude detection (43, 44) and for contributions of nonepithelial cells to inflammatory responses. Thus many have addressed this issue in vitro on CF vs. non-CF primary airway epithelial cells and also on CF and cystic fibrosis transmembrane conductance regulator (CFTR)-corrected CF airway epithelial cell lines.

Many investigators have observed higher expression and secretion of proinflammatory mediators in CF cells than in paired CFTR-expressing cells in the control state (no bacteria), and this hyperinflammatory state of CF cells is exacerbated by exposure to *Pseudomonas aeruginosa* (PA) or *Hemophilus influenzae* (6, 7, 11, 12, 15, 34, 49, 50, 59). This has been observed repeatedly for the IB3 (CF, ΔF508/W1282X) and C38 or S9 (CFTR-corrected; see Refs. 12, 50, and 59) pairs and also for the 9HTEo$^-$/pCep [wild-type (wt) CFTR] vs. 9HTEo$^-$/pCepR (overexpress R domain and do not secrete $Cl^-$; see Refs. 7, 34, and 59) pairs. The CF cells also bind more bacteria than the non-CF or CFTR-corrected cells (7, 11, 19). Consistent with these findings, a recent study showed that a specific CFTR blocker caused, after 6 days of treatment, an increase in proinflammatory signaling and interleukin (IL)-8 secretion of CFTR-expressing but not CF airway epithelial cells (35), indicating that inhibition of CFTR channel function was sufficient to increase proinflammatory signaling. In contrast, CFT1-ΔF508 cells exhibit smaller expression and secretion of proinflammatory cytokines than CFTR-corrected CFT1-CFTR cells in the control state and also during exposure to different strains of PA (29, 40). Ribeiro and colleagues (41) have shown that control (no bacteria) primary CF bronchial cells were hyperinflammatory during short-term (up to 11 days) culturing, but this property was lost during longer-term (>30 day) culturing. This exaggerated response of CF cells appeared to arise from sustained effects of extended exposure to inflammatory conditions characteristic of the CF lungs and not from the differential effects of CFTR expression. However, Ribeiro et al. (41) did not test specific bacterial products on the IL-8 responses of long-term CF vs. non-CF cells, although they did show that CF supernatants that likely contained such bacterial products had similar effects on long-term CF and non-CF cells. Others demonstrated different expression of only 24 out of 22,283 genes in CF vs. non-CF primary cells in control conditions (64) and no difference in intercellular adhesion molecule (ICAM)-1 and IL-8 during 6 h of bacterial exposure (2). Similarly, non-CF and CF primary cells responded similarly to cytokines a toll-like receptor (TLR) 2

Address for reprint requests and other correspondence: T. E. Machen, Dept. of Molecular and Cell Biology, 231 LSA, Univ. of California-Berkeley, Berkeley, CA 94720-3200 (e-mail: tmachen@berkeley.edu).

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agonist and to *Staphylococcus aureus* filtrate, although there small differences in responses to PA when cells were incubated in serum-containing medium (4). Responses of primary CF vs. non-CF airway epithelia to bacteria under conditions that would assure elimination of effects of prior CF exposure remain to be tested.

A potential problem with in vitro experiments on cell lines is that selection and passaging of cells transfected with CFTR, some by stable integration into the genome and some by episomal expression (see Ref. 2), may generate cells with different genetic backgrounds. This problem can be overcome by using one CF cell line and expressing CFTR using an adenovirus, as done two times previously. Aldallal et al. (2) showed that CF (IB3 and primary human airway epithelia) and CFTR-corrected CF cells exhibited similar expression of ICAM-1 and secretion of IL-8 under control conditions or during exposure to *H. influenzae* or PA strain PAO1 or to several proinflammatory cytokines. Pizurki et al. (36) found that CF15 cells [polarized human nasal CF (ΔF508/ΔF508) cells (24)] bound more PA than CFTR-corrected (using an adenovirus) CF15 cells, but CF15 and CFTR-corrected CF15 cells produced similar amounts of IL-8 in control conditions and during exposure to IL-1β.

The present experiments used the adenoviral approach to evaluate the roles of wt- and ΔF508CFTR in PA-triggered early innate immune responses of the CF15 cell line (21). Experiments were performed with confluent, polarized monolayers that were previously shown to elicit physiological restoration of CFTR expression in the plasma membrane and transepithelial $Cl^-$ transport after adenoviral correction (48). The role of PA binding in inflammatory initiation (7, 11) was tested by measuring binding of green fluorescent protein (GFP)-labeled bacteria to CF and CFTR-corrected cells. IL-8 secretion and nuclear factor (NF)-κB activity were measured because these are key intermediates in the proinflammatory responses of airway epithelia (11, 13). We tested multiple strains of PA to address the differential responsiveness of CF vs. CFTR-expressing airway epithelia (40). We also tested flagellin, the critical bacterial activator of airway epithelial cell innate immune responses (1, 17, 31, 52, 65), to eliminate PA-secreted factors that may alter epithelial responses (8). IL-8 secretion was measured from primary human CF and non-CF bronchial cells that had been passaged to eliminate problems associated with short-term growth of primary CF airway epithelia (41).

Retention of misfolded ΔF508CFTR in the endoplasmic reticulum (ER) could trigger ER stress (62, 63), and it has been proposed that such stress alters ER cytosolic $Ca^{2+}$ ($Ca_i$) handling (59) and increases inflammatory signaling (28, 59). $Ca^{2+}$ entry in CF cells may also increase in response to hyperpolarized membrane potential in CF (28, 39, 61). We tested for ER stress by measuring the activity of IRE1α, an ER membrane protein receptor for ER-unfolded proteins that has both endonuclease and kinase activities that are activated when misfolded proteins accumulate in the ER (62, 63). ΔF508CFTR with a GFP tag was overexpressed in CF15 cells and also in HeLa cells, which do not express CFTR. Finally, CFTR- and ΔF508CFTR effects on $Ca_i$ signaling and NF-κB activity were tested. Although PA and flagellin activate NF-κB, they do not increase $Ca_i$ in CF15 cells (13, also see Ref. 25) so we stimulated cells with flagellin plus either ATP (purinergic agonist) or thapsigargin (inhibitor of $Ca^{2+}$-ATPase in the ER) to increase $Ca_i$. Potential effects of wtCFTR to depolarize membrane voltage, decrease $Ca^{2+}$ entry, and reduce flagellin + ATP or flagellin + thapsigargin activation of NF-κB were also maximized by stimulating with forskolin to increase cellular cAMP concentration and activate protein kinase A.

## MATERIALS AND METHODS

*Reagents.* Unless otherwise specified, reagents and chemicals were obtained from Sigma (St. Louis, MO). CFTR-adenovirus (adenovirus 5, CMV promoter; "adv-CFTR"), lacZ-adenovirus (adv-lacZ) and NF-κB-driven luciferase (adv-NF-κBluc; see Ref. 48) were purchased from the University of Iowa Vector Core (2, 53). Adenoviruses to express wtCFTR or ΔF508CFTR with GFP appended to the COOH termini (wtCFTR-GFP and ΔF508CFTR-GFP; see Ref. 54) were purchased from the University of Pennsylvania Vector Core (James Wilson).

*CF15, HeLa, and Calu-3 cell culture and adenovirus infections.* CF15 CF airway cells, a continuous SV-40 large T antigen-transformed nasal epithelial cell line homozygous for ΔF508 CFTR (21, 36), were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM-F-12) media containing 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 10 ng/ml epidermal growth factor, 1 μM hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM triiodothyronine, 180 μM adenine, and 5.5 μM epinephrine. All CF15 cells were grown in the absence of the antibiotic resistance marker G418.

For some experiments, cells were passaged at a 1:5–1:10 dilution, and the remaining cell suspension was seeded directly on cover glasses or on a tissue culture plate (BD Falcon, Bedford, MA). In other experiments, cells were passaged on Transwell membranes (Corning/Costar) and then grown until cells formed confluent monolayers. Control experiments were performed in Ussing chambers to assure filter-grown cells attained confluence and were polarized. As reported previously (48), CF15 cells on filters had transepithelial resistances ($R_T$) greater than 200 $\Omega \cdot cm^2$ and exhibited amiloride-sensitive, serosa-positive currents of 10–25 $\mu A/cm^2$, consistent with $Na^+$ absorption through the epithelial $Na^+$ channel (ENaC). Forskolin treatment increased this apparent $Na^+$ absorption but did not increase anion currents (data not shown), consistent with their CF genotype.

Calu-3 cells, a human gland epithelial cell line homozygous for wt CFTR (27), were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin. Passaging and growth on filters or in plastic tissue culture dishes was the same as that described for CF15 cells.

HeLa cells were cultured in DMEM media containing 10% FBS and 1% penicillin/streptomycin at 37°C with 5% $CO_2$ and 95% humidity.

CF15 cells, Calu-3 cells, or HeLa cells were infected with adv-CFTR, adv-lacZ, adv-wtCFTR-GFP, adv-ΔF508CFTR-GFP, and/or adv-NF-κB-luc (MOI = 100) in media for 48 h at 37°C. Epithelia grown on filters were infected on apical and basal sides. Cells grown on plastic or cover glasses were infected by additions to the media. Previous experiments showed that multiplicity of infection (MOI) = 100 for 48 h gave a maximal number of cells infected (80–100%) and increases in $Cl^-$ secretion (36, 48). Experiments with adv-wtCFTR-GFP and adv-ΔF508CFTR-GFP showed similar frequency of cellular expression.

*Primary human non-CF and CF bronchial cells.* Bronchial epithelial cells were isolated from cadavers or lungs removed at the time of transplantation (60) from one non-CF and CF individual using methods described previously. Briefly, epithelial cells were obtained by protease digestion and then grown and passaged one time, as described previously (45). These P1 ("first passage") cells were suspended in a 1:1 mixture of DMEM-F-12 containing 5% FBS and seeded at $5 \times 10^5$ cells/$cm^2$ on Transwell polycarbonate inserts (no.

3401; Costar, Corning, NY). Growth surfaces were coated with human placental collagen. The day after plating, the DMEM-F-12 over the filters was replaced with Gray's medium, a medium whose composition is fully described elsewhere (45, 60). Once the cells had attained confluence, they were grown with an air-liquid interface (i.e., medium was only added to the basolateral surfaces). Previous experiments have shown that these monolayers contain epithelial cells with no obvious contamination from myeloid cells (45). For measurements of electrical $R_T$, 500 μl of PBS were added to the mucosal surface, and a "chop-stick" voltmeter (Millicell ERS; Millipore Products, Bedford, MA) was used to measure $R_T$. Experiments were performed 3–4 wk after plating (>14 days after attaining confluence). These cultures attained minimum $R_T$ >100 $\Omega \cdot cm^2$ (usual $R_T$ = 400–500 $\Omega \cdot cm^2$) and maintained a dry mucosal surface.

*P. aeruginosa.* PA strains PAK, PAO1 (serotype 05), PAC1R (serotype 03), and PA2192 (mucoid clinical isolate; see Refs. 8–10 and 55) were grown overnight in LB culture as described previously (17, 20, 26). Before experiments, PA were washed three times with PBS and resuspended in CF15 media minus antibiotics and supplements at a concentration of $10^8$ colony-forming units/ml (optical density at 600 nm = 0.05). GFP-expressing strains PAK and PAO1 (PAK-GFP, PAO1-GFP) were generated and then grown and used as described previously (17).

*Enzyme-linked immunosorbent assay of IL-8 secretion.* Samples were routinely collected from the basal chamber of cells grown on filters and in some experiments from media bathing cells grown on plastic. Enzyme-linked immunosorbent assay (ELISA) methods have been published previously (13, 17). Briefly, samples were collected, centrifuged (5 min 1,000 *g*), stored at −20°C, and then thawed, diluted in Assay Diluent (BD Pharmingen, San Diego, CA), run in triplicate per the manufacturer's protocol (OptEIA Human IL8 Set; BD Pharmingen), and read at 450 nm with an $EL_X808$ Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT).

*NF-κB-regulated luciferase.* A recombinant adenoviral vector expressing a luciferase reporter gene driven by NF-κB transcriptional activation (adv-NF-κBluc) was used for functional studies as described previously (47). Adenoviral stocks were stored in 10 mM Tris with 20% glycerol at −80°C. The virus was added to CF15 cell monolayers alone or along with any other viruses at 100 MOI and returned to the incubator for 48 h. Monolayers were then washed three times to remove viruses and then exposed to PA or flagellin for 4 h. Monolayers were then washed and processed using the luciferase assay system with Reporter Lysis Buffer (Promega, Madison, WI) to measure NF-κB-mediated transcriptional induction according to the manufacturer's protocol. Measurements of luciferase activity (relative light units) were performed in triplicate for each sample and normalized to the protein concentration (Bradford assay). These averages were then expressed relative to the average control values (absence of bacteria), which was set equal to 1.0.

*PAK-GFP and PAO1-GFP binding.* CF15 or adenovirus-treated CF15 monolayers were incubated with either PAK-GFP (MOI = 100) or PAO1-GFP (MOI = 10) for 1 h, washed three times, and fixed and stained for NF-κB (anti-p65 antibody; Santa Cruz Biotechnology) immunofluorescence to permit visualization of the cells in the fluorescence microscope. PA binding occurred in areas in which epithelial cells had overgrown in multilayers as described previously (17). Individual bacteria and epithelial cells were counted from confocal images (17), and bacterial binding was expressed as number of PA bound per 100 epithelial cells.

*$Ca_i$ measurements.* Cells grown on cover glasses were incubated in the original growth media containing 1–10 μM fura 2-AM for 40–60 min at room temperature and then washed three times with Ringer solution to remove the extra dye. Fura 2-AM (1–3 μM) was used to load nonconfluent cells, whereas 5–10 μM fura 2-AM was used to load confluent cells. For $Ca_i$ measurements, epithelial cells were incubated in solutions containing (in mM): 145 NaCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2.4 $K_2HPO_4$, 0.6 $KH_2PO_4$, 10 HEPES, and 10 glucose (pH 7.4). $Ca^{2+}$-free Ringer was composed of the same solution without added $Ca^{2+}$. Treatments of cells with ATP were made by diluting stock solutions 1,000× in Ringer solution.

Fluorescence ratio imaging measurements of $Ca_i$ were performed using methods that have been reported previously (13, 20). Briefly, a Nikon Diaphot inverted microscope and charge-coupled device camera collected emission (>510 nm) images during alternate excitation at 350 ± 5 and 380 ± 5 nm using a filter wheel (Lambda-10, Sutter Instruments, Novato, CA). Axon Imaging Workbench 4.0 (Axon Instruments, Foster City, CA) controlled both filters and collection of data. Calibration of fura 2 signals was performed as described previously (13, 14), assuming the apparent dissociation constant for fura 2 was 224 nM (14). In situ calibration of fura 2 was carried out by treating the cells with ionomycin (10 μM) and then perfusing the cells with a $Ca^{2+}$-free external solution to determine $R_{min}$ and with a solution containing 2 mM $Ca^{2+}$ to determine $R_{max}$. $R_{min}$ and $R_{max}$ are the minimum and maximum fluorescence (>510 nm) ratios measured during 350 nm/380 nm excitation. All images were corrected for background (region without cells).

*Confocal microscopy of wtCFTR-GFP- and ΔF508CFTR-GFP-expressing cells.* CF15 and HeLa cells grown on cover glasses and infected as described above with wtCFTR-GFP and ΔF508CFTR-GFP were mounted live on the stage of an inverted microscope (Nikon TE2000) and observed with a spinning disk confocal attachment (488 nm excitation and 520–560 nm emission; Solamere Technology, Salt Lake City, UT). Images were collected from *z*-axis regions roughly in the middle of the cell nucleus.

*ER stress: IRE1α activity assessed by splicing of XBP1 RNA.* ER stress was measured using a method that measures the endonuclease activity of IRE1α. This ER membrane-bound enzyme is activated during increases in unfolded proteins in the ER lumen or sustained decreases of ER $Ca^{2+}$ concentration or redox potential (3, 62, 63). Two RT-PCR-based assays for activation of IRE1α were performed using primers up- and downstream of the 26-bp exon of XBP1 mRNA. RT-PCR was performed on RNA isolated from CF15 and HeLa cells and also on PST-I digests of PCR products. CF15 and HeLa cells were either left untreated or treated with adenoviruses to generate expression of CFTR, CFTR-GFP, and ΔF508CFTR-GFP. The cells were also treated with thapsigargin (1 μM, 4 h) as a positive control (63).

ER stress was quantitated from intensities of four bands of RT-PCR products that were designated as follows: "Band α," the largest product (~500 bp), resulted from full-length XBP-I; "Band β" ran slightly faster because this band was full-length XBP1 − 26 bp; PST-I-treatment of the RNA cleaves XBP-1 (Band α) to produce two smaller bands (Band γ, 300 bp; Band δ, 200 bp). XBP1 PCR products from control cells with inactive IRE1α will have prominent Band α and no or little Band β. The Band β-to-Band α ratio measured (using ImageJ 1.37v, including background subtraction) should be small. PST-I digests of XBP1 PCR products from control cells will show little or no Band α or β but prominent Bands γ and δ, and the Band β/(Bands α + γ + δ) ratio should also be small. In contrast, XBP1 products from ER-stressed cells with active IRE1α will have prominent Band β, no or little Band α, and the Band β/Band α ratio will be larger than control cells. PST-I digests of XBP1 PCR products from ER-stressed cells will show prominent Band β and less or no Bands α, γ, and δ, and the Band β/(Bands α + γ + δ) ratio should be larger than in control cells without ER stress.

Assays for IRE1α activity have been described previously (3). Briefly, CF15 and HeLa cells grown in culture dishes over night were infected with the adenoviruses (MOI = 100) or left in culture medium. Cells were cultured for 2 days and incubated with medium or with thapsigargin (1 μM) for 4 h. Total RNA was isolated (RNeasy Isolation Kit; Qiagen). First-strand cDNA was generated by incubating 2 μg RNA with 2.5 mM dNTPs, 1 pmol random hexamer primers, 20 units RNase inhibitor, and 200 units SuperScript II reverse transcriptase (Invitrogen) for 1 h at 42°C. XBP1 DNA was PCR-amplified

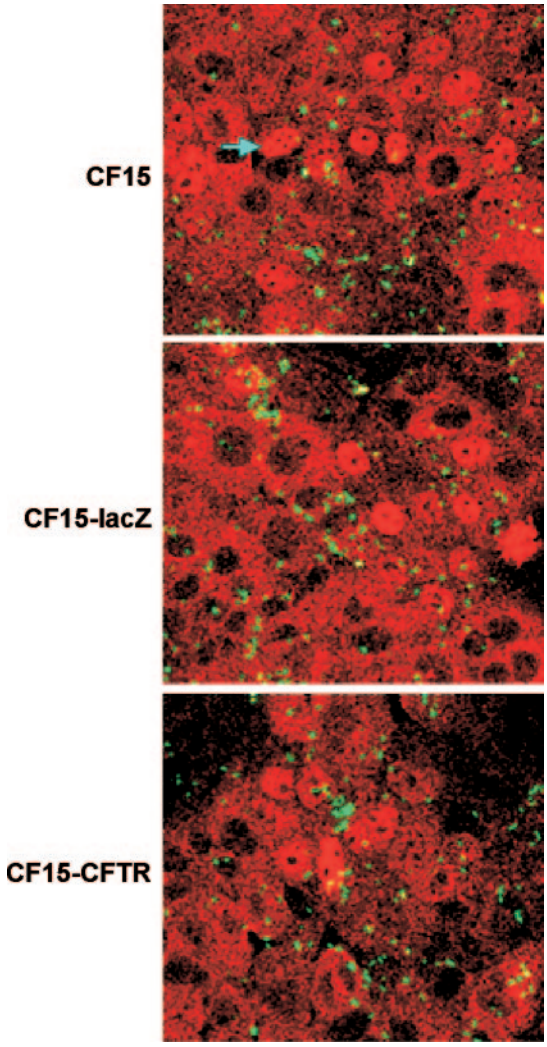


Fig. 1. PAK-green fluorescent protein (GFP) binding to CF15, CF15-lacZ, and CF15-cystic fibrosis transmembrane conductance regulator (CFTR) cells. PAK-GFP [multiplicity of infection (MOI) = 100] were added to apical surfaces of confluent layers grown on filters for 1 h. Cells were washed, fixed, and stained for nuclear factor (NF)-κB (p65) to provide contrast and examined for PAK-GFP binding. Typical confocal images of bacteria bound to multilayer regions are shown for CF15 (*A*), CF15-lacZ (*B*), and CF15-CFTR (*C*). Blue arrow denotes NF-κB that migrated to the nucleus.

using 2 μl first-strand cDNA, 300 nM forward primer (5′-AAACAGAGTAGCAGCTCAGACTGC-3′), 300 nM reverse primer (5′-TCCTTCTGGGTAGACCTCTGGGAG-3′), 1 μl 10 mM dNTPs, and 2.6 units RedTaq DNA polymerase (Sigma). A thermal-cycler program consisted of 94°C for 10 min; 94°C for 20 s, 65°C for 20 s, and 72°C for 30 s for 35 times; and 72°C for 5 min. 18S RNA was assayed under identical conditions using specific primers (Ambion). PCR products were analyzed on a 3% agarose gel. Products were also purified using the Qiaquick PCR Purification Kit (Qiagen) and digested with PST-I (5 U/μg PCR product; Fermentas) for ≥2 h before agarose gel analysis.

*Statistics.* Unpaired or paired *t*-tests were used to compare groups and effects, depending on the experiments (StatView; Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant. Data have been presented as averages ± SD or including values from all individual experiments; *n* refers to the no. of experiments (for luciferase and IL-8 assays, this refers to the no. of different wells or filters sampled; for $Ca_i$ measurements, *n* refers to the no. of different cover slips examined; and for XBP-1 assays, *n* refers to the no. of different cell preparations examined).

## RESULTS

*Role for CFTR in controlling P. aeruginosa binding?* Confluent monolayers of CF15 cells, and cells treated with adv-CFTR or adv-lacZ, were exposed to PAK-GFP (MOI = 100) for 1 h, washed, and then fixed and mounted on slides for counting. Similar experiments were also performed with PAO1-GFP, although PAO1 were added at MOI = 10 to test whether binding properties were affected by bacterial dose. Because PA bound almost exclusively to CF15 cells that grew in multiple layers, random multilayer regions were chosen for PA counting. There were no significant effects of CFTR expression on PA binding (Fig. 1 and Table 1). The smaller number of PAO1-GFP vs. PAK-GFP bound was likely due to using MOI = 100 for PAK and MOI = 10 for PAO1.

*Role for CFTR in NF-κB activation, IL8 secretion, and PA binding?* NF-κB luciferase assays were performed on control monolayers and on cells exposed on the apical surface to PAO1, PAK, PAC1R, or PA2192 (MOI = 100) or to flagellin ($10^{-7}$ g/ml) for 4 h. Samples were also taken from the basolateral solution at *time 0* and then after 4 h to measure IL-8 secretion.

NF-κB activity of control cells was slightly (35%) higher in CF15 than in CF15-lacZ cells (Fig. 2*A*). However, there was no significant difference between CF15 and CF15-lacZ cells in IL-8 secretion (Fig. 2*B*). Additional of strains PAO1, PAK, PAC1R or PA2192, or flagellin to the apical surfaces of CF15 monolayers increased both NF-κB activity (Fig. 2*A*) and IL-8 secretion (Fig. 2*B*), but there were no differences in responses of CF15 vs. CF15-lacZ or of CF-lacZ vs. CF15-CFTR. There was also no effect of CFTR expression on responses to flagellin. Compared with PAK and PAO1, PA2192 and PAC1R elicited larger stimulations of NF-κB (Fig. 2*A*) and IL-8 (Fig. 2*B*), indicating that, as shown previously (40), there were strain-dependent differences in PA activating innate immune responses. However, unlike previous work (40), there were no differences in NF-κB or IL-8 responses attributable to CFTR expression (Fig. 2, *A* and *B*).

Non-CF and CF primary human airway epithelial cells both secreted IL-8 in the basolateral solution even in the absence of any stimulation, but there was no significant difference between non-CF and CF cells in controls or in response to flagellin (Fig. 3).

Table 1. *Effects of CFTR expression on Pseudomanas aeruginosa binding*

| | PAK-GFP | *n* (no. PA/100 cells) | PAO1-GFP | *n* (no. PA/100 cells) |
|---|---|---|---|---|
| CF15 | 135±17 | 3 | 24±26 | 17 |
| CF15-lacZ | 145±43 | 3 | 11±9 | 16 |
| CF15-CFTR | 120±41 | 3 | 16±15 | 15 |

Data were expressed as average number ± SD of bacteria bound/100 epithelial cells; *n*, total no. of different fields inspected (2 experiments for PAK-GFP experiments and 3 experiments for PAO1-GFP). GFP, green fluorescent protein; PA, *P. aeruginosa*; CFTR, cystic fibrosis transmembrane regulator. CF15, CF15-lacZ, and CF15-CFTR monolayers were infected on the apical surface with PAK-GFP {100 multiplicity of infection (MOI)} or PAO1-GFP (10 MOI) for 1 h and then washed, fixed, stained, and examined using confocal microscopy. There were no significant differences in PAK or PAO1 binding among the cells (unpaired *t*-test, $P > 0.05$).

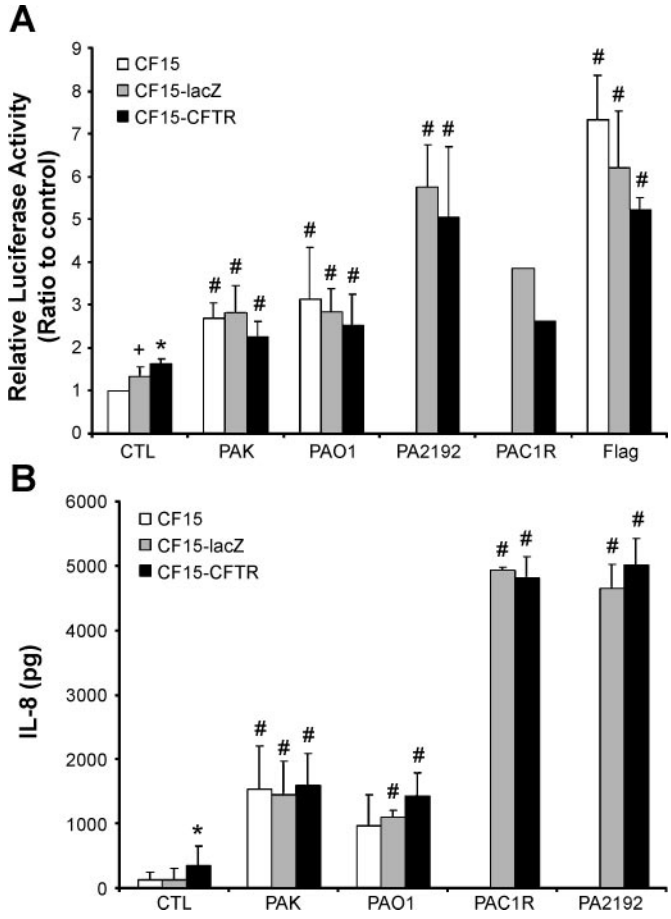


Fig. 2. *Pseudomonas aeruginosa* (PA)-induced activation of NF-κB and interleukin (IL)-8 expression and secretion of CF15, CF15-lacZ, and CF15-CFTR cells. Confluent epithelial monolayers expressing NF-κB-regulated luciferase were exposed for 4 h to PAO1, PAK, PAC1R, or PA2192 (MOI = 100) or to flagellin (flag, $10^{-7}$ g/ml). In addition, samples were collected from the basal side at the start of the experiment and after 4 h and analyzed for IL-8. *A*: PA-induced NF-κB activation. Bars show averages ± SD of luciferase activities. For controls, luciferase was expressed relative to CF15. For other treatments, luciferase activities were expressed relative to the controls for each cell type. Results are averages of 4 experiments for each comparison, except PAC1R, where $n = 2$. *B*: PA-induced IL-8 secretion. Bars show averages ± SD of differences in IL-8 in controls and during exposure of cells to PAK, PAO1, PA2192, or PAC1R. Results are averages of 4 experiments for each comparison, except PAC1R, where $n = 2$. Statistical comparisons: $^{+}$CF15 < CF15-lacZ, paired *t*-test, $P < 0.02$; *CF15-lacZ < CF15-CFTR cells in control (no treatment) condition, paired *t*-test, $P < 0.001$; and #responses of PA- or flagellin-treated cells larger than the same cells in absence of bacteria, paired *t*-test, $P < 0.05$. There were no significant differences between CF15-lacZ and CF15-CFTR cells during treatments with PA or flagellin.

*Role for wtCFTR or ΔF508CFTR in triggering ER stress?* A complication in the interpretation of data in Figs. 1–3 is that CF15 cells may express only low levels of ΔF508CFTR protein that are insufficient to trigger an ER stress response and, consequently, not change $Ca_i$ signaling. This issue was addressed by expressing ΔF508CFTR tagged with COOH-terminal GFP (54), which enabled detection of transduced cells and evaluation of the subcellular localization of ΔF508CFTR-GFP. Similar experiments were performed in HeLa cells, which do not express CFTR and therefore will presumably not have a stressed ER, to determine whether any activation of IREα in CF15 cells resulted from an already overloaded ER. CF15 and HeLa cells were infected with adv-ΔF508CFTR-GFP and also adv-wtCFTR and adv-wtCFTR-GFP for 1 day followed by washing and two further days of growth; these cells were then processed for measurements of IRE1α activity.

Representative confocal images of CF15 and HeLa cells expressing wtCFTR-GFP and ΔF508CFTR-GFP are shown in Fig. 4. Each construct transduced 70–90% of the cells. wtCFTR-GFP appeared prominently in the plasma membrane and also in a spot near the nucleus, likely the Golgi, in both CF15 (Fig. 4*A*) and HeLa (Fig. 4*B*) cells. Lower-level expression throughout the cytosol, likely the ER, was also observed. In contrast, ΔF508CFTR-GFP was not observed in the plasma membrane or Golgi but was found in the ER in both CF15 (Fig. 4*C*) and HeLa (Fig. 4*D*) cells.

IRE1α activity was determined in CF15 and HeLa cells from XBP1 PCR products and the same products that had been subjected to PST-I digestion. 18S RNA was used as a loading control. Results from cells expressing wtCFTR, wtCFTR-GFP, or ΔF508CFTR-GFP −/+ thapsigargin are shown in *lanes 1–8* for CF15 cells (Fig. 5, *A–C*) and HeLa cells (Fig. 5, *E–G*). Values for the Band β/Band α ratio for control PCR products and Band β/(Bands α + γ + δ) for PST-I-digests were similar in magnitude, so these were averaged. Data were plotted for CF15 cells in Fig. 5*D* and for HeLa cells in Fig. 5*H*. Values for relative IRE1α activities were normalized to the values measured in CF15 cells or HeLa cells under control conditions.

Control CF15 cells exhibited a prominent Band α and a very faint Band β (Fig. 5*A*, *lane 1*). PST-I treatment yielded Bands γ and δ, whereas Bands α and β were now only faintly visible (Fig. 5*B*, *lane 1*). Similar patterns were observed for CF15 cells that had been transduced with wtCFTR, wtCFTR-GFP, or ΔF508CFTR-GFP (Fig. 5, *A* and *B*, *lanes 3*, *5*, and *7*, respectively). Control HeLa cells exhibited strong Band α and absence of Band β (Fig. 5*E*, *lane 1*), and PST-I digests (Fig. 5*F*, *lane 1*) showed no Bands α and β and prominent Bands γ and δ. Expression of wtCFTR or ΔF508CFTR-GFP had essentially

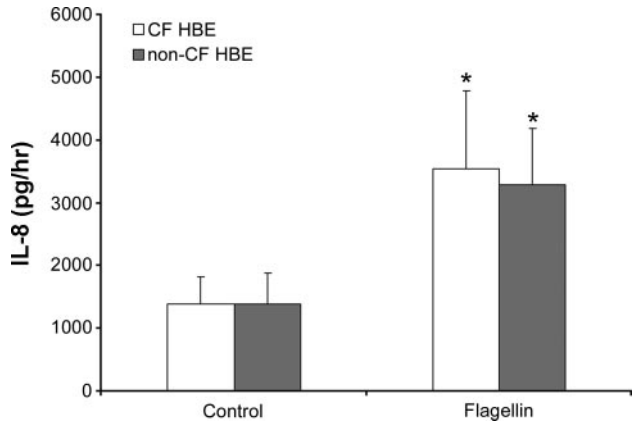


Fig. 3. IL-8 secretion by human primary CF and non-CF bronchial epithelia. Bronchial epithelial cells grown on filters were left untreated for 3 h while sampling for IL-8 measurements [enzyme-linked immunosorbent assay (ELISA)] each hour to obtain a baseline rate of secretion in the basal solution. Next, the cells were treated with flagellin ($10^{-7}$ g/ml), and IL-8 was sampled again. Average ± SD ($n = 8$ wells) rates of IL-8 secretion during *control hours 1–3* and during *flagellin hours 2–4* of are shown. Statistical comparison: *response of flagellin-treated cells larger than controls, paired *t*-test, $P <$ 0.001. There were no significant differences between non-CF and CF cells in control or during flagellin.

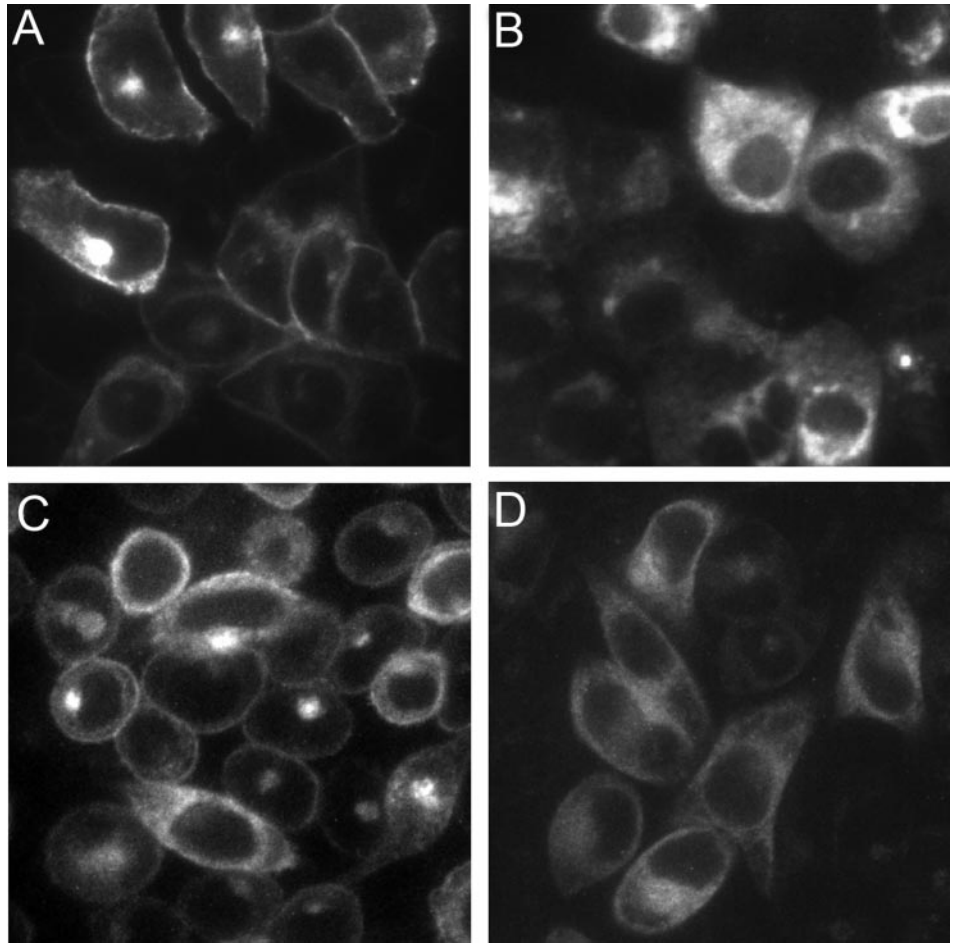


Fig. 4. Wild-type (wt) CFTR-GFP and ΔF508CFTR-GFP expression in CF15 and HeLa cells. CF15 (*A* and *B*) and HeLa (*C* and *D*) cells grown on cover glasses were infected with adenoviruses expressing either wtCFTR-GFP (*A* and *C*) or ΔF508CFTR (*B* and *D*). Confocal observations were made through the middle region of the nucleus. Images typical of three experiments each.

no effect on these patterns (Fig. 5, *E* and *F*, *lanes 3* and *7*). Expression of wtCFTR-GFP elicited a small increase in Band β of controls (Fig. 5*E*, *lane 5*). Thapsigargin increased Band β and decreased Band α for CF15 and HeLa cells and for cells transduced with wt- or ΔF508CFTR (Fig. 5*A*, *lanes 2*, *4*, *6*, and *8* and Fig. 5*E*, *lanes 2*, *4*, *6*, and *8*). PST-I digests of the thapsigargin-treated cells showed low-intensity Band β, even lower-intensity Band α, and higher-intensity Bands γ and δ for control CF15 and HeLa cells and for the same cells transduced with wtCFTR, wtCFTR-GFP, or ΔF508CFTR-GFP (Fig. 5*B*, *lanes 2*, *4*, *6*, and *8*; Fig. 5*F*, *lanes 2*, *4*, *6*, and *8*).

PST-I treatment did not totally cleave Band α in any of the preparations except the control HeLa cells (Fig. 5*F*, *lane 1*), even when PST-I digestion was performed for 12 h (data not shown). Band α from *lanes 1–8* of both CF15 and HeLa cells was isolated, purified, and sequenced and shown to be full-length XBP-1 containing the PST-I restriction site and suggested that the PST-I digestion was incomplete.

Quantitation showed that IRE1α activity was insignificantly ($P > 0.1$) higher in control CF15 cells compared with control HeLa cells (Fig. 5*D*, *lane 1* vs. Fig. 5*H*, *lane 1*). Compared with control CF15 cells, IRE1α activity was insignificantly altered by wtCFTR ($P > 0.12$), wtCFTR-GFP ($P > 0.43$), or ΔF508CFTR-GFP ($P > 0.10$; Fig. 5*D*, *lane 1* vs. *lane 3*; see Refs. 5 and 7). Similar results were obtained in HeLa cells, although wtCFTR-GFP ($P = 0.02$) and ΔF508CFTR-GFP ($P = 0.02$) both caused significant increases compared with control (Fig. 5*H*, *lanes 5* and *7* vs. *1*). This may have resulted from some virus-dependent rather than CFTR-dependent effect in the HeLa cells because the wtCFTR adenovirus did not significantly alter IRE1α activity compared with controls (Fig. 5*H*, *lane 1* vs. *3*, $P > 0.28$), and there was no significant difference in IRE1α activity between HeLa cells infected with adv-wtCFTR-GFP and adv-ΔF508CFTR-GFP ($P > 0.1$). In contrast, the ER stress-agonist thapsigargin caused >20-fold increases in IRE1α activity of CF15 and HeLa cells in control conditions or during expression of wt- or ΔF508CFTR (Fig. 5*D*, *lanes 2*, *4*, *6*, and *8*; Fig. 5*H*, *lanes 2*, *4*, *6*, and *8*).

*Role for CFTR in controlling $Ca_i$ and NF-κB of cells with activated $Ca_i$ and CFTR?* CF15 cells infected with adv-lacZ or adv-CFTR were left untreated to determine baseline $Ca_i$ and then treated with flagellin (which had no effect on $Ca_i$; see Ref. 13) or with ATP. A representative single cell response to ATP in CF15 cells is shown in Fig. 6, and averages for CF15-lacZ and CF15-wtCFTR cells are summarized in Table 2: control $Ca_i = \sim 100$ nM, and ATP caused a rapid increase in $Ca_i$ ("first peak") resulting from the rapid release of ATP from the ER (13), whereas, as shown previously (13), the "second peak" and "plateau" were mostly due to ATP-induced $Ca^{2+}$ entry across the plasma membrane in the cells. CFTR expression had no apparent effect on either baseline $Ca_i$ or in the first peak or plateau phases of the $Ca_i$ responses to ATP (Table 2). CFTR expression had a small effect on the second peak $Ca_i$ response, but this second peak was variable from preparation to prepa-

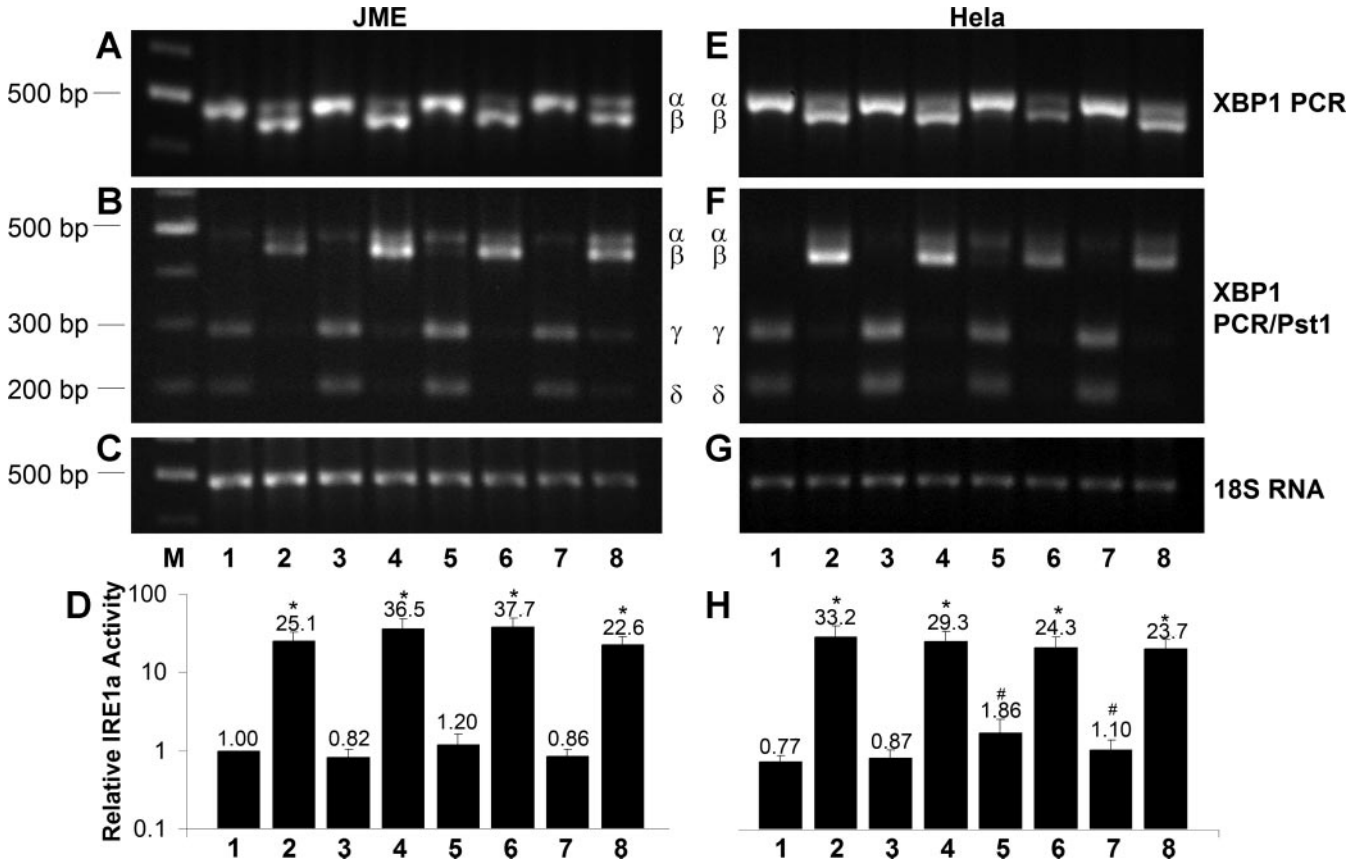


Fig. 5. IRE1α activity in CF15 and HeLa cells infected with wtCFTR, wtCFTR-GFP, and ΔF508CFTR and effects of thapsigargin. CF15 cells or HeLa cells expressing CFTR, CFTR-GFP, or ΔF508CFTR were left untreated or treated with thapsigargin (1 μM, 4 h). XBP1-specific RT-PCR products were analyzed by gel electrophoresis (*A*, CF15 cells; *E*, HeLa cells) and after PST-I digestion (*B*, CF15 cells; *F*, HeLa cells). PCR of 18S RNA was performed as a control (*C*, CF15 cells; *G*, HeLa cells). *Lane M* refers to molecular weight markers and *lanes 1–8* to treatments with adenoviruses (adv) and thapsigargin [control/no adv (*lane 1*), control/no adv. + thapsigargin (*lane 2*), adv-wtCFTR (*lane 3*), adv-wtCFTR + thapsigargin (*lane 4*), adv-wtCFTR-GFP (*lane 5*), adv-wtCFTR-GFP + thapsigargin (*lane 6*), adv-ΔF508CFTR-GFP (*lane 7*), and adv-ΔF508CFTR-GFP + thapsigargin (*lane 8*)]. Quantitation of data in *lanes 1–8* (*D*, CF15 cells; *H*, HeLa cells) in terms of relative IREα activity (average ± SD for 3 different experiments each and for 1–2 different gels in each case) was performed as described in the text. Note *y*-axis is a log scale. Averages for each lane shown above bars. Statistics in *D* and *H*: *different ($P < 0.001$) for thapsigargin-treated cells vs. controls in each condition and #different ($P < 0.02$) for HeLa cells expressing wtCFTR-GFP or ΔF508CFTR-GFP vs. control. All other lanes were insignificantly different ($P > 0.10$) from control CF15 or control HeLa cells.

ration, so it seems that this small effect may have resulted from subtle differences in this second peak rather than a difference resulting from CFTR expression.

As a further test of the potential role of CFTR in controlling innate immune responses through changes in $Ca_i$, we compared NF-κB responses of CF15-lacZ vs. CF15-CFTR and of CF15-ΔF508CFTR vs. CF15-wtCFTR during treatments that both activated NF-κB (flagellin) and also raised $Ca_i$ (ATP and thapsigargin). We used both PAK and flagellin for these experiments because some previous experiments showed differences in $Ca_i$ responses to PA and flagellin in CF vs. CFTR-corrected cells (38, 59). Previous experiments showed that $Ca_i$ synergized with flagellin to activate innate immune responses (13), and, since cell membrane voltage is hyperpolarized in CF (28), it might have been expected that flagellin + ATP or flagellin + thapsigargin would cause larger activation in lacZ- or ΔF508CFTR-GFP- vs. wtCFTR-GFP-expressing cells. CF15-lacZ and CF15-CFTR cells (Fig. 7*A*) behaved similarly during flagellin ± ATP; CF15-ΔF508CFTR-GFP and CF15-wtCFTR-GFP cells (Fig. 7*B*) also responded similarly to flagellin and flagellin + ATP. There was a larger stimulation of NF-κB by flagellin + thapsigargin for CF15-wtCFTR than for CF15-ΔF508CFTR cells (Fig. 7*B*). PAK also stimulated similar responses in CF15-ΔF508CFTR-GFP and CF15-wtCFTR-GFP cells (Fig. 7*B*). Thus CF15 cells or CF15 cells overexpressing ΔF508CFTR responded similarly to wtCFTR-corrected cells during flagellin + ATP. Similarly, bronchial epithelial cells from non-CF and CF individuals secreted IL-8 in the basolateral solution at similar rates during flagellin + ATP: 2,933 ± 880 pg/h in non-CF ($n = 8$) and 1,867 ± 1,343 pg/h in CF cells ($n = 6$, $P > 0.3$).

We also compared responses in forskolin-treated cells to determine the potential role of activated CFTR in controlling membrane voltage, $Ca_i$, and NF-κB. In contrast to the stimulatory effect of ATP on flagellin-stimulated NF-κB, there was no difference in NF-κB activation during flagellin vs. flagellin + forskolin (Fig. 7*A*). This lack of effect of forskolin was also observed in Calu-3 cells: luciferase (relative to controls) for flagellin = 4.9 ± 2.5 and flagellin + forskolin = 4.8 ± 3.6, $P > 0.8$ ($n = 4$). Forskolin had an inhibitory effect on flagellin + ATP-stimulated NF-κB activity (Fig. 7*A*). Two other experiments showed similar inhibitory effects of forsko-

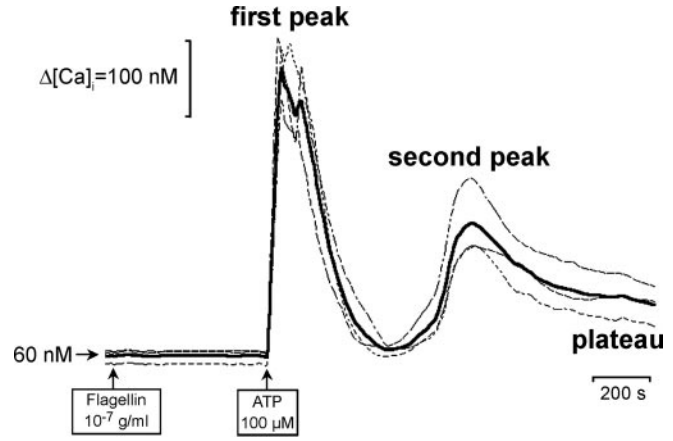


Fig. 6. Effects of ATP on cytosolic $Ca^{2+}$ ($Ca_i$) in CF15 cells. Cells were first treated with flagellin ($10^{-7}$ g/ml), which had no effect on $Ca_i$. Subsequent treatment with ATP (100 μM) caused a rapid increase in $Ca_i$ (first peak) followed by a second peak and plateau. A typical response (of 6 similar) for CF15 cells is shown. Summary shown in Table 2.

lin on flagellin + thapsigargin-stimulated NF-κB activity in CF15-lacZ and CF15-CFTR cells and also in the CFTR-expressing Calu-3 cell line (Table 3). Thus forskolin inhibited effects of flagellin + $Ca_i$-raising agonists, but this effect did not depend on the presence or activity of CFTR.

## DISCUSSION

*Methodology: CF15 cells and adenoviruses for introducing CFTR.* The CF15 cell line is valuable for making comparisons between control and CFTR-expressing cells because the cells grow to confluence, express tight junctions, exhibit $R_T > 200$ $\Omega \cdot cm^2$, and generate amiloride-inhibitable cation absorption characteristic of active apical ENaC and basolateral $Na^+$-$K^+$-ATPase (48). The cells also exhibit $Na^+/H^+$ and $Cl^-/HCO_3^-$ exchange activity in the basolateral but not the apical membranes (E. Wunderlich and T. E. Machen, unpublished observations) and $Ca^{2+}$-activated Cl channels in the apical but not the basolateral membrane (E. Wunderlich, T. E. Machen, and B. Illek, unpublished observations). Exposure to apical PA for 4 h caused IL-8 secretion to increase from 600 to 6,000 pg/ml in individual experiments, which was equal to or larger than that measured in other related cell types (24, 38), indicating that CF15 cells responded to PA by secreting IL-8 at normal rates.

Table 2. *CFTR expression on baseline $Ca_i$ and ATP-induced $Ca_i$ responses in CF15 cells*

| | $Ca_i$, nM | | | |
|---|---|---|---|---|
| | Baseline | ATP 1st Peak | ATP 2nd Peak | ATP Pateau |
| CF15-lacZ | 91±35* | 790±263* | 539±245† | 294±147‡ |
| CF15-CFTR | 99±33* | 742±400* | 247±136† | 243±130‡ |

Data are averages ± SD; $n = 5$ coverslips. Cytosolic $Ca^{2+}$ ($Ca_i$) was measured in 20–30 cells each for CF15-lacZ and CF15-CFTR monolayers on cover slips during control and then during ATP (100 μM), which caused 1st peak, 2nd peak, and plateau responses as shown in Fig. 6. Responses were similar in cells treated with ATP alone or ATP following flagellin ($10^{-7}$ g/ml), and data here include measurements from both types of experiments. *$P > 0.1$, $n = 11$; †$P < 0.05$, $n = 5$; and ‡$P > 0.1$, $n = 10$.

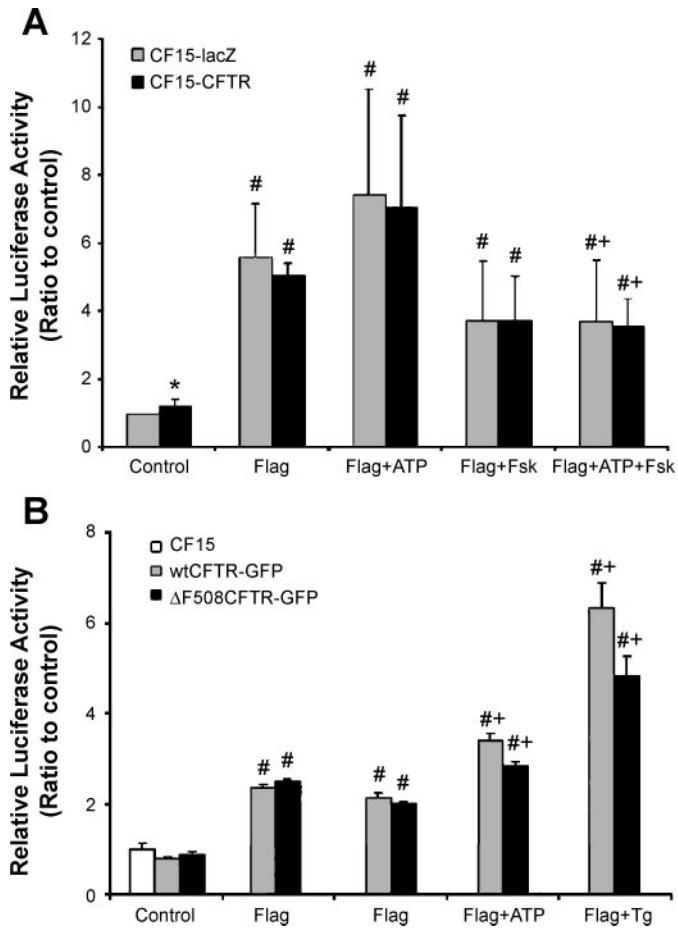


Fig. 7. Effects of PAK, flagellin, ATP, and forskolin on NF-κB activity in CF15 cells expressing lacZ, wtCFTR, wtCFTR-GFP, and F508CFTR-GFP. *A*: NF-κB-luciferase-expressing cells were exposed for 4 h to flagellin (flag, $10^{-7}$ g/ml), flagellin + ATP (flag + ATP, 100 μM) or flagellin + ATP + forskolin (flag + ATP + fsk, 1 μM) on the apical surface. For controls, luciferase was expressed relative to CF15-lacZ. For the other treatments, luciferase activities were expressed relative to CF15-lacZ or CF15-CFTR controls. Results are averages ± SD ($n = 4$–10) for each comparison. Statistical comparisons: *NF-κB activity of CF15-CFTR > CF15-lacZ, $P < 0.04$; #response of flagellin-, flagellin + ATP-, flagellin + forskolin-, and flagellin + ATP + forskolin-treated cells > controls, paired *t*-test, $P < 0.002$; and +response of flagellin + ATP + forskolin < flagellin + ATP, paired *t*-test, $P < 0.01$. There was no significant difference ($P > 0.1$) between CF15-lacZ and CF15-CFTR cells during treatments with flagellin, flagellin + ATP, flagellin + forskolin, or flagellin + ATP + forskolin. There were also no significant differences of flagellin vs. flagellin + forskolin ($P > 0.1$). *B*: CF15, CF15-wtCFTR-GFP, or CF15-ΔF508CFTR-GFP cells expressing NF-κB-luciferase were left untreated or exposed for 4 h to PAK ($10^8$ colony-forming units/ml), flagellin (flag, $10^{-7}$ g/ml), flagellin + ATP (flag + ATP, 100 μM), or flagellin + thapsigargin (flag + tg, 1 μM) on the apical surface. For untreated controls, luciferase was expressed relative to CF15-lacZ. For the other treatments, luciferase was expressed relative to the controls CF15-wtCFTR-GFP or CF15-ΔF508CFTR-GFP. Bars show averages ± SD for 3 experiments each. Statistical comparisons: #response of PAK-, flagellin-, flagellin + ATP-, and flagellin + thapsigargin-treated cells > controls, paired *t*-test, $P < 0.05$ for all comparisons of wtCFTR-GFP vs. ΔF508CFTR and +response of flagellin < flagellin + ATP and flagellin < flagellin + tg, paired *t*-test $P < 0.01$. Luciferase activities were not different for CF15-wtCFTR-GFP vs. CF15-ΔF508CFTR-GFP, $P > 0.06$ for any treatment for all comparisons of wtCFTR-GFP vs. ΔF508CFTR.

Table 3. *Effects of forskolin and thapsigargin on flagellin-activated NF-κB responses in CF15-lacZ and CF15-CFTR and Calu-3 cells*

| Treatment | Expt. No. | Cells | | |
|---|---|---|---|---|
| | | CF15-lacZ | CF15-CFTR | Calu-3 |
| Control | 1 | 1.0 | 1.0 | 1.0 |
| | 2 | 1.0 | 1.0 | 1.0 |
| Flag + Tg | 1 | 18.8 | 15.0 | 9.8 |
| | 2 | 17.4 | 15.5 | 3.4 |
| Flag + Tg + Fsk | 1 | 12.3 | 11.5 | 4.1 |
| | 2 | 13.3 | 11.2 | 2.2 |

Monolayers expressing nuclear factor (NF)-κB-regulated luciferase were exposed to flagellin (Flag, $10^{-7}$ g/ml), thapsigargin (Tg, 0.1 μM), and/or forskolin (Fsk, 1 μM) for 4 h and then assayed. Data are luciferase activities normalized to total cell protein and then expressed relative to controls measured in two paired experiments each on CF15-lacZ, CF15-CFTR, and Calu-3 cells.

As detailed here and elsewhere (48), adenoviruses transduced 70–90% of CF15 cells. Inflammation assays indicated that there were only small effects on NF-κB activity and IL-8 secretion that could be attributed to adenovirus alone. In addition, PA binding and NF-κB and IL-8 responses were equivalent in CF15 and CF15-lacZ cells. These results indicated that adenovirus expression alone had little effect on innate immune defense properties of the cells and was thus a valuable method for making comparisons between CF vs. CFTR-corrected CF epithelia. Expression of wtCFTR-GFP resulted in apical membrane expression, and wtCFTR expression in CF15 cells increased forskolin-stimulated, DPC-inhibitable $Cl^-$ secretion (48). Thus the adenoviral method was a benign approach to expressing CFTR and other genes.

*Similar inflammatory responses and (lack of) ER stress of CF15 and CFTR-corrected cells.* A major conclusion from these studies was that NF-κB-activated luciferase activities and IL-8 secretion were similar for CF15 cells expressing lacZ, wtCFTR, or ΔF508CFTR both in the control state and also during treatment with mulitiple strains of PA or flagellin. IL-8 secretion was also similar in control non-CF vs. CF primary bronchial cells. PA binding was also unaltered by expression of wtCFTR.

It might have been expected that, if there were ER stress resulting from accumulation of ΔF508CFTR, then IREα would have been active in control CF15 cells and also in CF15 cells expressing ΔF508CFTR-GFP. However, CF15 cells exhibited low IRE1α activity that was insignificantly different from that of HeLa cells. Although ΔF508CFTR-GFP was retained in the ER in both CF15 and HeLa cells, whereas wtCFTR-GFP was found in the plasma membrane and at lower levels in the ER, there were only small (<20%) differences in IRE1α activity among these cells. Although there were significant stimulatory effects on IRE1α activity of HeLa cells expressing wtCFTR-GFP and ΔF508CFTR-GFP, these small effects likely resulted from virus-dependent rather than CFTR-dependent effects: IRE1α activity was the same for wtCFTR vs. controls and for wtCFTR-GFP vs. ΔF508CFTR-GFP.

In contrast to the lack of effect of any of the CFTR constructs, thapsigargin elicited typically robust (40-fold) activation of IRE1α, which occurred similarly in controls and cells expressing wt- or ΔF508CFTR. Although it has often been assumed that ER stress contributes to exaggerated inflammatory signaling in CF (57), the lack of effect of ΔF508CFTR or wtCFTR on ER stress was not entirely unexpected: there is only 25% difference in ER retention of ΔF508- vs. wtCFTR (23), and the misfolded portion of ΔF508CFTR is in the cytosol, whereas Bip, which regulates IRE1α (62, 63), is present in the ER lumen and does not interact with CFTR (24). Recent experiments (37) have similarly shown that overexpression of CFTR also has no effect on ER stress of Calu-3 cells. Expression of both CFTR and ΔF508CFTR in vivo will likely occur at lower levels than those induced by the adenoviruses. Thapsigargin activation of IRE1α, which activates NF-κB signaling (63), could explain the elevated NF-κB activity in cells treated with flagellin + thapsigargin.

It might have been expected if ER handling of $Ca^{2+}$ had been altered in CF that baseline $Ca_i$ would have been increased, and responses to ATP would have been altered (28, 58). However, CFTR expression did not alter either baseline $Ca_i$ or responses to ATP. Possible explanations for the differences in the present vs. previous data (58) is use of stable cell lines vs. adenoviral expression or differences in the $Ca_i$ probes fluo 3 (nonratiometric; see Ref. 58) vs. fura 2 (ratiometric; present experiments). The lack of $Ca_i$ responses to flagellin and PA in CF15 (13) and $9HTEo^-$ and $16HBE14o^-$ (25) cells compared with $1HAEo^-$ and 16HBE cells (1, 32, 38) may represent cell type differences. Whatever the explanation for differences, it is clear that increases in $Ca_i$ are not required for airway epithelia to respond to PA or flagellin, although increases in $Ca_i$ amplify responses to flagellin and PA (13).

In addition to there being no effect of CFTR in controlling $Ca_i$ signaling, there were no CFTR-dependent differences in NF-κB responses to flagellin + agonists known to raise $Ca_i$ (ATP and thapsigargin), even under conditions in which CFTR should have been maximally activated (forskolin). An implication of these studies is that any CFTR-dependent effects to depolarize membrane potential (28) were small enough that they did not alter $Ca^{2+}$ entry in the cells.

*Forskolin decreases flagellin-activated NF-κB.* Forskolin reduced NF-κB responses to flagellin + ATP or flagellin + thapsigargin in CF15 and Calu-3 cells, indicating that increases in cAMP and protein kinase A (PKA) reduce inflammatory signaling in airway epithelia. This negative regulation of flagellin- and TLR 5 (65) signaling is consistent with previous experiments in lymphocytes showing that TLR 4-activated signaling was reduced by agonists that activated PKA (32). An implication of these and previous (13) results is that the responses of airway epithelial cells to PA and flagellin (activate TLR 5) will be increased by agonists that raise $Ca_i$ (e.g., purinergic agonists and bradykinin) and decreased by agonists that raise cAMP (e.g., vasoactive intestinal peptide, adrenergic agonists and $PGE_2$, and *P. aeruginosa* exotoxin Y).

*Hyperinflammation in CF?* Our results demonstrated that expression of either CFTR or ΔF508CFTR in CF15 cells had no effects on ER stress, $Ca_i$, or inflammatory responses. We acknowledge that the different results here compared with others who show exaggerated innate immune responses in CF may result from our using a CF cell line that fails to reproduce the complexities of the in vivo phenotype. However, it seems more likely that other technical problems may explain the exaggerated inflammatory responses in previous experiments. For example, it is difficult to appropriately match stable CF and

non-CF primary cells or CF vs. CFTR-corrected cell lines. Ribeiro et al. (41) found persistent changes in morphology and $Ca_i$ signaling in primary CF cells that disappeared after prolonged culture, indicating that these effects resulted from prior exposure of the CF cells to the inflamed CF lung environment rather than to the absence of CFTR. Consistent with this idea and with the findings here, there are now several studies on primary cells (2, 4, 64) showing that, with careful matching, there are only minor CFTR-dependent differences in inflammatory gene expression and cytokine secretion. Different responses between stable CF vs. CFTR-corrected cell lines may result from undetected genetic differences unrelated to CFTR. The apparent proinflammatory effects of 6-day treatment of non-CF cells with CFTR blocker (35) could result from long-term effects of the blocker unrelated to channel function. We have found that the cell-permeant CFTR blocker CFTRinh-172 (35) caused inconsistent increases in NF-κB activation in Calu-3 cells, whereas the cell-impermeant CFTR blocker GlyH101 *decreased* NF-κB activity (Fu and Machen, unpublished observations). It is also difficult to make precise matches of cell growth and confluence in cell lines, both of which can affect bacterial binding (7, 19, 26, 46, 53) and perhaps inflammatory responses.

Overall, our results indicate that exaggerated innate immune responses in CF airways in vivo result not from the epithelial cells being hyperinflammatory due to ER stress and exuberant $Ca_i$ and NF-κB signaling (12) but more likely from the absence of CFTR's $Cl^-$ and $HCO_3^-$ (18, 27, 42) conductance properties and consequent effects to reduce ASL volume and change its composition (22, 58). These changes in ASL volume and composition in CF impair the mucociliary escalator and cough from eliminating PA. According to this view, CF airway epithelia respond appropriately to the large number of accumulated bacteria by producing large amounts of cytokines and recruiting large numbers of neutrophils to the airways.

## ACKNOWLEDGMENTS

We thank Walt Finkbeiner for providing cells; David Raleigh for help with IL-8 experiments; and Sheldon Miller, Jonathan Widdicombe, Horst Fischer, Paul McCray, and Paul Quinton for discussions.

## GRANTS

Research was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01DK-51799 (to T. E. Machen) and Cystic Fibrosis Foundation Grants MACH03, MACH07 (to T. E. Machen); Tobacco-Related Disease Research Program Grants 8DT01660 (to K. Hybiske) and 8IT0052 (to T. E. Machen); and by Cystic Fibrosis Research Inc.

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