

Cystic fibrosis (CF) is a genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. CFTR is a chloride channel that regulates ion transport in multiple epithelial tissues. The absence of functional CFTR leads to the impairment of mucociliary clearance, the first line of defense against inhaled bacteria

P. aeruginosa inhibits endocytic recycling of CFTR in polarized human airway epithelial cells

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The most common mutation in the CFTR gene is F508-CFTR. F508-CFTR is partially functional as a Cl− channel. Thus current research efforts have focused on identification of drugs that restore the presence of CFTR in the apical membrane, the objective of the present study was to determine whether P. aeruginosa affects CFTR-mediated Cl− secretion in polarized human airway epithelial cells. We report herein that a cell-free filtrate of P. aeruginosa reduces CFTR-mediated transepithelial Cl− secretion by inhibiting the endocytic recycling of CFTR and thus the number of WT-CFTR and ΔF508-CFTR Cl− channels in the apical membrane of polarized human airway epithelial cells. These data suggest that chronic infection with P. aeruginosa may interfere with therapeutic strategies aimed at increasing the apical membrane expression of ΔF508-CFTR.

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mediated Cl\(^{-}\) secretion in polarized human airway epithelial cells. We report herein that a cell-free filtrate of \textit{P. aeruginosa} reduced CFTR-mediated transepithelial Cl\(^{-}\) secretion across polarized human airway epithelial cells by inhibiting the endocytic recycling of CFTR and thus the number of wild-type (WT)-CFTR and \(\Delta F508\)-CFTR Cl\(^{-}\) channels in the apical membrane in polarized human airway epithelial cells. These data indicate that therapeutic strategies based on the restoration of Cl\(^{-}\) transport in the CF airway may be compromised by chronic infection with \textit{P. aeruginosa}.

**MATERIALS AND METHODS**

**Cell lines and cell culture.** Human airway epithelial cells (Calu-3) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in MEM containing 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, 2 \(\mu\)M t-glutamine, 1 \(\mu\)M sodium pyruvate, and 10% FBS in a 5% CO\(_2\)–95% air incubator at 37°C as described previously (44). To establish polarized monolayers, Calu-3 cells were seeded onto Transwell permeable supports (0.4-\(\mu\)m pore size, 1 \(\times\) 10\(^{6}\)/24-mm diameter; Corning, Corning, NY) coated with Vitrogen plating medium containing DMEM (JRH Biosciences, Lenexa, KS), human fibronectin (10 \(\mu\)g/ml; Collaborative Biomedical Products, Bedford, MA), 1% Vitrogen 100 (Collagen, Palo Alto, CA), and BSA (10 \(\mu\)g/ml; Sigma-Aldrich, St. Louis, MO) (44). Cells were grown in an air-liquid interface culture at 37°C for 14–21 days. Parental human bronchial epithelial CFBE410– cells (\(\Delta F508\)/\(\Delta F508\)), originally immortalized and characterized by Gruenert and colleagues (7, 12), were stably transduced with either WT-CFTR or \(\Delta F508\)-CFTR (generous gift from Dr. J. P. Clancy, Dept. of Pediatrics, University of Alabama at Birmingham, Birmingham, AL; Ref. 3). CFBE410– cells were maintained in MEM supplemented with 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, 2 \(\mu\)M t-glutamine, 10% FBS, and 1 \(\mu\)g/ml blasticidin (WT-CFTR) or 2 \(\mu\)g/ml puromycin (\(\Delta F508\)/CFTR) in a 5% CO\(_2\)-95% air incubator at 37°C. To establish polarized monolayers, 1 \(\times\) 10\(^{6}\) CFBE410– cells were seeded onto 12-mm Snapwell or 24-mm Transwell permeable supports (0.4-\(\mu\)m-pore size; Corning) and grown in an air-liquid interface culture at 37°C for 6–9 days and at 27°C for 36 h to increase trafficking and expression of \(\Delta F508\)-CFTR in the apical membrane (14, 19, 22). Madin-Darby canine kidney (MDCK) cells stably expressing green fluorescent protein (GFP)-WT-CFTR or GFP-\(\Delta F508\)-CFTR fusion protein were established and maintained in culture in a 5% CO\(_2\)-95% air incubator at 37°C in MEM complete medium containing 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, 2 \(\mu\)M t-glutamine, 10% FBS, and 150 \(\mu\)g/ml G418 as described previously (50, 51). Addition of GFP to the NH\(_2\) terminus of CFTR had no effect on CFTR localization, trafficking, and function as a Cl\(^{-}\) channel or on CFTR degradation (51). MDCK cells were seeded onto Transwell permeable supports (0.2 \(\times\) 10\(^{6}\) cells on 6.5-mm- or 24-mm-diameter supports with 0.4-\(\mu\)m-pore size) and grown in culture at 37°C for 7–10 days as polarized monolayers. Sodium butyrate (5 mM; Sigma-Aldrich) was added to the MDCK cell culture medium 16–18 h before experiments to stimulate CFTR expression (52).

\textit{P. aeruginosa} cultures. Lysogeny broth was inoculated with \textit{P. aeruginosa} strain UCBPP-PA14 (PA14), a relatively recent laboratory strain isolated from a burn patient (59), from a glycerol stock and incubated at 37°C using rotation until the bacterial count reached optical density (OD)\(_{600}\) of 1.5, corresponding to a bacterial count of \(5 \times 10^{8}\) colony-forming units (CFU)/ml (16–18 h of culture). Bacteria were harvested by centrifuging cultures at 4,800 \(\times\) g for 10 min at 4°C. After being washed with PBS at pH 7.4 and 4°C to eliminate products secreted into the extracellular environment, bacteria were resuspended in PBS to a stock concentration of \(5 \times 10^{8}\) CFU/ml. In addition, \textit{P. aeruginosa} strain PA01 (67) was cultured as described above. Heat-killed bacteria were prepared by incubating the PBS-resuspended cultures at 95°C for 10 min.

**Bacteria-free \textit{P. aeruginosa} filtrates.** Bacterial cultures grown as described above were centrifuged at 4,800 \(\times\) g for 10 min at 4°C. Supernatants were harvested and filter sterilized at 4°C using a Sterilfiltr 0.2-\(\mu\)m filter apparatus (Millipore, Bedford, MA), which resulted in bacteria-free filtrates. For Ussing chamber experiments, the bacteria-free filtrates were concentrated 10\(^{\times}\) at 4°C using a Centrifloc filter device with a 30-kDa molecular mass cutoff (YM30; Millipore) to minimize the volume of lysogeny broth (filtrate vehicle) used during the experiment. In addition, heat-inactivated filtrates were prepared using incubation at 60°C for 45 min.

**Ussing chamber measurements.** Monolayers grown on 6.5-mm-diameter Transwell or 12-mm-diameter Snapwell permeable supports as described above were mounted in an Ussing-type chamber (Jim’s Instruments, Iowa City, IA, or Physiologic Instruments, San Diego, CA) and bathed in solutions at pH 7.4 that were maintained at 37°C and stirred using bubbling with 5% CO\(_2\)-95% air. Short-circuit current (\(I_{sc}\)) was measured by clamping the transepithelial voltage across the monolayers to 0 mV using a voltage clamp (model VCC MC6; Physiologic Instruments) as described previously (29, 44, 53). Current output from the clamp was digitized using an analog-to-digital converter (iWorx, Dover, NH). Data collection and analysis were performed using LabScribe version 1.6 software (iWorx).

\(I_{sc}\) was stimulated with 100 \(\mu\)M 8-(4-chlorophenylthio) (CPT)-AMP in Calu-3 and MDCK cells or 20 \(\mu\)M forskolin in WT-CFTR CFBE410– cells added to the apical and basolateral bath solution or with 50 \(\mu\)M genistein in \(\Delta F508\)-CFTR CFBE410– cells added only to the apical bath solution. Different activation protocols were used to stimulate \(I_{sc}\) in CFBE410– cells expressing WT-CFTR and \(\Delta F508\)-CFTR because of the different activation profiles of WT-CFTR and the temperature-rescued \(\Delta F508\)-CFTR in these cell lines (3). To determine the effects of \textit{P. aeruginosa} on the CFTR-mediated Cl\(^{-}\) currents, 5 \(\times\) 10\(^{6}\) or 5 \(\times\) 10\(^{7}\) CFU/ml of the appropriate strain of washed (PBS resuspended) \textit{P. aeruginosa} was added to the apical side of the monolayers and incubated at 37°C in a CO\(_2\) incubator. After incubation, the baseline and peak stimulated \(I_{sc}\) were measured. Data are expressed as net stimulated \(I_{sc}\), which was calculated by subtracting the baseline \(I_{sc}\) from the peak stimulated \(I_{sc}\). To determine the effects of the bacteria-free PA14 filtrate on CFTR-mediated Cl\(^{-}\)secretion, the monolayers were bathed in sterile bath solution and \(I_{sc}\) was stimulated with CPT-cAMP. The 10\(^{\times}\) concentrated PA14 filtrate was diluted 1:10 by addition to the apical bath solution, and the change in stimulated \(I_{sc}\) was measured. Data are expressed as the change in stimulated \(I_{sc}\) after addition of filtrate to the apical bath solution. Glibenclamide (200 \(\mu\)M) was added to the apical bath solution to inhibit CFTR-mediated \(I_{sc}\).

Intact Calu-3 monolayers were bathed in MEM (−FBS). To measure CFTR-mediated Cl\(^{-}\) currents across the apical membrane in Calu-3 cells, basolateral membranes were permeabilized with nystatin (200 \(\mu\)g/ml), and an apical-to-basolateral Cl\(^{-}\) concentration gradient was established (16, 26). A low-C\(^{1+}\), high-Na\(^{+}\), high-glucuronate basolateral bath solution was used to prevent cell swelling due to the increased basolateral Cl\(^{-}\) permeability under these conditions as described previously (15, 16, 26). The basolateral bath solution contained (in mM) 115 Na\(^{+}\)-glucuronate, 5 NaCl, 25 NaHCO\(_3\), 3.3 KH\(_2\)PO\(_4\), 0.8 K\(_2\)HPO\(_4\), 1.2 MgCl\(_2\), 1.2 CaCl\(_2\), and 10 glucose. The apical bath solution contained (in mM) 120 NaCl, 25 NaHCO\(_3\), 3.3 KH\(_2\)PO\(_4\), 0.8 K\(_2\)HPO\(_4\), 1.2 MgCl\(_2\), 1.2 CaCl\(_2\), and 10 mannitol (44). Mannitol was substituted for glucose in the apical bath solution to eliminate the contribution of the Na\(^{+}\)-glucose cotransporter to \(I_{sc}\) as described previously (16). Successful permeabilization of the basolateral membrane was based on the recording of a current consistent with apical-to-basolateral flow of negative charge (16). CFBE410– cells were bathed in solutions with apical-to-basolateral Cl\(^{-}\) gradient in the presence of amiloride (100 \(\mu\)M) in the apical bath solution to inhibit Na\(^{+}\) absorption through ENaC (29). The apical bath solution...
Determination of CFTR expression in the apical plasma membrane. To determine the effect of *P. aeruginosa* on the apical membrane expression of CFTR, polarized epithelial cells grown on 24-mm-diameter Transwell permeable supports were incubated at 37°C in the presence or absence of membrane-bound aldehyde fixative. After being incubated, the apical membrane proteins were selectively biotinylated using sulfo-succinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) or sulfo-succinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin) (EZ-Link; Pierce Biotechnology, Rockford, IL) and isolated using streptavidin agarose beads, and the apical membrane CFTR was detected using Western blot analysis as described previously (69). Determination of the apical membrane expression of GFP was performed by biotinylating the appropriate plasma membrane MAb (Upstate Biotechnology, Lake Placid, NY); and goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA). All purchased antibodies were used at the concentrations recommended by the respective manufacturers.

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Determination of CFTR internalization from the apical plasma membrane. To determine whether *P. aeruginosa* caused internalization of CFTR from the apical membrane, studies were conducted as described previously (69). Briefly, apical membrane proteins in polarized MDCK cells were first biotinylated at 4°C using EZ-Link sulfo-NHS-SS-biotin. Subsequently, polarized cells were incubated with warm (37°C) PA14 filtrate added to the apical medium, and the disulfide bonds on sulfo-NHS-SS-biotinylated proteins remaining in the apical membrane were reduced using GSH added to the apical medium. At this point, only proteins internalized from the apical membrane remained biotinylated.

Endocytic assay. Studies were conducted to determine whether PA14 decreased the apical membrane expression of CFTR by inhibiting the recycling of CFTR from endosomes to the apical membrane, according to a method described in detail previously (69). Briefly, apical membrane proteins were biotinylated using EZ-Link sulfo-NHS-SS-biotin at 4°C, and the endocytic vesicles were loaded with biotinylated proteins by incubation with warm vehicle (37°C lysogeny broth). Cells were cooled immediately to 4°C, and the disulfide bonds on sulfo-NHS-SS-biotinylated proteins in the apical membranes were reduced using GSH. Subsequently, cells were either lysed or incubated at 37°C with warm (37°C) vehicle or PA14 filtrate to allow internalized, biotinylated proteins to recycle to the apical membrane. Cells were then immediately cooled again to 4°C, and the disulfide bonds on sulfo-NHS-SS-biotinylated proteins in the apical membranes were reduced using GSH. Recycling of endocytosed CFTR was calculated as the difference between the amount of biotinylated CFTR after the first and second GSH treatments.

RESULTS

*P. aeruginosa* inhibits CFTR-mediated Cl<sup>-</sup> secretion in Calu-3 human airway epithelial cells. Studies were conducted to examine the effects of PA14, a relatively recent laboratory isolate (59), on electrogenic Cl<sup>-</sup> secretion across polarized human airway epithelial cells endogenously expressing WT-CFTR. Harvested bacteria were washed with PBS to eliminate products secreted into the extracellular environment and subsequently resuspended in PBS at a stock concentration of 5 × 10<sup>8</sup> CFU/ml. Polarized (filter grown) Calu-3 cells were incubated at 37°C in a CO<sub>2</sub> incubator in the absence of antibiotics. Washed (PBS resuspended) PA14 bacteria (5 × 10<sup>7</sup> CFU/ml) were added to the apical side of the monolayers, and I<sub>sc</sub> was measured as described in MATERIALS AND METHODS. PA14 reduced the glibenclamide-sensitive, CPT-CAMP-stimulated I<sub>sc</sub> after 4–6 h (Fig. 1, A and B). Killing PA14 by incubation at 95°C for 10 min resulted in loss of this activity (Fig. 1, A and C). The effect of PA14 was not observed after exposing the apical side of the monolayers to PA14 for <3 h (data not shown). Additional studies were conducted to determine the effects of another strain of *P. aeruginosa*, PA01, which has been passaged in the laboratory for many years. Thus the gene expression profiles and/or genomic content of PA01 and those of PA14, a relatively recent laboratory isolate, are likely to differ (27, 43, 75). Neither live nor heat-killed PA01 significantly decreased the glibenclamide-sensitive CPT-CAMP-stimulated I<sub>sc</sub> (data not shown). Additional studies were conducted to determine the effects of another strain of *P. aeruginosa*, PA01, which has been passaged in the laboratory for many years. Thus the gene expression profiles and/or genomic content of PA01 and those of PA14, a relatively recent laboratory isolate, are likely to differ (27, 43, 75). Neither live nor heat-killed PA01 significantly decreased the glibenclamide-sensitive CPT-CAMP-stimulated I<sub>sc</sub> (data not shown). Additional studies were conducted to determine the effects of another strain of *P. aeruginosa*, PA01, which has been passaged in the laboratory for many years. Thus the gene expression profiles and/or genomic content of PA01 and those of PA14, a relatively recent laboratory isolate, are likely to differ (27, 43, 75). Neither live nor heat-killed PA01 significantly decreased the glibenclamide-sensitive CPT-CAMP-stimulated I<sub>sc</sub> (data not shown). Additional studies were conducted to determine the effects of another strain of *P. aeruginosa*, PA01, which has been passaged in the laboratory for many years. Thus the gene expression profiles and/or genomic content of PA01 and those of PA14, a relatively recent laboratory isolate, are likely to differ (27, 43, 75). Neither live nor heat-killed PA01 significantly decreased the glibenclamide-sensitive CPT-CAMP-stimulated I<sub>sc</sub> (data not shown). Additional studies were conducted to determine the effects of another strain of *P. aeruginosa*, PA01, which has been passaged in the laboratory for many years. Thus the gene expression profiles and/or genomic content of PA01 and those of PA14, a relatively recent laboratory isolate, are likely to differ (27, 43, 75).
cell monolayers (25, 68). Thus studies were conducted to determine whether PA14 altered $R_b$. Calu-3 cells were incubated with washed PA14 bacteria for 6 h as described above, and $R_j$ was measured as described in MATERIALS AND METHODS. As shown in Fig. 2B, $R_j$ did not differ in the vehicle-treated and PA14-treated Calu-3 monolayers. These data confirm the findings of another study that demonstrated that addition of *P. aeruginosa* to the apical solution of Calu-3 monolayers at concentrations similar to those used in our study did not affect $R_b$ for at least 6 h (41). Together, our data indicate that a relatively recent laboratory isolate of *P. aeruginosa*, strain PA14, inhibits CFTR-mediated Cl⁻ secretion across the apical membrane of polarized human airway epithelial cells endogenously expressing WT-CFTR.

*P. aeruginosa* inhibits transepithelial Cl⁻ secretion in WT-CFTR- and ΔF508-CFTR-expressing human airway epithelial cells (CFBE410−). Studies were conducted to confirm that *P. aeruginosa* decreases transepithelial Cl⁻ secretion in polarized human airway epithelial cells. To this end, studies were conducted in CFBE410− cells stably expressing either WT-CFTR or ΔF508-CFTR. To increase trafficking and the apical membrane expression of ΔF508-CFTR, cells were grown for 36 h at 27°C, a temperature that, at least for some cells, increases the expression of ΔF508-CFTR in the plasma membrane (14, 19, 22). To control for any possible effects of reduced temperature on the results, WT-CFTR-expressing cells were also grown at 27°C for 36 h. CFBE410− cells stably expressing either WT-CFTR or ΔF508-CFTR were incubated at 27°C in a CO₂ incubator in the absence of antibiotics. Washed PA14 bacteria (5 × 10⁷ CFU/ml) were added to the apical side of the monolayers. PA14 inhibited the glibenclamide-sensitive, forskolin-stimulated $I_{sc}$ in CFBE410− cells stably expressing...
WT-CFTR after 4–6 h of incubation (Fig. 3A). The effect was not observed after exposing the apical side of the monolayers to PA14 for <3 h. To determine whether the inhibition of $I_{sc}$ was reversible, CFBE410− cells stably expressing WT-CFTR were first incubated with washed PA14 bacteria as described above. After 6 h of incubation, the monolayers were washed and subsequently incubated at 37°C in a CO2 incubator with sterile medium containing antibiotics. As shown in Fig. 3B, 1 h after washing the bacteria from the apical side of the CFBE410− monolayers, the glibenclamide-sensitive, forskolin-stimulated $I_{sc}$ partially recovered to control values. As shown in Fig. 3C, PA14 also inhibited the glibenclamide-sensitive, genistein-stimulated $I_{sc}$ in CFBE410− cells stably expressing rescued ΔF508-CFTR after 4–6 h of incubation (Fig. 3C). The effect was not observed after exposing the apical side of the monolayers to PA14 for <3 h. One hour after washing the bacteria from the apical side of the CFBE410− monolayers, the glibenclamide-sensitive, genistein-stimulated $I_{sc}$ partially recovered to control values (Fig. 3D).

To determine whether the effects of P. aeruginosa on WT-CFTR and ΔF508-CFTR were limited to human airway epithelial cells, we examined the effects of P. aeruginosa on CFTR-mediated Cl− secretion across polarized MDCK cells stably expressing either WT-CFTR or ΔF508-CFTR. As demonstrated in Fig. 4, PA14 (after 4–6 h incubation at 37°C with $5 \times 10^6$ CFU/ml washed PA14 bacteria added to the apical side of the monolayers) had no effect on parental MDCK cells, which do not express CFTR, but inhibited CPT-cAMP-stimulated $I_{sc}$ in the WT-CFTR- and ΔF508-CFTR-expressing cells. Together, these data demonstrate that P. aeruginosa inhibits CFTR-mediated Cl− secretion in polarized human airway epithelial cells and in polarized kidney epithelial cells stably expressing WT-CFTR or ΔF508-CFTR.

P. aeruginosa inhibits expression of CFTR in the apical membrane. P. aeruginosa could inhibit CFTR-mediated Cl− secretion across the apical plasma membrane by 1) inactivating CFTR Cl− channels in the membrane and/or 2) decreasing the apical membrane expression of CFTR. To determine whether P. aeruginosa decreased the expression of CFTR in the apical plasma membrane, polarized MDCK cells were incubated with washed PA14 or PA01 bacteria ($5 \times 10^6$ CFU/ml added to the apical medium in the absence of antibiotics) and the apical membrane expression of CFTR was measured using cell surface biotinylation. PA14 decreased the apical membrane expression of CFTR after 4–6 h of incubation (Figs. 5A and 4B and Table 1). In contrast, neither heat-killed PA14 nor live PA01 had a significant effect on the apical membrane expression of CFTR (Figs. 5A and 4B). The effect of PA14 was not observed after exposing the apical side of the monolayers to PA14 for <3 h. As shown in Fig. 5C, PA14 had no effect on the apical membrane expression of gp114, the basolateral membrane expression of Na+/K+-ATPase and the transferrin receptor, or the expression of ezrin in cell lysates. The expression of ΔF508-CFTR in the apical membrane of MDCK cells was too low to examine using cell surface biotinylation; thus the effect of PA14 on the apical membrane expression of ΔF508-CFTR was not examined.

![Figure 3](http://apc.cell.physiology.org/)

**Fig. 3.** Summary of Ussing chamber studies performed to determine the effects of P. aeruginosa on Cl− secretion in CFBE410− cells stably expressing either wild-type (WT)-CFTR (A and B) or ΔF508-CFTR (C and D). Expression of ΔF508-CFTR in the apical membrane was increased by reducing the temperature in the chambers (27°C) as described in MATERIALS AND METHODS. Cells were incubated with PA14 added to the apical side of the monolayer. A: PA14 decreased the glibenclamide-sensitive, forskolin-stimulated $I_{sc}$ across the CFBE410− monolayer stably expressing WT-CFTR after 4–6 h of incubation. B: to determine whether the inhibition of stimulated $I_{sc}$ was reversible in the WT-CFTR-expressing CFBE410− cells, the monolayers were washed after 6 h of incubation with PA14 and subsequently incubated at 37°C with sterile medium containing antibiotics for 1 h (PA14+Wash). The glibenclamide-sensitive, forskolin-stimulated $I_{sc}$ was measured and net stimulated $I_{sc}$ was calculated as described in MATERIALS AND METHODS. C: PA14 also decreased the glibenclamide-sensitive, genistein-stimulated $I_{sc}$ across the CFBE410− monolayer stably expressing ΔF508-CFTR after 4–6 h of incubation. D: to determine whether inhibition of stimulated $I_{sc}$ was reversible in ΔF508-CFTR-expressing CFBE410− cells, the monolayers were washed after 6 h of incubation with PA14 and subsequently incubated at 37°C with sterile medium containing antibiotics for 1 h (PA14+Wash). The glibenclamide-sensitive, genistein-stimulated $I_{sc}$ was measured and the net stimulated $I_{sc}$ was calculated as described in MATERIALS AND METHODS. n = 6–9 experiments/group; *P < 0.05.

![Figure 4](http://apc.cell.physiology.org/)

**Fig. 4.** Summary of Ussing chamber studies performed to determine the effects of P. aeruginosa on Cl− secretion in MDCK cells stably expressing either WT-CFTR or ΔF508-CFTR or parental (nontransfected) cells. Expression of ΔF508-CFTR was induced with sodium butyrate as described in MATERIALS AND METHODS. Cells were incubated with PA14 added to the apical side of the monolayers. PA14 reduced the glibenclamide-sensitive, CPT-cAMP-stimulated $I_{sc}$ partially recovered to control values (Fig. 3D).
ΔF508-CFTR could not be measured in this cell line. As shown in Table 1, PA14 also decreased the apical plasma membrane expression of WT-CFTR in Calu-3 and CFBE410− cells and decreased the expression of temperature-rescued ΔF508-CFTR in CFBE410− cells. These data indicate that *P. aeruginosa* inhibits CFTR-mediated Cl− secretion at least in part by decreasing the expression of CFTR Cl− channels in the apical membrane.

Bacteria-free filtrate of *P. aeruginosa* inhibits apical membrane expression of CFTR. *P. aeruginosa* could exert its inhibitory effect on the apical membrane expression of CFTR either directly by binding to epithelial cells and/or to CFTR or indirectly via products secreted into the extracellular medium. To discriminate between these possibilities, studies were conducted to test the effect of the bacteria-free PA14 filtrate on the apical membrane expression of CFTR. Secretion of many bacterial factors into the extracellular environment increases with the age of bacterial culture and is highest during the late stationary phase. Thus it can be predicted that if a secreted product or products inhibited the apical membrane expression of CFTR, its concentration would be highest, and therefore its effect would be strongest, in bacterial filtrates prepared from stationary phase PA14 cultures. To test this prediction, cell-free PA14 filtrates harvested from the lag, log, and stationary phase cultures were added to the apical side of polarized MDCK monolayers and the apical membrane expression of CFTR was measured using cell surface biotinylation as described in MATERIALS AND METHODS. The small inhibitory effects of the lag and log phase filtrates on the apical membrane expression of CFTR dramatically increased in the stationary phase filtrates after only 10-min incubation with MDCK cells (Fig. 6A) and were independent of the filtrate’s total protein content (data not shown). These data indicate that a product or products secreted predominantly during the stationary phase rapidly (within minutes) inhibit the apical membrane expression of CFTR. Thus, in the experiments described below, the cell-free PA14 filtrate was harvested from PA14 cultures grown for 16–18 h, corresponding to the late stationary phase. Heating the filtrate at 60°C for 45 min resulted in loss of its inhibitory effect on the apical membrane expression of CFTR observed with sterile medium at 37°C for 10 min before being biotinylated. Disappearance of CFTR from the apical membrane during incubation with PA14 filtrate was followed by partial recovery of CFTR expression in the apical membrane observed 10 min after washing off the PA14 filtrate (Fig. 7A). These results strongly suggest that PA14 may affect trafficking of CFTR in the apical membrane. To determine whether *P. aeruginosa* caused internalization of CFTR from the apical membrane, the apical membrane proteins in polarized MDCK

Table 1. Effects of the *P. aeruginosa* strain PA14 and the bacteria-free PA14F on apical membrane expression of CFTR in different polarized epithelial cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PA14</th>
<th>PA14F</th>
</tr>
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<tbody>
<tr>
<td>WT-CFTR in MDCK</td>
<td>10.0±4.8*</td>
<td>15.0±4.5*</td>
</tr>
<tr>
<td>WT-CFTR in Calu-3</td>
<td>19.0±6.6*</td>
<td>7.5±5.0*</td>
</tr>
<tr>
<td>WT-CFTR in CFBE410−</td>
<td>30.5±9.5*</td>
<td>29.2±13.9*</td>
</tr>
<tr>
<td>ΔF508-CFTR in CFBE410−</td>
<td>54.8±6.9*</td>
<td>47.4±7.1*</td>
</tr>
</tbody>
</table>

Data are expressed as %vehicle control (means ± SE). PA14F, PA14 filtrate. Cells were incubated with PA14 (4–6 h) or PA14 filtrate (10 min) added to the apical side of the monolayers. n = 6–12 experiments/group; *P < 0.05.
cells were first biotinylated, and subsequently PA14 filtrate was added to the apical medium as described in MATERIALS AND METHODS. As shown in Fig. 7B, incubation with PA14 filtrate resulted in internalization of the apical membrane CFTR.

Additional studies were conducted to determine the effects of PA14 filtrate on CFTR mediated Cl− secretion. Because PA14 filtrate caused rapid internalization of CFTR from the apical membrane, we performed studies designed to monitor the acute effect of PA14 filtrate on the CPT-cAMP-stimulated \( I_{sc} \) as described in MATERIALS AND METHODS. Polarized Calu-3 cells were bathed in a sterile bath solution, and \( I_{sc} \) was stimulated with CPT-cAMP. Subsequently, 10× concentrated PA14 filtrate prepared to minimize the volume of lysogeny broth (filtrate vehicle) used during the experiment was diluted 1:10 by being added to the apical bath solution as described in MATERIALS AND METHODS. PA14 filtrate reduced the glibenclamide-sensitive, CPT-cAMP-stimulated \( I_{sc} \) in 10 min (Fig. 8). It is important to note that vehicle alone increased the CPT-cAMP-stimulated \( I_{sc} \) under the same experimental conditions. After heat inactivation, PA14 filtrate lost its inhibitory effect and behaved similarly to vehicle, causing an increase in CPT-cAMP-stimulated \( I_{sc} \) (Fig. 8). Together these data are consistent with the view that \( P. \ aeruginosa \) secretes a heat-sensitive product or products that reduce the number of CFTR Cl− channels in the apical membrane by accelerating CFTR internalization.

**Bacteria-free \( P. \ aeruginosa \) filtrate does not increase CFTR endocytosis.** Endocytosis and endocytic recycling determine, at least in part, the expression of CFTR in the apical membrane (31, 42, 47). Thus \( P. \ aeruginosa \) could accelerate internalization and reduce the apical membrane expression of CFTR either by stimulating endocytosis of CFTR from the apical membrane or by inhibiting endocytic recycling of CFTR back to the apical membrane or both. We have determined the effect of the PA14 filtrate on CFTR endocytosis as described in MATERIALS AND METHODS. As shown in Fig. 9A, CFTR endocytosis was linear between 0 and 3 min in vehicle-treated cells; thus the data reported are from the 3-min time point. The PA14 filtrate did not increase CFTR endocytosis (Fig. 9, B and C).
endocytic recycling of CFTR was linear between 0 and 3 min in cells treated with vehicle; thus the data reported are from the 3-min time point. The PA14 filtrate decreased endocytic recycling of CFTR (Fig. 10, B and C). Thus *P. aeruginosa* reduces the apical membrane expression of CFTR by rapidly inhibiting the recycling of CFTR from an endosomal pool back to the apical plasma membrane.

**DISCUSSION**

The major observation in this study is that *P. aeruginosa* secretes a heat-sensitive product that reduces CFTR-mediated transepithelial Cl\(^{-}\) secretion across polarized human airway epithelial cells by inhibiting the endocytic recycling of CFTR and thus the number of WT-CFTR and ΔF508-CFTR Cl\(^{-}\) channels in the apical membrane in polarized human airway epithelial cells.

It is generally accepted that the major effect of *P. aeruginosa* on airway epithelial cells is mediated by an interaction with the basolateral membrane. For example, *P. aeruginosa* access to the basolateral membrane as a result of epithelial injury and/or incomplete polarization leads to increased bacterial binding, invasion, activation of proinflammatory effectors, and cytotoxicity (21, 35, 41). However, compelling evidence indicates that the interaction of *P. aeruginosa* with the apical membrane of polarized human airway epithelial cells also activates signaling pathways (1, 33, 34, 57). For example, the addition of *P. aeruginosa* to the apical surface of polarized human airway epithelial cells elicits specific changes in gene expression (33). Because the inhibition of CFTR-mediated Cl\(^{-}\) secretion was at least partially reversible and was not associated with a change in *R\(_{p}\)* it is reasonable to conclude that the effects of *P. aeruginosa* on CFTR did not result from an injury to the monolayers. Thus, emerging data, including our observations in the present study, indicate that *P. aeruginosa* affects the human airway epithelial cell function even before an established endobronchial injury. To our knowledge, we have demonstrated for the first time in the present study that *P. aeruginosa* added to the apical side, which is the side of the airway epithelium initially exposed in vivo to pathogens (2,
Fig. 10. Endocytic recycling assays were performed to determine the mechanism by which PA14 filtrate decreased expression of CFTR in the apical membrane. The apical membrane proteins in polarized MDCK cells stably expressing WT-CFTR were biotinylated at 4°C. Because the endocytosis of CFTR increased linearly until 3 min (see Fig. 9), the endocytic vesicles were loaded with biotinylated proteins by being incubated with warm vehicle (37°C lysogeny broth) for 3 min. Cells were then cooled to 4°C, and the disulfide bonds on sulfo-NHS-sulfo-NHS-biotinylated proteins in the apical membranes were reduced using GSH. Subsequently, cells were either lysed or incubated with warm (37°C) vehicle or PA14 filtrate to allow internalized biotinylated proteins to recycle to the apical membrane. Cells were then cooled again to 4°C, and the disulfide bond on sulfo-NHS-SH-sulfo-NHS-biotinylated proteins in the apical membranes was reduced using GSH. Recycling of endocytosed CFTR was calculated as the difference between the amount of biotinylated CFTR after the first and second GSH treatments. As demonstrated by the representative Western blot image shown in A, CFTR recycling was linear between 0 and 3 min in the vehicle-treated cells. Thus data reported are from the 3-min time point. Representative Western blot image shown in B and summary of the results of these studies (C) demonstrate that PA14 filtrate inhibited the endocytic recycling of CFTR. n = 3 experiments/group; *P < 0.05.

58), decreases transepithelial Cl⁻ secretion by inhibiting the endocytic recycling of CFTR. However, we cannot rule out the possibility that PA14 affects the biophysical properties of CFTR in addition to its effect on CFTR trafficking.

Studies have demonstrated that P. aeruginosa uses asialo-GM₁ for binding (34, 39) and CFTR for ingestion by epithelial cells (57). Data reported herein describe another mechanism of interaction between P. aeruginosa and epithelial cells, which, unlike the first two mechanisms, does not require a direct contact of the bacterium with epithelial cells and/or CFTR. Studies have demonstrated that P. aeruginosa also affects amiloride-sensitive Na⁺ transport in polarized epithelial cells. However, it is unlikely that the effects on CFTR-mediated Cl⁻ transport and amiloride-sensitive Na⁺ transport could be mediated by the same factors, because inhibition of Na⁺ transport is mediated by heat-stable hemolysin (68) and a nonsecreted product (20). In addition, it is unlikely that rhamnolipids could mediate the effect on CFTR, because rhamnolipids inhibit Iₘ by affecting Na⁺ rather than Cl⁻ transport and are secreted by PA01 (25), a strain that has no effect on CFTR-mediated Cl⁻ transport.

The effect of P. aeruginosa on CFTR-mediated Cl⁻ secretion across polarized human airway epithelial cells in the present study may be clinically relevant. Strain PA14 as well as clinical isolates of P. aeruginosa (6 isolated from patients with CF and 6 isolated from patients without CF; Swiatecka-Urban A, Su JR, and Stanton BA, unpublished observations) decreased CFTR-mediated Cl⁻ transport in polarized human airway epithelial cells at concentrations comparable to those in patients with CF (13, 63, 72) and in severely ill patients without CF (56). Thus our study suggests that similar inhibition of Cl⁻ transport may occur in patients infected with P. aeruginosa. In patients without CF, the decline in CFTR-mediated Cl⁻ secretion may be transient. In patients with CF, chronic infection with P. aeruginosa may compromise future therapy designed to restore CFTR-mediated Cl⁻ transport and mucociliary clearance. Because such therapy may not work in patients with established bacterial colonization and/or chronic pulmonary infection, treatment may have to be initiated before the onset of airway bacterial colonization. Alternatively, combined therapy may require the inhibition of the secreted product or products that accelerate internalization of CFTR from the apical membrane as well as the following: 1) promotion of ER exit and plasma membrane expression of ΔF508-CFTR, 2) activation of ΔF508-CFTR in the apical plasma membrane, and 3) increase in the half-life of ΔF508-CFTR in the apical plasma membrane.

In summary, our data provide the first direct evidence that P. aeruginosa inhibits CFTR-mediated Cl⁻ secretion across polarized human airway epithelial cells expressing either WT-CFTR or rescued ΔF508-CFTR by specifically inhibiting the endocytic recycling of CFTR Cl⁻ channels. Inhibition of this effect may be necessary to allow pharmacological restoration of CFTR-mediated Cl⁻ transport in the airways of patients with CF.

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