Much of the pulmonary disease in cystic fibrosis is associated with polymorphonuclear leukocyte-dominated airway inflammation caused by bacterial infection. Respiratory epithelial cells express the polymorphonuclear chemokine interleukin-8 (IL-8) in response to ligation of asialylated glycolipid receptors, which are increased on damaged or regenerating cells and those with cystic fibrosis transmembrane conductance regulator mutations. Because both Pseudomonas aeruginosa and Staphylococcus aureus, the most common pathogens in cystic fibrosis, bind asialylated glycolipid receptors such as asialoGM1, we postulated that diverse bacteria can activate a common epithelial signaling pathway to elicit IL-8 expression. P. aeruginosa PA01 but not pil mutants and S. aureus RN6930 but not the agr mutant RN6911 stimulated increases in [Ca\(^{2+}\)], in 1HAEo− airway epithelial cells. This response stimulated p38 and ERK1/2 mitogen-activated protein kinase (MAPK) signaling cascades resulting in NF-κB activation and IL-8 expression. Ligation of the asialoGM1 receptor or thapsigargin-elicited Ca\(^{2+}\) release activated this pathway, whereas P. aeruginosa lipopolysaccharide did not. The rapid kinetics of epithelial activation precluded bacterial invasion of the epithelium. Recognition of asialylated glycolipid receptors on airway epithelial cells provides a common pathway for Gram-positive and Gram-negative organisms to initiate an epithelial inflammatory response.

The pathogenesis of pulmonary infection in cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, has been a topic of great interest. Early in the disease process CF patients often have intermittent airway infection because of Staphylococcus aureus, followed by Pseudomonas aeruginosa infection, which eventually becomes chronic. These infections are associated with an excessive inflammatory response, characterized by the accumulation of large amounts of the polymorphonuclear leukocyte (PMN) chemokine IL-8, as well as PMNs and their toxic products in the airways (1). Failure to clear inhaled pathogens, because of a combination of factors such as diminished defensin activity (2), obstruction because of inspissated mucus (3), or other cystic fibrosis transmembrane conductance regulator-associated defects (4), results in a large bacterial burden. Cells with cystic fibrosis transmembrane conductance regulator mutations, as well as damaged or regenerating epithelial cells, express increased numbers of asialylated glycolipids such as asialoGM1 (5, 6) with a GalNAcβ1–4Gal moiety that acts as a receptor for many pulmonary pathogens including S. aureus and P. aeruginosa (7, 8). The binding of asialoGM1 on airway epithelial cells by P. aeruginosa stimulates nuclear translocation of NF-κB and activates transcription of IL-8 (9), the major airway chemokine in the lung for PMNs, initiating the epithelial immune response to this pathogen. This response can be stimulated by intact P. aeruginosa, the isolated P. aeruginosa adhesin type IV pilin, or an antibody to the pilin receptor asialoGM1 (9, 10).

The molecular mechanisms involved in bacterial induction of epithelial cell signaling have been examined for several mucosal pathogens. Pathogenic Neisseria, which also express type IV pili, but not pil mutants, activate cervical epithelial cell lines by increasing [Ca\(^{2+}\)], through an interaction with CD46 (11). The Ca\(^{2+}\) increase is because of influx associated with the translocation of a Neisseria porin into the eukaryotic cells and induces apoptosis, which accompanies Neisseria invasion of these epithelial cells (12). Invasive pathogens in the gut such as Salmonella also stimulate NF-κB-dependent gene expression through Ca\(^{2+}\)-dependent signaling (13). However, airway epithelial cells with intact tight junctions are highly resistant to bacterial invasion (14) and apoptosis (15). In diseases such as CF in which there is prominent stimulation of epithelial cytochrome expression (1), the bacterial burden is clearly in the airway lumen and not intracellular (4), indicating that superficial interactions between specific bacterial gene products and epithelial receptors are sufficient to activate epithelial cell signaling. There is ample evidence that epithelial cells express IL-8 in response to a variety of pulmonary pathogens through NF-κB-dependent transcription. However, the more proximal components of the epithelial cell signaling cascade, the nature of the receptor, and the kinases involved in signal transduction have not been previously characterized. As perturbation of airway epithelial cells by various stimuli can initiate Ca\(^{2+}\) oscillations (16) we postulated that bacterial ligands might...
activate epithelial responses by stimulating alterations in [Ca\(^{2+}\)]\(_{i}\), and causing activation of Ca\(^{2+}\)-dependent kinases.

In the studies presented, we sought to determine whether the common pulmonary pathogens *S. aureus* and *P. aeruginosa*, which are known to recognize asialylated glycolipid receptors, stimulate epithelial proinflammatory responses by the activation of a common pathway and to identify the components of the major signaling cascades involved. We demonstrate that these organisms, but not mutants lacking specific adhesins that ligate asialoGM1 moieties on the epithelial surface, generate increases in Ca\(^{2+}\) from intracellular stores to activate MAPKs, stimulate NF-\(\kappa\)B translocation, and initiate IL-8 production.

**MATERIALS AND METHODS**

**Cell Lines**—1HAEo cells, SV40 immortalized human airway epithelial cells whose properties have been well characterized (9), obtained from D. Gruenert (University of California, San Francisco, CA) were grown in 96-well plates in DMEM-F-12 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml gentamicin, 4 \(\mu\)g/ml amphotericin B. 9HTEo cells were obtained from P. Davis (Case Western Reserve University) and grown in 6-well plates in DMEM-F-12 as above. Unless specified, reagents were purchased from Sigma. CHO-K1 cells obtained from ATCC were grown in 96-well plates in DMEM-F-12 supplemented with 10% FCS. Weaned CHO-K1 cells, SV40 immortalized human airway epithelial cells whose properties have been well characterized (9), obtained from D. Gruenert (University of California, San Francisco, CA) were grown in 96-well plates in DMEM-F-12 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml gentamicin, 4 \(\mu\)g/ml amphotericin B. 9HTEo cells were obtained from P. Davis (Case Western Reserve University) and grown in 6-well plates in DMEM-F-12 as above. Unless specified, reagents were purchased from Sigma. CHO-K1 cells obtained from ATCC were grown in 96-well plates in DMEM + 10% FCS.

**Bacterial Strains and Culture Conditions**—PAO1, a motile, pilated, nonmucoid strain of *P. aeruginosa*, whose genome has been sequenced, was grown in M9 or LB media. PAO algC has a mutation blocking phosphoglucomutase expression that results in defective LPS and alginate biosynthesis (17). PAO/np (npA) lacks expression of pilin (10). *S. aureus* strains RN6390 and RN6911 (agr) were grown in CYPG media and diluted as above (18).

**Ca\(^{2+}\) Imaging**—Subconfluent monolayers of 1HAEo cells grown on glass coverslips were loaded with 5 \(\mu\)M Fura-2/AM (Molecular Probes, Eugene, OR) and 0.02% pluronic acid at room temperature for 45 min and then washed twice with PBS. Ratios were imaged with a Zeiss Axiovert microscope and analyzed using VPROBE software. Frames were collected at 6-s intervals following the application of bacteria. Fields were examined for each condition. After a 60-min incubation the monolayers were washed three times in PBS and solubilized in 0.2% NaOH, and scintillations were counted. Binding competition studies were performed by incubating the monolayers with bacteria in DMEM + 10% bovine serum albumin with or without rabbit polyclonal antibody to 270 \(\mu\)g/ml asialoGM1 (anti-asialoGM1) (Wako, Richmond, VA) or with monoclonal anti-human epithelial antigen (250 \(\mu\)g/ml) (Dako, Carpenteria, CA) for 1 h at 37°C, 5% CO\(_2\). Assays using *S. aureus* were done in the presence of 0.1% FCS.

**IL-8 Assays**—IL-8 was measured by enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN) following a 60-min exposure of confluent monolayers of 1HAEo cells, weaned from serum for 24 h, in 96-well plates to 1–4 \(\times\) 10\(^6\) cfu/ml of the various bacterial strains preincubated for 60 min with the reagents indicated (9). The cells were washed and sterilized with gentamicin, and supernatants were harvested either 4 or 18 h later for IL-8 enzyme-linked immunosorbent assay. Duplicate wells were treated with trypan blue to assess epithelial viability during the assay, which was >75%. Assays using *S. aureus* were done in the presence of 0.1% FCS.

**Statistical Analysis**—Each IL-8 or luciferase data point was determined in quintuplicate, a mean and standard deviation was calculated, and statistical significance was evaluated using a one-way analysis of variance with Dunnett’s post test (GraphPad Instat version 3.00; GraphPad Software, San Diego, CA) to test the null hypothesis that there was no difference in the amount of the outcome variable (IL-8 production or luciferase units) under each test condition as compared with the untreated control. For bacterial binding studies, means and standard deviations were compared using an unpaired, two-tailed t test.

**Western Hybridizations**—Epithelial cells grown in 6-well plates to 90% confluence were weaned from serum overnight. The cells were stimulated with thapsigargin, *P. aeruginosa* PAO1, or *S. aureus* (in DMEM + 0.1% FCS) for the time intervals indicated, washed, and lysed by the addition of 0.5% Triton X-100 in PBS for 45 min. Aliquots of the cell lysates (5–20 \(\mu\)g of protein) were combined with 4 \(\times\) NuPage (Novex, San Diego, CA) sample and reducing agent and heated to 70°C for 10 min. Proteins were separated by electrophoresis on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes and blocked in 5% skim milk overnight. Immunodetection was done with mouse monoclonal anti-phosphorylated or anti-nonphosphorylated p44/p42 MAPK (Thr-202/Tyr-204) (Santa Cruz Biotechnology; Santa Cruz, CA), anti-phosphorylated p38 MAPK (Tyr-180/Tyr-182), or anti-actin (New England Biolabs, Beverly, MA). An anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody and detected with Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA).

**Activation of NF-\(\kappa\)B Detected by Luciferase Reporter Constructs**—1HAEo cells were grown in 6-well plates to 80–90% confluence, washed once with PBS, and replated with serum and antibiotic free DMEM-F-12. Cells were transiently transfected using LipofectAMINE 2000 reagent (Life Technologies, Inc., Gaithersburg, MD), 1 ng of plasmid DNA of pNP-\(\kappa\)B-luciferase (Strategene, La Jolla, CA), which contains five NF-\(\kappa\)B binding sites upstream of a luciferase reporter gene, and a constitutively active TK promoter (Promega, Madison, WI) to control for transfection efficiency and incubated at 37°C in 5% CO\(_2\) for 8 h. Cells were washed twice, and selected wells were pretreated with 5 \(\mu\)g BAPTA/AM (Molecular Probes, Eugene, OR) and resuspended in serum and antibiotic free DMEM-F-12 for 1 h. Cells were stimulated with antibody to asialoGM1 (100 \(\mu\)g/ml), 15 \(\mu\)g thapsigargin, or 10 \(\mu\)g/ml of PAO1 in serum and antibiotic free DMEM-F-12 for 2 h or with *S. aureus* RN6390 in DMEM-F-12 + 0.1% FCS, washed, lysed, and harvested using passive lysis buffer (Promega, Madison, WI). Luciferase assays were performed using the reagents and protocol for the dual luciferase reporter assay system (Promega, Madison, WI) and analyzed with a luminometer. After standardization for transfection efficiency, data were plotted as the mean of quadruplicate samples and are representative of at least two independent experiments.

To directly demonstrate that asialoGM1 provides the required receptor to initiate NF-\(\kappa\)B activation, CHO cells, which do not normally express apically exposed asialoGM1, were incubated with various concentrations of purified asialoGM1 (Fluka, Milwaukee, WI) for 2 h at 37°C, and incorporation was demonstrated by flow cytometry using anti-asialoGM1 (100 \(\mu\)g/ml) as a primary antibody and goat anti-rabbit conjugated to fluorescein isothiocyanate (Zymed Laboratories Inc., South San Francisco, CA) as a secondary antibody. In subsequent experiments, CHO cells were incubated with 250 \(\mu\)g asialoGM1 for 2 h, transiently transfected with the NF-\(\kappa\)B reporter system as above, and exposed to anti-asialoGM1 (100 \(\mu\)g/ml), 10 \(\mu\)g/ml of *S. aureus*, or 10 \(\mu\)g/ml P. aeruginosa. Analysis of NF-\(\kappa\)B activation was carried out as above. Experiments using *S. aureus* were performed in the presence of 0.1% FCS.
Fig. 2. Spectrophotometric monitoring of Ca\(^{2+}\) transients in 1HAEo- cells loaded with Fura-2/AM and exposed to the stimuli indicated. A, *P. aeruginosa* PAO1, anti-asiagoM1, and *P. aeruginosa pilA* mutant PAO/NP (10). B, purified PAO1 pilin (5) and anti-human epithelial antigen (HEA). C, PAO algC (LPS mutant) and *P. aeruginosa* LPS (100 µg/ml). D, *S. aureus* wild type RN6390 and *S. aureus* RN6911 (agr). E, composite tracings of multiple cells stimulated with antibody to asiagoM1.
RESULTS

**Pulmonary Pathogens Stimulate Ca\(^{2+}\) Increases in 1HAEo—Cells**—Cellular responses to external stimuli often involve Ca\(^{2+}\)-dependent signaling cascades. We tested the ability of *P. aeruginosa*, *S. aureus*, and corresponding mutant strains lacking genes expected to be involved in adherence to stimulate Ca\(^{2+}\) increases in human respiratory epithelial cells. The addition of *P. aeruginosa* PAO1 to Fura-2/AM-loaded 1HAEo—cells prompted a 100 nM rise in [Ca\(^{2+}\)], with return to baseline within 5 min (Fig. 1). Single cell recordings demonstrated that similar 100 nM increases in [Ca\(^{2+}\)], could be elicited by PAO1, anti-asialoGM1, or purified pilin, but not by the mutant PAO/NP (piaA), which does not recognize this receptor (5) (Fig. 2). Antibody recognition of human epithelial antigen on the cells did not elicit Ca\(^{2+}\) fluxes nor did purified *P. aeruginosa* LPS alone or in the presence of 0.1% serum. A PAO algC mutant that lacks phosphomannomutase activity required for both LPS and alginate synthesis (17) activated a more prolonged Ca\(^{2+}\) response than the parental strain. *P. aeruginosa* PAO1 and antibody to asialoGM1 also stimulated Ca\(^{2+}\) responses in respiratory epithelial cells derived from nasal polyps in primary culture (data not shown). *S. aureus* RN6390 activated Ca\(^{2+}\) fluxes of similar amplitude as PAO1 (Fig. 2D) and was also able to initiate Ca\(^{2+}\) oscillations following the initial stimulus. The agr mutant RN6911 did not activate Ca\(^{2+}\) fluxes. Representative tracings from single cells activated by anti-asialoGM1 demonstrate the cell to cell variations in the Ca\(^{2+}\) response (Fig. 2E).

**Bacterial Recognition of AsialoGM1 Receptors**—The receptor for *P. aeruginosa* pilin is the GalNAcβ1-4Gal moiety of asialylated glycolipids such as asialoGM1 (10). Although other adhesins are also expressed, pilin is the major ligand for this receptor and is responsible for much of the induction of IL-8 expression (5). A dose-response relationship of the binding of *P. aeruginosa* to cells with increasing amounts of surface asialoGM1 has been described (19). In addition, some data suggest that *S. aureus* might compete with *P. aeruginosa* for receptors on the surface of epithelial cells (20). We postulated that the activation of Ca\(^{2+}\) responses by *S. aureus*, similarly, was because of ligation of the asialoGM1 receptor. Binding competition studies were done to see whether antibody to asialoGM1 inhibited bacterial attachment to airway epithelial cells and whether *P. aeruginosa* could directly compete with *S. aureus* for epithelial binding. In the presence of anti-asialoGM1, 60% of PAO1 binding was displaced as compared with 40% of *S. aureus* RN6390 binding (p < 0.0002 and p < 0.004, respectively). Control assays done with the same concentration of antibody to human epithelial antigen resulted in no inhibition of binding by either species (data not shown). The *S. aureus* agr mutant RN6911, which did not activate Ca\(^{2+}\) fluxes, was not displaced by antibody to asialoGM1. Radiolabeled *P. aeruginosa* PAO1 could be displaced by *S. aureus* RN6390 as effectively as by unlabeled PAO1 present in 5-fold excess. An equivalent inoculum of the *S. aureus* mutant RN6911 did not compete as efficiently suggesting that it has less affinity for common receptors. Labeled *S. aureus* was also able to compete with *P. aeruginosa* for binding (data not shown).

**Activation of IL-8 by Adherent Bacteria through Ca\(^{2+}\)-Dependent Signaling Pathways**—Epithelial cells produce the PMN chemokine IL-8 in response to adherent *P. aeruginosa* through activation of NF-kB and transcription of the IL-8 gene (9, 10). *S. aureus* strains similarly stimulate IL-8 expression (Fig. 3). The wild type *S. aureus* strain RN6390 was associated with a greater IL-8 response than the *agr* mutant. To determine whether the activation of IL-8 expression is a direct consequence of [Ca\(^{2+}\)], release, epithelial cells were pretreated with reagents expected to alter Ca\(^{2+}\) signaling prior to and during *P. aeruginosa* stimulation, and IL-8 production was measured (Fig. 4). Although chelation of extracellular Ca\(^{2+}\) with EGTA (21) did not inhibit IL-8 expression, epithelial cells pretreated with BAPTA/AM (22) to chelate intracellular Ca\(^{2+}\) had significantly decreased IL-8 responses (p < 0.001 for each). NiCl\(_2\), which functions as an external Ca\(^{2+}\) channel blocker (23), did not significantly inhibit the induction of IL-8 expression nor did 100 μM verapamil (24) have a major inhibitory effect. Compounds expected to affect Ca\(^{2+}\)/calcineurin-dependent signaling pathways such as cyclosporin A (25) or FK506 (25) decreased the IL-8 response (p < 0.001 for each) although the maximal effect required overnight incubation with the epithelial cells. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), an inhibitor of Fe\(^{2+}\)/Zn\(^{2+}\)-dependent enzymes including calcineurin (26), significantly decreased PAO1-induced expression of IL-8, as well (p < 0.001).

Although *P. aeruginosa* may activate epithelial IL-8 expression through a Ca\(^{2+}\)-dependent signaling system, we were interested in establishing whether fluxes in [Ca\(^{2+}\)], alone, in the absence of a bacterial stimulus, were sufficient to evoke IL-8 expression. Airway epithelial cells treated with thapsigargin (27), an inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-dependent ATPase that transiently increases [Ca\(^{2+}\)], or with okadaic acid, a PP2A phosphatase inhibitor that affects Ca\(^{2+}\)-calmodulin-dependent kinase activity (28), produced a dose-dependent increase in IL-8 production (p < 0.01 and p < 0.001, respectively) (Fig. 4B) suggesting that activation of IL-8 expression can be...
mediated solely through increases in [Ca\textsuperscript{2+}].

P. aeruginosa and S. aureus Stimulate IL-8 Expression through Ca\textsuperscript{2+}-dependent Activation of MAPKs—Many signaling cascades activate the nuclear translocation of NF-κB and other transcriptional activators involved in IL-8 transcription. NF-κB translocation can be initiated by Ca\textsuperscript{2+}-dependent signals through the stimulation of MAPKs (29). We tested the involvement of two of the major families of MAPKs in the activation of IL-8 expression induced by ligation of the asialoGM1 receptor by bacteria or antibody (Fig. 5). Stimulation of epithelial IL-8 expression by anti-asialoGM1 (Fig. 5A), P. aeruginosa (Fig. 5B), or S. aureus (Fig. 5C) was inhibited by either PD98059, a specific inhibitor of the MAPK/ERK1 kinase (30), or SB202190, which inhibits the p38 kinase (31). Inhibition of S. aureus stimulation of the cells by PD98059 or SB202190 was significant with \( p < 0.05 \) and \( p < 0.01 \), respectively, and for all the other stimuli \( p < 0.001 \). Moreover, the Ca\textsuperscript{2+} dependence of the IL-8 response was indicated by the ability of BAPTA/AM to significantly inhibit the induction of IL-8 by S. aureus, P. aeruginosa, or anti-asialoGM1 (\( p < 0.001 \) for each).

To determine whether the proposed pathway is active in human airway epithelial cells in general, the kinetics of MAPK activation following exposure of an unrelated cell line to stimuli that increased [Ca\textsuperscript{2+}], and caused IL-8 production was examined. In lysates of 9HTEo cells exposed to thapsigargin, phosphorylated ERK (p44/p42) was briefly detectable by Western hybridization within 15 min of stimulation and was gone by 30 min (Fig. 6). Stimulation of the same cell line with P. aeruginosa also activated ERK1/2 briskly and transiently. 1HAEo cells responded similarly to P. aeruginosa with phosphorylation of the p44/p42 ERK (Fig. 7A). In the presence of 0.1% FCS, which is required for staphylococcal binding, phosphorylated ERKs were detectable in unstimulated cells. There was a rapid response to S. aureus RN6390 with dephosphorylation of the ERK kinase, as compared with the total (phosphorylated
and nonphosphorylated) p44/p42 kinase in the cell. Addition of anti-asialoGM1 to 1HAEo cells in 96-well plates were pretreated with the compounds indicated for 60 min prior to a 1-h exposure to (A) anti-asialoGM1 (Ab-aGM1), (B) P. aeruginosa PAO1, and (C) S. aureus RN6390. The epithelial cell were washed, sterilized with gentamicin, and refed with media containing the compound being tested, and culture supernatants were assayed for IL-8 18 h later. Control wells were treated with each of the compounds without bacterial stimulation and were assayed.

**DISCUSSION**

Respiratory epithelial cells function as an important component of the normal host defense against bacterial infection. Pathogens that elude superficial innate defenses (antimicrobial peptides, mucin, and cilary activity) and express appropriate adhesins to recognize GalNAcβ1–4Gal binding sites on the epithelial cells.

**FIG. 5.** IL-8 expression in epithelial cells stimulated by anti-asialoGM1 or bacteria: effects of MAPK inhibitors and Ca^{2+} blockers. Confluent monolayers of 1HAEo cells were treated with the compounds indicated for 60 min prior to a 1-h exposure to (A) anti-asialoGM1 (Ab-aGM1), (B) P. aeruginosa PAO1, and (C) S. aureus RN6390. The epithelial cells were washed, sterilized with gentamicin, and refed with media containing the compound being tested, and culture supernatants were assayed for IL-8 18 h later. Control wells were treated with each of the compounds without bacterial stimulation and were assayed.

**FIG. 6.** Activation of ERK p44/p42 MAPKs following stimulation of 9HTEo airway epithelial cells detected by Western hybridization. Cells treated with thapsigargin (15 μM) to acutely increase [Ca^{2+}], or P. aeruginosa PAO1 were lysed at timed intervals (min) and screened for the presence of phosphorylated ERKp44/p42. neg. and pos. indicate controls for p-ERK.

**FIG. 7.** Activation of NF-κB following S. aureus or P. aeruginosa stimulation of epithelial cells—To demonstrate that stimulation of epithelial cells by S. aureus, as well as P. aeruginosa, results in the activation of NF-κB as an antecedent to IL-8 transcription, we used an NF-κB-luciferase reporter construct to monitor the effects of the adherent bacteria (Fig. 8A). Exposure of 1HAEo airway epithelial cells to antibody to asialoGM1, thapsigargin, P. aeruginosa, or S. aureus RN6390 stimulated 4- to 5-fold activation of the reporter construct (p < 0.001 for each) (Fig. 8A). As predicted by the effects of Ca^{2+} chelation on IL-8 expression, cells pretreated with BAPTA/AM had less activation of NF-κB in response to ligation of the asialoGM1 receptor by anti-asialoGM1 (p < 0.05), P. aeruginosa, or S. aureus (p < 0.001 for each).

To further demonstrate that bacteria activate NF-κB through ligation of an asialoGM1 receptor, the signaling pathway was reproduced in CHO-K1 cells, which normally do not have detectable asialoGM1 on their surface. Exogenous asialoGM1 was incorporated into the surface membranes of the CHO cells in a dose-dependent fashion as documented by flow cytometry (Fig. 8B). The CHO cells were transiently transfected with the pNF-κB reporter construct and stimulated with antibody to asialoGM1 (Fig. 8C), P. aeruginosa (Fig. 8D), or S. aureus (Fig. 8E). In response to either anti-asialoGM1 or P. aeruginosa PAO1 there was a 5- to 6-fold increase (p < 0.001) in reporter activity, as compared with controls lacking the asialoGM1 receptor. S. aureus increased reporter activity 2- to 3-fold (p < 0.001). The endogenous activation of NF-κB was higher in the CHO-K1 cells exposed to S. aureus, likely as a result of the 0.1% FCS required for staphylococcal binding.

**DISCUSSION**

Respiratory epithelial cells function as an important component of the normal host defense against bacterial infection. Pathogens that elude superficial innate defenses (antimicrobial peptides, mucin, and cilary activity) and express appropriate adhesins to recognize GalNAcβ1–4Gal binding sites on the
epithelial surface, rapidly stimulate increases in $[\text{Ca}^{2+}]_i$ that serve to initiate MAPK activation and the transcription of IL-8, a major PMN chemokine. The presence of this common bacterial receptor was predicted by Krivan et al. (7), who recognized that many pulmonary pathogens bind to the GalNAc$\beta1-4$Gal moiety present on asialylated glycolipids including asialoGM1 in vitro. The association of asialylated glycolipid receptors and cystic fibrosis transmembrane conductance regulator mutations has been documented by several investigators working with cell lines (9, 20) and primary tissues (5). The increased activation of this pathway in CF patients who have bacterial contamination of the lower airways and greater numbers of available receptors is likely to be a major contributing factor to the chronic airway inflammation characteristic of this disease. However, this pathway is also of significance in the normal lung, as well, providing a general mechanism for defense of the airway in response to inhaled pathogens.

Diverse bacterial ligands are recognized by common epithelial receptors. Incorporation of the asialoGM1 glycolipid into the membranes of CHO cells, which ordinarily lack superficial asialoGM1 moieties, provided receptor function and mediated the activation of NF-κB in response to ligation of the receptor by antibody, P. aeruginosa, or S. aureus. Although bacteria have many superficial ligands that may interact with epithelial components, there is clearly some specificity for the recognition of the GalNAc$\beta1-4$Gal disaccharide of asialoGM1 and similar glycolipids that provides a common receptor for diverse respiratory pathogens (7) and initiates this epithelial proinflammatory response. P. aeruginosa pili have been shown to bind specifically to the asialylated glycolipids and stimulate the activation of NF-κB translocation and IL-8 expression (9). Despite expression of multiple virulence factors, nonpiliated P. aeruginosa were unable to activate the $\text{Ca}^{2+}$ fluxes required to stimulate an IL-8 response. Other superficial components of P. aeruginosa, particularly LPS, may activate epithelial signaling cascades; however, the recognition of the asialylated glycolipids appears to be a major stimulus for the expression of IL-8 (9). Although P. aeruginosa LPS can induce mucin (muc-2) expression through activation of a c-Src-Ras-MAPK/ERK1/2-MAPK-pp90rsk-NF-κB cascade in colonic epithelial cells (32), LPS from the same strain of P. aeruginosa did not evoke the $[\text{Ca}^{2+}]_i$ changes in respiratory cells that induced NF-κB and IL-8 expression. Moreover, a P. aeruginosa mutant with defective LPS O-side chains was, nonetheless, able to activate $\text{Ca}^{2+}$ oscillations and previously has been shown to stimulate IL-8 expression (9). Thus, P. aeruginosa activation of IL-8 expression by airway epithelial cells is independent of LPS.

The staphylococcal ligand, while undefined, appears to be an agr-dependent cell surface structure like the S. aureus adhesins that recognize extracellular matrix components. Unlike wild type S. aureus RN6390, the mutant RN6911 did not compete with anti-asialoGM1 or with P. aeruginosa PAO1 for binding nor did it activate immediate increases in $[\text{Ca}^{2+}]_i$. The binding of RN6911 did evoke some IL-8 expression indicating that agr-independent adhesins function through other pathways. As pathogens often express multiple ligands, independent binding events are likely, which may result in similar epithelial responses.

The surface components of the Gram-positive staphylococci differ significantly from those of the Gram-negative P. aeruginosa, and it is unlikely that either the binding affinities or the binding kinetics of the two pathogens are identical. However, evidence for the activation of a common asialoGM1 pathway is further supported by the similar kinetics of the host response. Both species, as well as antibody to asialoGM1, initiated immediate changes in $[\text{Ca}^{2+}]_i$, followed by phosphorylation of MAPKs within 15 min and NF-κB activation. This was too rapid a response to involve epithelial ingestion of organisms, as hours are required for the internalization of P. aeruginosa (4). Although bacterial invasion of gut epithelial cells may be required before some signaling pathways are activated (13, 33), for airway cells a superficial ligand-receptor was sufficient to
initiate Ca\(^{2+}\) signaling and an IL-8 response.

Stimulation of the 1HAEo− cells initiated immediate increases in [Ca\(^{2+}\)], that came from predominantly intracellular stores. The Ca\(^{2+}\) response and ensuing IL-8 expression were inhibited by BAPTA/AM but not by EGTA at the level of MAPK and NF-κB activation, and blocking external Ca\(^{2+}\) channels with NiCl\(_2\) did not inhibit the IL-8 response. The ability of thapsigargin, which causes an immediate increase in [Ca\(^{2+}\)], to activate MAPK phosphorylation and stimulate IL-8 expression is also consistent with this hypothesis. The Ca\(^{2+}\) response did not appear to be because of influx from the media, as was the case for Neisseria (12). Activation of Ca\(^{2+}\) fluxes of similar magnitude and duration, followed by IL-8 expression, were observed in an unrelated 9HTEo− cell line, nasal polyp cells in primary culture, as well as Calu-3 cells (data not shown). The amplitude of the Ca\(^{2+}\) fluxes, 100−150 nM, is less than that of excitatory cells but is similar to the responses of gastrointestinal cells, which activate NF-κB through Ca\(^{2+}\) signaling following Salmonella infection (13), suggesting that this may represent a common mechanism to initiate protection of mucosal surfaces.

The Ca\(^{2+}\) signal in airway cells, whether initiated by bacterial ligation of asialoGM1, antibody recognition of the receptor, or thapsigargin, was sufficient to activate p38 and ERK MAPKs. Both families of MAPKs participate as biochemical inhibition of either was sufficient to inhibit the IL-8 response. Ca\(^{2+}\)-dependent cytokine signaling is often mediated by calcineurin-dependent pathways. However, the relatively modest inhibitory effects of cyclosporin and FK506 on IL-8 expression suggest that calcineurin is not involved to any great extent. The 6-fold stimulation of IL-8 by the phosphatase inhibitor okadaic acid (which does not affect calcineurin) is more consistent with signaling through other Ca\(^{2+}\)-dependent protein kinases and phosphatases (28). This portion of the signaling cascade of airway epithelial cells may be similar to that of HL-60 cells, a promyelocytic cell line, which responds to okadaic acid by tyrosine phosphorylation of p44, degradation of IκB-α, activation of NF-κB, and IL-8 transcription (34). There is likely to be redundancy within this proinflammatory pathway, as well as cross-talk with other intracellular signaling mechanisms at a variety of levels. Inhibition of one component of the pathway, one of the MAPKs, for example, may not have equivalent consequences on the net production of IL-8 as interference with other, even downstream, component. This Ca\(^{2+}\)-dependent-MAPK-NF-κB signaling system, although not previously described in respiratory cells, is similar to that delineated in professional immune cells and neuronal cells (34, 35). However, the more proximal components of the pathway, the glycolipid receptors and possibly associated G-proteins involved in signaling, remain to be identified. The association of glycosphingolipids and G-protein-coupled receptors has been well documented, and the participation of glycolipid rafts and inositol 1,4,5-trisphosphate receptor in the spatial organization of several signaling pathways has been demonstrated (36–38).

**FIG. 8.** Activation of NF-κB following stimulation of 1HAEo− or CHO cells. 1HAEo− or CHO cells were transiently transfected with an NF-κB reporter construct and a renilla luciferase control vector. Following stimulation with antibody to asialoGM1, thapsigargin, or bacteria luciferase activity was measured and compared with unstimulated control wells or wells pretreated with BAPTA/AM. Each condition was done in quadruplicate, and each experiment was repeated three times. A, exposure of transfected 1HAEo− cells to anti-aGM1, P. aeruginosa PAO1, or S. aureus RN6390 resulted in activation of the transcription factor NF-κB as measured by the luciferase reporter system. This activation was consistently inhibited by pretreatment with BAPTA/AM. B, treatment of CHO cells with increasing concentrations of purified asialoGM1 (aGM1) leads to incorporation into the membrane as measured by flow cytometry. Detection was done using anti-aGM1 as the primary antibody and goat anti-rabbit conjugated to fluorescein isothiocyanate as the secondary antibody. Percentage of cells positive for fluorescence is measured on the y axis. C–E, CHO cells with or without incubation with 250 μM aGM1 for 2 h and exposed to (C) anti-aGM1, (D) P. aeruginosa, or (E) S. aureus. Higher endogenous activation of cells in E is attributed to the presence of 0.1% FCS in the media as described above.
The asialoGM1 signaling pathway in the normal respiratory epithelium provides a mechanism to rapidly elicit a PMN response through IL-8 expression when pathogens contact the surface of epithelial cells. In the normal airway, mucosal glycolipid conjugates are fully sialylated, and inhaled organisms rarely encounter GalNAcβ1→4Gal-glycolipid binding sites on the epithelial mucosa, even if they are able to withstand the antibacterial activities of airway surface fluid and mucociliary clearance. Damaged epithelial cells, or those undergoing regeneration, provide asialylated receptors (6). In cystic fibrosis, defective sialylation (20) results in increased numbers of epithelial receptor sites and increased IL-8 production induced by adherent bacteria. This is a major contributing factor to the general proinflammatory milieu in CF airways (39). Although the airway epithelial response to infection seems appropriate in the normal host, it may be useful to modulate components of this signaling cascade in specific pathological conditions such as CF, to prevent undesirable consequences of excessive inflammation in the lung.

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Cystic Fibrosis Pathogens Activate Ca\textsuperscript{2+}-dependent Mitogen-activated Protein Kinase Signaling Pathways in Airway Epithelial Cells

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