

# Bafilomycin A<sub>1</sub> inhibits rhinovirus infection in human airway epithelium: effects on endosome and ICAM-1

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**Suzuki, Tomoko, Mutsuo Yamaya, Kiyohisa Sekizawa, Masayoshi Hosoda, Norihiro Yamada, Satoshi Ishizuka, Katsutoshi Nakayama, Masaru Yanai, Yoshio Numazaki, and Hidetada Sasaki.** Bafilomycin A<sub>1</sub> inhibits rhinovirus infection in human airway epithelium: effects on endosome and ICAM-1. *Am J Physiol Lung Cell Mol Physiol* 280: L1115–L1127, 2001.—To examine the effects of bafilomycin A<sub>1</sub>, a blocker of vacuolar H<sup>+</sup>-ATPase, on rhinovirus (RV) infection in the airway epithelium, primary cultures of human tracheal epithelial cells were infected with RV14. Viral infection was confirmed by showing that viral RNA in the infected cells and the viral titers in the supernatants of infected cells increased with time. RV14 infection upregulated the production of cytokines and mRNA of intercellular adhesion molecule (ICAM)-1 in epithelial cells. Bafilomycin A<sub>1</sub> reduced the viral titers of RV14 and inhibited the production of cytokines and ICAM-1 before and after RV14 infection. Bafilomycin A<sub>1</sub> reduced susceptibility of epithelial cells to RV14 infection. RV14 increased activated nuclear factor- $\kappa$ B in the cells, and bafilomycin A<sub>1</sub> reduced the activated nuclear factor- $\kappa$ B. Bafilomycin A<sub>1</sub> decreased the number of acidic endosomes in the epithelial cells. These results suggest that bafilomycin A<sub>1</sub> may inhibit infection by RV14 by not only blocking RV RNA entry into the endosomes but also reducing ICAM-1 expression in the epithelial cells. Bafilomycin A<sub>1</sub> may therefore modulate airway inflammation after RV infection.

intercellular adhesion molecule-1; asthma; common cold; airway inflammation; vacuolar adenosine 5'-triphosphatase

PERSPECTIVE STUDIES HAVE INDICATED that asthma attacks are associated with infections of various viruses including rhinoviruses (RVs) and respiratory syncytial viruses (18, 24). Studies using PCR-based diagnostics have emphasized the importance of RVs by demonstrating that RVs are responsible for 80–85 and 45% of the asthma flairs in 9- to 11-yr-old children and adults, respectively, with RV being the most commonly implicated pathogen (18, 24). In contrast to a variety of other respiratory pathogens (e.g., influenza virus and adeno-

virus), cell cytotoxicity does not appear to play a major role in the pathogenesis of RV infection (32), but the clinical and pathological features of RV infection are, to a great extent, due to the elaboration of a variety of inflammatory mediators by the host (47).

The vacuolar H<sup>+</sup>-ATPases (V-ATPases) are a family of ATP-driven proton pumps responsible for the acidification of a variety of intracellular compartments in eukaryotic cells (10). V-ATPases provide the acidic environment required for dissociation of internalized ligand-receptor complexes within endosomes, for activating the secreted lysosomal enzymes, for facilitating protein processing and degradation by acid-dependent proteases, and for assisting the degradation of the bone matrix (10). Furthermore, exposure of influenza virus to a low pH within the endosomes by V-ATPases induces the formation of a fusion pore between the viral and endosomal membranes that permits entry of the viral RNA (40). The specific V-ATPase inhibitor bafilomycin A<sub>1</sub> (5) blocks the infection of influenza virus and RV in HeLa cells and Madin-Darby canine kidney (MDCK) cells (3, 26, 28) and inhibits the uncoating of RV type 2 and type 14 (RV14) from late endosomes (2, 29). However, the role of V-ATPases in RV infection in human airway epithelial cells, the primary target for respiratory viruses, has not been elucidated.

Infection of epithelial cells with RV induces production of several cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)- $\alpha$ , and granulocyte-macrophage colony-stimulating factor (34, 36). These cytokines are known to mediate a wide variety of proinflammatory and immunoregulatory effects (1) and may play an important role in the pathogenesis of RV infections. Acidic pH with V-ATPases appears to play a role in the production of proteins in the cells (42). However, the effects of V-ATPase inhibitors on the production of inflammatory mediators and the major RV receptor, intercellular adhesion molecule-1 (ICAM-1), in RV infection have not been investigated (27, 36, 47).

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We therefore investigated whether inhibitors of V-ATPases have inhibitory effects on RV infection in cultured human tracheal epithelial cells and determined the mechanisms responsible for the inhibition of RV infection. We also studied the effects of V-ATPase inhibitors on the production of cytokines and ICAM-1 by RV infection.

## METHODS

**Medium components.** Reagents for the cell culture medium were Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum (FCS),  $\gamma$ -globulin-free fetal calf serum (GGFCS; all from GIBCO BRL, Life Technologies, Palo Alto, CA), trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, amphotericin B (all from Sigma, St. Louis, MO), Ultrosor G serum substitute (USG; BioSeptra, Marlborough, MA), 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA; Molecular Probes, Eugene, OR), and bafilomycin A<sub>1</sub> (Wako Pure Chemical Industries, Osaka, Japan).

**Human embryonic fibroblast cell culture.** Human embryonic fibroblast cells were cultured in MEM containing 10% FCS supplemented with  $5 \times 10^4$  U/l of penicillin and 50 mg/l of streptomycin in a Roux-type bottle (Iwaki Glass, Chiba, Japan) sealed with a rubber plug (25). Confluence was achieved at 7 days, at which time the cells were collected by trypsinization (0.05% trypsin and 0.02% EDTA). The cells ( $1.5 \times 10^5$  cells/ml) suspended in MEM containing 10% FCS were then plated in glass tubes (15  $\times$  105 mm; Iwaki Glass) that were sealed with rubber plugs and cultured at 37°C.

**Human tracheal epithelial cell culture.** Tracheae for cell culture were obtained 3–6 h after death from 80 patients (age  $63 \pm 4$  yr; 39 women and 41 men) under a protocol passed by the Tohoku University (Sendai, Japan) Ethics Committee. Forty of the patients were smokers. None had a respiratory illness including bronchial asthma, and they died of acute myocardial infarction ( $n = 22$ ), congestive heart failure ( $n = 6$ ), malignant tumor other than lung cancer ( $n = 23$ ), rupture of aortic aneurysm ( $n = 5$ ), liver cirrhosis ( $n = 3$ ), renal failure ( $n = 4$ ), leukemia ( $n = 5$ ), malignant lymphoma ( $n = 3$ ), cerebral bleeding ( $n = 6$ ), and cerebral infarction ( $n = 3$ ).

Isolation and culture of the human tracheal surface epithelial cells were performed as previously described (44). In brief, the surface epithelium was scored into longitudinal strips and pulled off the submucosa. The tracheal surface epithelial cells were isolated by digestion with protease (0.4 mg/ml; Sigma type XIV) dissolved in phosphate-buffered saline (PBS) at 4°C overnight. The cells were pelleted (200 g for 10 min) and suspended in DMEM-Ham's F-12 medium (50:50 vol/vol) containing 5% FCS. Cell counts were made with a hemocytometer, and estimates of viability were done with trypan blue and by measuring the amount of lactate dehydrogenase in the medium as previously reported (36). Protein concentrations were measured with the method previously reported (44). The cells were then plated at  $5 \times 10^5$  viable cells/ml ( $2 \times 10^5$  cells/cm<sup>2</sup>) in round-bottom glass tubes (15-mm diameter and 105-mm length; Iwaki Glass) coated with human placental collagen (44). The glass tubes were sealed with rubber plugs, kept stationary at a slant of  $\sim 5^\circ$ , and cultured at 37°C. This medium was replaced by DMEM-Ham's F-12 medium containing 2% USG on the first day after the cells were plated. The cell culture medium was supplemented with  $10^5$  U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B. We

used primary cultures of human tracheal epithelial cells on days 6–14 of culture for the experiments.

We confirmed cilia beating on the epithelial cells and the absence of fibroblasts in glass tubes using an inverted microscope (MIT-2, Olympus, Tokyo, Japan) from the beginning of the cell culture to the end of the experiments. Furthermore, to determine whether cultured cells can form tight junctions, we performed parallel cultures of human tracheal epithelial cells on Millicell CM inserts (0.45- $\mu$ m pore size and 0.6-cm<sup>2</sup> area; Millipore Products Division, Bedford, MA) to measure electrical resistance and short-circuit current with Ussing chamber methods (44). When the cells cultured under these conditions become differentiated and form tight junctions without contamination by fibroblasts, they have values of  $>40 \Omega \cdot \text{cm}^2$  for resistance and  $>10 \mu\text{A}/\text{cm}^2$  for short-circuit current (44). Therefore, cultured human tracheal epithelial cells were judged as cells able to form tight junctions and were used for the following experiments when cells on the Millicell CM inserts had a high resistance ( $>40 \Omega \cdot \text{cm}^2$ ) and a high short-circuit current ( $>10 \mu\text{A}/\text{cm}^2$ ). We observed whether the human tracheal epithelial cells made a dome formation to confirm that cells on solid glass supports form tight junctions, and we found that the human tracheal epithelial cells make a dome formation when the cells made confluent cell sheets on days 5–7 of culture as described by Widdicombe et al. (41).

To further exclude fibroblast contamination, the cells were examined with cytokeratin staining. The cells cultured in glass tubes were collected by trypsinization and cultured on glass slides overnight at 37°C in a CO<sub>2</sub> incubator. The cells were then fixed with 4% paraformaldehyde and 0.05% Triton X-100 in 0.1 M phosphate buffer for 15 min at room temperature. After being rinsed with PBS, the cells were incubated with 10% skim milk to block nonspecific binding of the first and second antibodies. The sections were overlaid with either LeukoStat anti-keratin antibody (MAK-5, Triton, Alameda, CA), anti-vimentin antibody (DAKO-VIMENTIN, DAKO, Santa Barbara, CA), or an isotype-matched mouse monoclonal antibody with an irrelevant specificity (Chemicon International) in PBS containing 1% bovine serum albumin. Anti-keratin and anti-vimentin antibodies label epithelial cells and fibroblast cells, respectively (11, 33). After an overnight incubation at 4°C and a wash with PBS, the sections were incubated with biotinylated horse anti-mouse immunoglobulins (Vector Laboratories) diluted with PBS for 1 h at room temperature followed by incubation with the avidin-biotin-peroxidase complexes (Vectastain ABC kit, Vector Laboratories) for 1 h at room temperature. The sections were developed by exposure of the substrate to 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) and were counterstained with methyl green.

We found from these experiments that nearly all the cells ( $>95\%$ ) from the monolayers of human tracheal epithelial cells in the tubes reacted with the anti-keratin antibody, but they did not react with the anti-vimentin antibody (data not shown). As a positive control, we stained the human embryonic fibroblast cells with anti-vimentin antibody and obtained positive signals (data not shown).

**Viral stocks.** RV14 was prepared in our laboratory from patients with common colds (25). RV can be distinguished from enterovirus, which also produces "entero-like" cytopathic effects on human embryonic fibroblasts, by inactivation of RV after treatment with acid (25). Furthermore, RV14 was identified with a microneutralization test with an antibody for RV14 as previously described (14). Stocks of RV14 were generated by infecting human embryonic fibroblast cells cultured in glass tubes in 1 ml of MEM supplemented

with 2% GGFCS,  $5 \times 10^4$  U/l of penicillin, and 50 mg/l of streptomycin at 33°C. The cells were incubated for several days in glass tubes in 1 ml of MEM supplemented with 2% GGFCS until the cytopathic effects were obvious, after which the cultures were frozen at -80°C, thawed, and sonicated. The virus-containing fluid so obtained was frozen in aliquots at -80°C. The content of the viral stock solutions was determined with the human embryonic fibroblast cell assay described in *Detection and titration of viruses*.

**Detection and titration of viruses.** RVs were detected by exposing confluent human embryonic fibroblast cells in glass tubes to serial 10-fold dilutions of virus-containing medium in MEM supplemented with 2% GGFCS. The glass tubes were then incubated at 33°C for 7 days, and the cytopathic effects of the viruses on the human embryonic fibroblast cells were observed with an inverted microscope (MIT, Olympus) as previously reported (25). The amount of specimen required to infect 50% of the human embryonic fibroblast cells [50% tissue culture infectious dose (TCID<sub>50</sub>)] was determined.

We examined in the preliminary experiments whether either a V-ATPase inhibitor, bafilomycin A<sub>1</sub>, or blockers of Na<sup>+</sup>/H<sup>+</sup> exchangers (NHEs), EIPA and FR-168888, affect RV14 titration in human embryonic fibroblasts. We performed titration of RV14 using DMEM-Ham's F-12 medium containing 2% USG and RV14 at concentrations ranging from 10<sup>1</sup> to 10<sup>5</sup> TCID<sub>50</sub> units/ml supplemented with either bafilomycin A<sub>1</sub> (0.1 μM) (22), EIPA (10 μM), or FR-168888 (10 μM; a gift from Fujisawa Pharmaceutical, Tokyo, Japan) (6, 43). The medium containing RV14 supplemented with either bafilomycin A<sub>1</sub> (0.1 μM), EIPA (10 μM), or FR-168888 (10 μM) was diluted 10-fold with MEM supplemented with 2% GGFCS and added to the human embryonic fibroblasts in glass tubes to observe the cytopathic effects. We found that neither bafilomycin A<sub>1</sub> (0.1 μM), EIPA (10 μM), nor FR-168888 (10 μM) influenced the cytopathic effects on human embryonic fibroblasts at any content of RV14.

**Viral infection of human tracheal epithelial cells.** The medium was removed from the confluent monolayers of human tracheal epithelial cells and replaced with 1 ml of DMEM-Ham's F-12 medium containing 2% USG. The human tracheal epithelial cells were infected with RV14 by exposure to culture supernatants of human embryonic fibroblasts infected with RV14. As a mock infection, epithelial cells were exposed to the supernatants from fibroblasts cultured in medium alone. In the preliminary experiments, we found that the culture supernatants of human embryonic fibroblasts without RV14 infection did not contain significant amounts of IL-1β and TNF-α and that they did not alter the levels of IL-1β and TNF-α in the supernatants of cultured human tracheal epithelial cells from baseline. RV14 was added at a concentration of 10<sup>5</sup> TCID<sub>50</sub> units/ml. After a 1-h incubation at 33°C, the viral solution was removed, and the cells were rinsed one time with 1 ml of PBS. The cells were then placed in DMEM-Ham's F-12 medium containing 2% USG supplemented with 10<sup>5</sup> U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B and cultured at 33°C with rolling in an incubator (HDR-6-T, Hirasawa, Tokyo, Japan) (36). To measure the time course of viral release for the first 24 h, we used four separate cultures from each trachea, and the culture supernatants were collected 1, 6, 12, or 24 h after RV14 infection. Furthermore, to measure the viral release for 1–3, 3–5, or 5–7 days, the human tracheal epithelial cells cultured in the tubes were rinsed with PBS, and fresh DMEM-Ham's F-12 medium containing 2% USG was added at 24 h after RV14 infection. The whole volume of the medium was then taken for the measurement of viral content, and the same volume of

fresh medium was replaced on *days 3 and 5*, and the whole volume of the medium was taken on *day 7*. The supernatants were stored at -80°C for the determination of viral content. Furthermore, cell-associated viral content was analyzed with sonicated human tracheal epithelial cells in MEM. The viral content in the supernatant and the cell-associated viral content are expressed as TCID<sub>50</sub> units per milliliter and TCID<sub>50</sub> units per 10<sup>6</sup> cells, respectively (36).

**Cytokine assays.** Because RV14 infection increased the production of various cytokines from primary cultures of human tracheal epithelial cells (36), we measured IL-1α, IL-1β, IL-6, IL-8, TNF-α, interferon (IFN)-α, IFN-β, and IFN-γ by specific enzyme-linked immunosorbent assays (ELISAs). Sensitivities of the assays were 25 pg/ml for the IFN-α ELISA kit (COSMO BIO, Tokyo, Japan); 10 pg/ml for the IL-1α, IL-1β (both from Ohtsuka, Tokushima, Japan), IL-6, and IL-8 (both from Toray, Tokyo, Japan) ELISA kits; 4 pg/ml for the TNF-α ELISA kit (Ohtsuka); 3 pg/ml for the IFN-γ ELISA kit (Genzyme); and 1 U/ml for the IFN-β ELISA kit (BioSource International, Camarillo, CA). In preliminary experiments, we found that the concentration of TNF-α in the culture medium was low (0–10 pg/ml). Therefore, we concentrated the culture medium by freeze-dry methods with a centrifugal vaporizer (Tokyo Rikakikai, Tokyo, Japan) before measuring the concentration of TNF-α. After the culture medium was freeze-dried, the pellet was dissolved in 200 μl of water and the concentration of TNF-α was measured. The value was normalized according to the medium volume.

We used an average value of replicate cultures from the same trachea ( $n = 3$ ) for the analysis of cytokine production.

**Northern blot analysis.** Northern blot analysis was done as previously described (30, 36). Equal amounts of total RNA (10 μg) extracted from human tracheal epithelial cells, as determined spectrophotometrically, were subjected to electrophoresis in a 1% agarose-formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Hybond-N+, Amersham Life Sciences). The membrane was hybridized with [<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham)-labeled human ICAM-1 cDNA (1.8-kb *Xba*I fragment; British Bio-technology, Oxon, UK) with a random-primer labeling kit (Random Primer, Takara). Hybridization with a radiolabeled probe was performed overnight at 42°C. After high-stringency washing was performed (1× saline-sodium citrate and 0.1% sodium dodecyl sulfate, 60°C), autoradiographic detection of the hybridized probe was performed by exposure to Kodak scientific imaging film for 48–72 h at -70°C. Quantification of the autoradiographic bands was accomplished with an image analyzer (Bio Imaging analyzer BAS-2000; Fuji Photo Film).

**Effects of bafilomycin A<sub>1</sub> on RV infection.** Confluent human tracheal epithelial cells were incubated with medium containing bafilomycin A<sub>1</sub> (0.1 μM) (26) or with medium alone for 60 min at 37°C. The monolayers were then exposed to RV14 (10<sup>5</sup> TCID<sub>50</sub> units/ml) in MEM containing bafilomycin A<sub>1</sub> (0.1 μM) or medium alone for 60 min before being rinsed and the addition of fresh DMEM-Ham's F-12 medium containing 2% USG, 10<sup>5</sup> U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B supplemented with either bafilomycin A<sub>1</sub> (0.1 μM) or the vehicle for bafilomycin A<sub>1</sub>. Whole volumes of the medium were taken for measurement of the viral content, and the same volume of fresh medium containing either bafilomycin A<sub>1</sub> (0.1 μM) or the vehicle for bafilomycin A<sub>1</sub> was replaced at various times after infection.

To study the concentration-response effects of bafilomycin A<sub>1</sub> on RV infection, concentration-response curves to bafilo-

mycin A<sub>1</sub> were obtained with concentrations ranging from 10 pM to 1 μM. To determine the minimum incubation period needed to cause inhibition of RV infection, incubation with bafilomycin A<sub>1</sub> (0.1 μM) was also performed for various times ranging from 10 min to 24 h after exposure to RV14 (10<sup>5</sup> TCID<sub>50</sub> units/ml). The cells were then cultured in fresh medium containing the vehicle for bafilomycin A<sub>1</sub>. To determine whether preincubation with bafilomycin A<sub>1</sub> inhibits RV infection, the cells were incubated with bafilomycin A<sub>1</sub> (0.1 μM) for either 1 or 24 h and washed. The cells were then exposed to RV14 (10<sup>5</sup> TCID<sub>50</sub> units/ml) containing the vehicle for bafilomycin A<sub>1</sub>. To determine whether coincubation of RVs with bafilomycin A<sub>1</sub> inhibits RV infection, the cells were treated with bafilomycin A<sub>1</sub> (0.1 μM) during a period of RV exposure (10<sup>5</sup> TCID<sub>50</sub> units/ml; 60 min). The cells were then cultured in fresh medium containing the vehicle for bafilomycin A<sub>1</sub>.

We also examined the effects of EIPA and FR-168888, blockers of NHEs (3, 38). Confluent human tracheal epithelial cells were incubated with medium containing either EIPA (10 μM) or FR-168888 (10 μM) from 60 min before RV exposure (10<sup>5</sup> TCID<sub>50</sub> units/ml) to the end of the experiments.

*Effects of bafilomycin A<sub>1</sub> on the susceptibility to RV infection.* To examine whether bafilomycin A<sub>1</sub> decreases the susceptibility to RV14 infection, confluent human tracheal epithelial cells were incubated with and without bafilomycin A<sub>1</sub> (0.1 μM) for either 1 or 24 h. The epithelial cells were then exposed to serial 10-fold dilutions of RV14 for 1 h at 33°C. The presence of RV14 in the supernatants collected for 1–3 days after infection was determined with the human embryonic fibroblast cell assay described in *Detection and titration of viruses* to assess whether infection occurred at each dose of RV14 used. This index of susceptibility to infection, defined as the minimum dose of RV14 that could induce infection, was compared with the susceptibility of control cells that were not preincubated with bafilomycin A<sub>1</sub> (34).

*Detection of RV RNA by RT-PCR.* Extraction of RNA from human tracheal epithelial cells cultured in glass tubes was performed as previously described (36), and pellets of RNA were dissolved in water and stored at -80°C before use.

PCR was also performed as previously described (36). Primer pairs for the RV were present at 2 ng/μl. Sequences of the PCR primer pairs used in the present study are described elsewhere (36). The PCR was performed in an automated thermal cycler (MJ Research, Watertown, MA), and 10 μl of the reaction mixture from each sample were removed at 30 cycles. Samples were separated on a 2% agarose gel (FMC BioProducts, Rockland, ME) and stained for 30 min with 1 μg/ml of ethidium bromide. The DNA bands were visualized on a ultraviolet (UV) illuminator and photographed with type 667 positive/negative film (Polaroid, Cambridge, MA).

For the RV, RNA expression in the human tracheal epithelial cells was examined before and 8, 24, 72, and 120 h after RV14 infection in the presence of either bafilomycin A<sub>1</sub> (0.1 μM), EIPA (10 μM), FR-168888 (10 μM), or the vehicle for the blockers (control).

*Effects of bafilomycin A<sub>1</sub> on cytokine production.* Because the maximal production of cytokines was obtained on day 3 for IL-1β, on day 1 for IL-6, on day 1 for IL-8, and on day 3 for TNF-α after exposure of human tracheal epithelial cells to RV14 (36), production of IL-1β, IL-6, IL-8, and TNF-α in the culture medium was measured at the maximal production of each cytokine after RV14 infection (10<sup>5</sup> TCID<sub>50</sub> units/ml) in the presence of either bafilomycin A<sub>1</sub> (0.1 μM) or the vehicle for bafilomycin A<sub>1</sub>. The amount of IL-1α, IFN-α, IFN-β, and IFN-γ released from human tracheal epithelial cells into the

culture medium was measured before and 1, 3, and 5 days after RV14 infection.

To confirm that increases in cytokine production by RV14 infection were due to the effects of RV14 infection and not to a contaminant present in the viral stock, the ability of UV-inactivated virus to induce cytokine production was also examined. UV inactivation was performed as previously described (16).

As previously demonstrated (36), endogenous IL-1β is associated with increased production of ICAM-1 after RV14 infection. Furthermore, IL-1β induces production of various cytokines in airway epithelial cells (4). Therefore, we examined the effects of IL-1β on the production of IL-6, IL-8, and TNF-α and the effects of bafilomycin A<sub>1</sub> on the IL-1β-induced production of these cytokines. To examine the effect of bafilomycin A<sub>1</sub> on the IL-1β-induced production of cytokines in human tracheal epithelial cells, epithelial cells were pre-treated with bafilomycin A<sub>1</sub> (0.1 μM) for 24 h and then treated with IL-1β (200 pg/ml) in the presence of bafilomycin A<sub>1</sub> (0.1 μM) for 24 or 48 h. The concentration of IL-1β was matched to a net increase in the culture medium after RV14 infection.

*Effects of bafilomycin A<sub>1</sub> on ICAM-1 mRNA expression.* To study the effects of bafilomycin A<sub>1</sub> on ICAM-1 mRNA expression, human tracheal epithelial cells were treated with bafilomycin A<sub>1</sub> (0.1 μM) for either 1 or 24 h before RV14 infection (10<sup>5</sup> TCID<sub>50</sub> units/ml). The expression of ICAM-1 mRNA in cells was examined with Northern blot analysis before and 8, 24, 72, and 120 h after RV14 infection. We also studied the effects of EIPA (10 μM) and FR-168888 (10 μM) on the expression of ICAM-1 mRNA 72 h after RV14 infection. We used an average value from replicate cultures of the same trachea (*n* = 3) for analysis of the intensity of the ICAM-1-to-β-actin bands.

As previously demonstrated (36), endogenous IL-1β is associated with increased production of ICAM-1 after RV14 infection. To examine the effect of bafilomycin A<sub>1</sub> on the IL-1β-induced production of ICAM-1 mRNA in human tracheal epithelial cells, epithelial cells were treated with IL-1β (200 pg/ml) in the presence and absence of bafilomycin A<sub>1</sub> (0.1 μM) for 48 h. The concentration of IL-1β was matched to a net increase in the culture medium after RV14 infection.

*Isolation of nuclear extracts.* Nuclear extracts were prepared with the methods previously described (27, 47). For the isolation of nuclear extracts, all procedures were performed on ice. Human tracheal epithelial cells were washed with ice-cold PBS, harvested by scraping into PBS, and pelleted in a 1.5-ml microfuge tube at 1,850 *g* for 5 min. After these procedures were repeated one more time, the pellet was suspended in one packed cell volume of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated for 15 min. Membrane lysis was accomplished by adding 25 μl of 10% Nonidet P-40 followed by vigorous agitation. The nuclei were then collected by centrifugation, resuspended in 50 μl of extract buffer (20 mM HEPES, 420 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF), and agitated vigorously at 4°C for 15 min. After removal of debris by centrifugation, the protein concentration of the nuclear extract was determined. The nuclear extracts were then stored at -70°C until used.

*Electrophoretic mobility shift assay.* Electrophoretic mobility shift assays (EMSAs) were performed as previously described (27, 47). The radiolabeled double-strand oligonucleotide probe for the nuclear factor-κB (NF-κB) site was prepared by annealing complementary oligonucleotides and by end labeling with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide ki-

nase. The radiolabeled probe used was composed of the sequence 5'-GATCGAGGGGACTTTCCCTAGC-3' (Stratagene, La Jolla, CA). The labeled probes were purified by CHROMA SPIN + TE-10 (CLONTECH, Palo Alto, CA) and diluted with buffer (10 mM Tris·HCl and 1 mM EDTA) to the desired concentration. Equivalent amounts of nuclear protein were incubated with 2 µg of salmon sperm DNA and 2–5 fmol (20,000 dpm) of the radiolabeled probe for 20 min at room temperature in 20 µl of a buffer containing 10 mM HEPES (pH 7.9), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.25 mM DTT, 0.25 mM PMSF, 0.1 mM EDTA, and 10% glycerol. Resolution was accomplished by electrophoresing 12 µl of reaction solution on 4% nondenaturing polyacrylamide gels in Tris-borate-EDTA buffer (89 mM Tris·HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.0) for 60 min at 150 V at room temperature. Autoradiographic detection of the hybridized probe was performed by exposure to Kodak scientific imaging film for 48–72 h at -70°C.

**Supershift EMSA.** Supershift assays were used to determine which members of the NF-κB family were involved in RV14-induced NF-κB-DNA binding. In these studies, EMSAs were performed as described in *Electrophoretic mobility shift assay* except that rabbit polyclonal antibodies against the NF-κB subunit proteins p65, p50, c-Rel, and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA) were included in the 1-h radiolabeled probe-extract binding reaction at 4°C (27, 47). Preimmune serum (Santa Cruz Biotechnology) was used to control for any nonspecific effects of these antisera.

**Measurement of intracellular pH.** Human tracheal epithelial cells cultured in flasks in DMEM-Ham's F-12 medium containing 2% USG and antibiotics were collected by trypsinization (0.05% trypsin and 0.02% EDTA) and suspended in the Krebs-Ringer phosphate with dextrose solution. Human tracheal epithelial cells ( $5 \times 10^5$  cells) suspended in an Eppendorf tube were incubated at 37°C for 10 min in the Krebs-Ringer phosphate with dextrose solution, which contained 1 µg/ml of 2',7'-bis-(carboxyl)-5-(and-6)-carboxylfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR). After BCECF loading, the cells were centrifuged at 200 *g* for 5 min at 37°C and resuspended in a thermostated stirred cuvette, and fluorescence was measured with excitation at 490 nm and emission at 525 nm with 5-nm slits in a fluorescence spectrophotometer (Hitachi F-2000, Tokyo, Japan) as previously described (9). Intracellular pH (pH<sub>i</sub>) was calibrated with nigericin and K<sup>+</sup> as previously described (37). We found in the preliminary experiments that sodium propionate induced a transient cytosolic acidification of the epithelial cells followed by a prolonged alkalization. Therefore, we examined the effect of bafilomycin A<sub>1</sub> (0.1 µM), EIPA (10 µM), and FR-168888 (10 µM) on the recovery of pH<sub>i</sub>. The recovery of pH<sub>i</sub> after the addition of sodium propionate is expressed as the initial recovery rate of pH<sub>i</sub>. The initial recovery rate of pH<sub>i</sub> was determined by measuring the slope of the line tangent to the initial upstroke of the curve and calculating the change in pH<sub>i</sub> for 1 min (20).

**Measurement of changes in acidic endosome distribution.** Human tracheal epithelial cells were cultured on glass-bottom microwell dishes (Mat Tek, Ashland, MA). To examine the distribution of acidic endosomes in human tracheal epithelial cells, we stained the endosomes in the cells with a dye, LysoSensor Green DND-189 (Molecular Probes), according to the manufacturer's instructions (13). LysoSensor Green DND-189 detects pH values in the 4.5–6.0 range (13). The cells were washed twice with PBS at room temperature, incubated for 15 min in PBS containing 1 µM LysoSensor Green DND-189 and 5 mM glucose, and observed under confocal fluorescent microscopy (Leica DMRXE) with a ×40

objective. Images of acidic endosomes and differential interference microscopy of human tracheal epithelial cells were recorded on a Leica TCS NT system every 6 s for 400 s and used for analysis.

**Preparation of the drugs.** Bafilomycin A<sub>1</sub>, EIPA, and FR-168888 were dissolved in 100% dimethyl sulfoxide to give stock solutions of 0.1 mM bafilomycin A<sub>1</sub> and 10 mM EIPA and FR168888 and stored at 4°C. The stock solution was diluted in the culture medium as necessary for each experiment.

**Statistical analysis.** The results are expressed as means ± SE; *n* is the number of donors from whom cultured epithelial cells were used. Statistical analysis was performed with two-way repeated-measures ANOVA. Subsequent post hoc analysis was made with Bonferroni's method. For all analysis, values of *P* < 0.05 were assumed to be significant.

## RESULTS

**Effects of bafilomycin A<sub>1</sub> on RV infection in human tracheal epithelial cells.** Exposing confluent tracheal epithelial cell monolayers to RV14 (10<sup>5</sup> TCID<sub>50</sub> units/ml) consistently led to infection. The collection of culture medium at differing times after viral exposure revealed no detectable virus 1 h after infection. RV14 was detectable in the culture medium 6 h after infection, and the viral content progressively increased between 6 and 24 h after infection (Fig. 1A). Evidence of continuous viral production was obtained by demonstrating that the viral titers of supernatants collected for 1–3, 3–5, and 5–7 days after infection each contained significant levels of RV14 (Fig. 1B). Treatment of the cells with bafilomycin A<sub>1</sub> (0.1 µM) significantly decreased the viral titers in supernatants from 12 h after infection (Fig. 1).

The inhibitory effects of bafilomycin A<sub>1</sub> on RV14 infection were concentration dependent, and the maximum effect was obtained at 0.1 µM (Fig. 2A). Likewise, the inhibitory effects of bafilomycin A<sub>1</sub> (0.1 µM) on RV14 infection were dependent on the incubation period after exposure to RV14, and the minimum incubation time to cause inhibition of RV14 infection was obtained at 30 min (Fig. 2B). When the cells were pretreated with bafilomycin A<sub>1</sub> (0.1 µM) for 24 h and bafilomycin A<sub>1</sub> was then removed, the viral titers of RV14 in the supernatants became the same as those in the cells without bafilomycin A<sub>1</sub> pretreatment 5 days after infection (Fig. 2C). Furthermore, when the cells were treated with bafilomycin A<sub>1</sub> for 1 h during the exposure to RV14, the viral titers of RV14 in the supernatants became the same as those in the cells without bafilomycin A<sub>1</sub> treatment 3 days after infection (Fig. 2C). However, bafilomycin A<sub>1</sub> (0.1 µM) failed to inhibit the viral titers of RV14 in the supernatants when the cells were pretreated with bafilomycin A<sub>1</sub> for 1 h before exposure to RV14 ( $4.1 \pm 0.2$  TCID<sub>50</sub> units/ml in the presence of bafilomycin A<sub>1</sub> vs.  $4.2 \pm 0.2$  TCID<sub>50</sub> units/ml in the absence of bafilomycin A<sub>1</sub> 1 day after infection; *P* > 0.20; *n* = 7).

The viral titers of the cell lysates also contained significant levels of RV14. The viral titers of cell-associated RV14 were  $0.0 \pm 0.0$  log TCID<sub>50</sub> units at 1 h,  $0.1 \pm 0.1$  log TCID<sub>50</sub> units at 6 h,  $0.8 \pm 0.1$  log TCID<sub>50</sub>

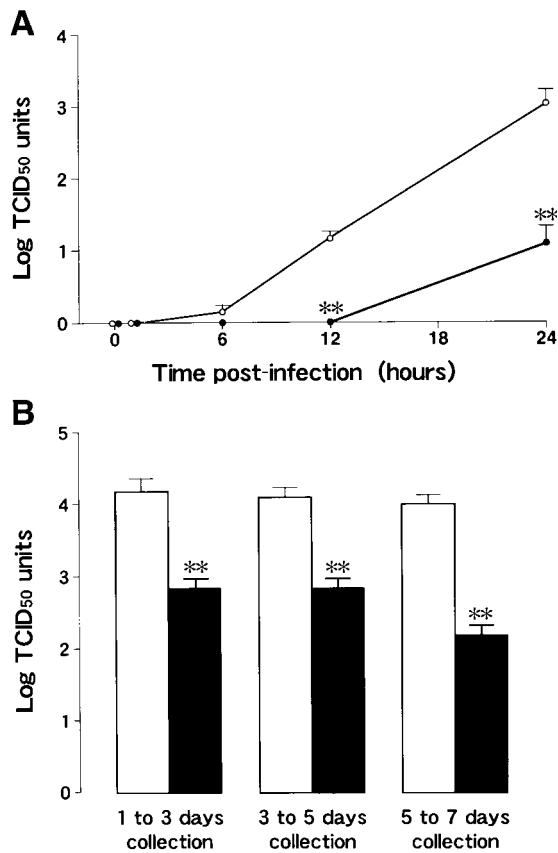


Fig. 1. A: viral titer in supernatants of human tracheal epithelial cells collected at sequential times during the 1st 24 h after exposure to  $10^5$  units of 50% tissue culture infectious dose (TCID<sub>50</sub>)/ml of rhinovirus (RV) 14 in the presence (●) and absence (○) of bafilomycin A<sub>1</sub> (0.1  $\mu$ M). B: viral titer in supernatants collected for indicated days after exposure to  $10^5$  TCID<sub>50</sub> units/ml of RV14 in the presence (solid bars) and absence (open bars) of bafilomycin A<sub>1</sub> (0.1  $\mu$ M). The cells were exposed to bafilomycin A<sub>1</sub> or vehicle for bafilomycin A<sub>1</sub> from 60 min before RV14 infection to day 7 after infection. Results are means  $\pm$  SE of separate experiments with cells from 7 different donors. \*\*Significant difference from viral infection alone,  $P < 0.01$ .

units at 12 h,  $2.2 \pm 0.2$  log TCID<sub>50</sub> units at 24 h,  $2.4 \pm 0.3$  log TCID<sub>50</sub> units at 3 days,  $2.2 \pm 0.2$  log TCID<sub>50</sub> units at 5 days, and  $1.8 \pm 0.2$  log TCID<sub>50</sub> units at 7 days ( $n = 7$  each). In the cell lysates, the viral titer levels also increased significantly with time ( $P < 0.05$  in each case by ANOVA). Bafilomycin A<sub>1</sub> (0.1  $\mu$ M) significantly decreased the cell-associated viral titers of RV14. The viral titers of cell-associated RV14 in bafilomycin A<sub>1</sub>-treated human tracheal epithelial cells were  $0.0 \pm 0.0$  log TCID<sub>50</sub> units at 1 h,  $0.0 \pm 0.0$  log TCID<sub>50</sub> units at 6 h,  $0.0 \pm 0.0$  log TCID<sub>50</sub> units at 12 h ( $P < 0.01$ ),  $0.7 \pm 0.1$  log TCID<sub>50</sub> units at 24 h ( $P < 0.05$ ),  $1.2 \pm 0.2$  log TCID<sub>50</sub> units at 3 days ( $P < 0.05$ ),  $1.1 \pm 0.2$  log TCID<sub>50</sub> units at 5 days ( $P < 0.05$ ), and  $1.0 \pm 0.1$  log TCID<sub>50</sub> units at 7 days ( $P < 0.05$ ;  $n = 7$  each).

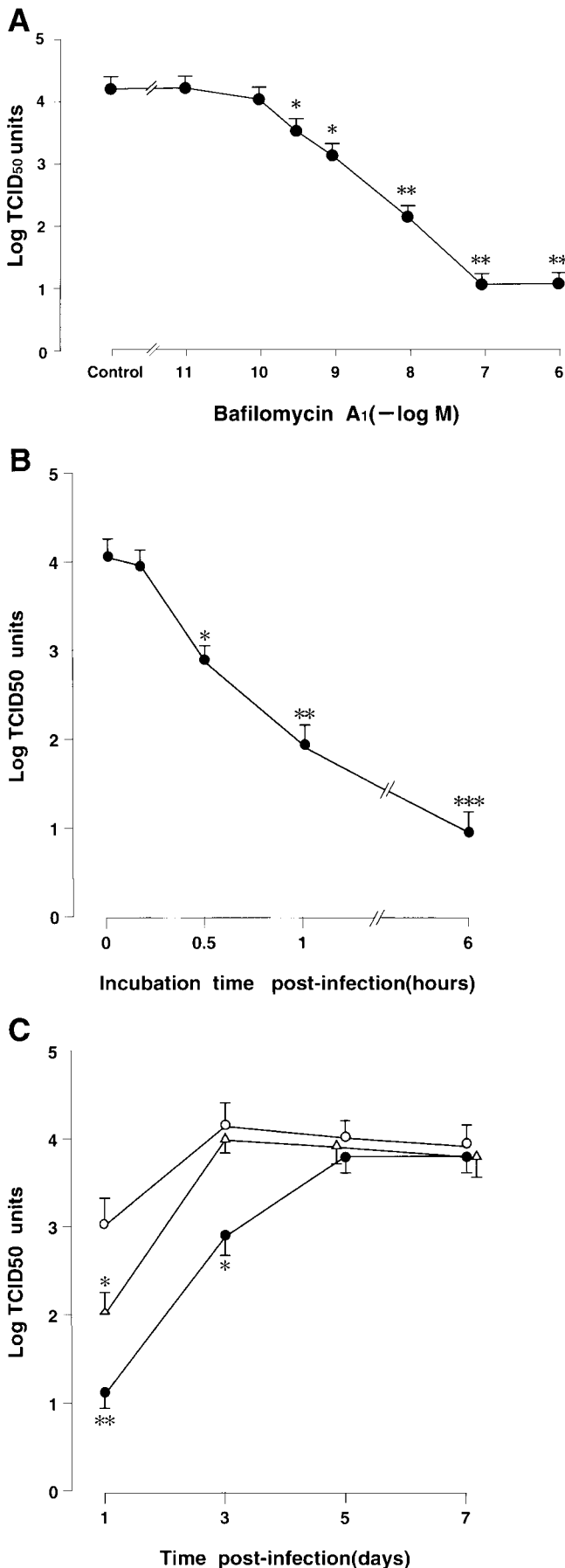
Human tracheal cell viability as assessed by the exclusion of trypan blue was consistently  $>96\%$  in the RV-infected and bafilomycin A<sub>1</sub> (0.1  $\mu$ M; 5 days)-treated cultures. Likewise, RV14 infection did not alter the amount of lactate dehydrogenase in the supernatants

( $30 \pm 3$  IU/l before vs.  $31 \pm 3$  IU/l 5 days after infection;  $P > 0.50$ ;  $n = 7$ ). Both RV14 infection ( $10^5$  TCID<sub>50</sub> units/ml; 5 days) and bafilomycin A<sub>1</sub> treatment (0.1  $\mu$ M; 5 days) also had no effect on cell number and total protein content when RV14 infection and bafilomycin A<sub>1</sub> treatment were performed after the cells had formed confluent cell sheets in the tubes. Cell counts after RV14 infection ( $1.8 \times 10^6 \pm 0.2 \times 10^6$  cells;  $P > 0.50$ ;  $n = 7$ ) and with bafilomycin A<sub>1</sub> treatment ( $1.8 \times 10^6 \pm 0.1 \times 10^6$  cells;  $P > 0.50$ ;  $n = 7$ ) were not significantly different from the control value ( $1.8 \times 10^6 \pm 0.1 \times 10^6$  cells;  $n = 7$ ). Total protein contents after RV14 infection ( $1,012 \pm 107$   $\mu$ g/ $10^6$  cells;  $P > 0.50$ ;  $n = 7$ ) and with bafilomycin A<sub>1</sub> treatment ( $1,023 \pm 155$   $\mu$ g/ $10^6$  cells;  $P > 0.50$ ;  $n = 7$ ) were not significantly different from the control value ( $1,015 \pm 124$   $\mu$ g/ $10^6$  cells;  $n = 7$ ). Exposure to bafilomycin A<sub>1</sub> for 5 days did not change the baseline electrical resistance and potential difference of the cultured human tracheal epithelial cells. The baseline electrical resistance 5 days after exposure to bafilomycin A<sub>1</sub> (0.1  $\mu$ M) was  $141 \pm 17$   $\Omega \cdot \text{cm}^2$  ( $P > 0.20$ ) compared with  $152 \pm 25$   $\Omega \cdot \text{cm}^2$  in control cells ( $n = 5$ ). The baseline potential difference 5 days after exposure to bafilomycin A<sub>1</sub> (0.1  $\mu$ M) was  $9.2 \pm 0.3$  mV compared with  $10.3 \pm 0.9$  mV in control cells ( $P > 0.20$ ;  $n = 5$ ). Likewise, EIPA (10  $\mu$ M) and FR-168888 (10  $\mu$ M) did not change the baseline electrical resistance. The baseline electrical resistance 5 days after exposure to EIPA (10  $\mu$ M) and FR-168888 (10  $\mu$ M) was  $148 \pm 22$  ( $P > 0.50$ ) and  $154 \pm 22$   $\Omega \cdot \text{cm}^2$  ( $P > 0.50$ ), respectively ( $n = 5$ ). EIPA (10  $\mu$ M) slightly decreased the potential difference, but FR-168888 (10  $\mu$ M) was without effect on the potential difference. The potential difference 5 days after exposure to EIPA (10  $\mu$ M) and FR-168888 (10  $\mu$ M) was  $7.4 \pm 1.1$  ( $P < 0.05$ ) and  $9.9 \pm 1.0$  mV ( $P > 0.50$ ), respectively ( $n = 5$ ).

Viral titers during the 1–3 days after RV14 infection in supernatants of the cells treated with EIPA (10  $\mu$ M;  $2.9 \pm 0.2$  log TCID<sub>50</sub> units/ml;  $P < 0.05$ ;  $n = 7$ ) and FR-168888 (10  $\mu$ M;  $3.1 \pm 0.2$  log TCID<sub>50</sub> units/ml;  $P < 0.05$ ;  $n = 7$ ) significantly decreased compared with that in control cells ( $4.2 \pm 0.3$  log TCID<sub>50</sub> units/ml;  $n = 7$ ), although the inhibitory effects of EIPA and FR-168888 were smaller than those of bafilomycin A<sub>1</sub> (0.1  $\mu$ M;  $1.1 \pm 0.2$  log TCID<sub>50</sub> units/ml;  $P < 0.05$ ;  $n = 7$ ).

**Effects of bafilomycin A<sub>1</sub> on the susceptibility to RV infection.** Pretreatment of human tracheal epithelial cells with bafilomycin A<sub>1</sub> (0.1  $\mu$ M) for 24 h decreased the susceptibility of cells to RV14 infection, increasing by 10-fold the minimum dose of virus necessary to cause infection ( $3.7 \pm 0.2$  log TCID<sub>50</sub> units/ml;  $P < 0.01$ ;  $n = 7$ ) compared with that in medium alone ( $2.1 \pm 0.2$  log TCID<sub>50</sub> units/ml;  $n = 7$ ). In contrast, 1 h of pretreatment with bafilomycin A<sub>1</sub> did not increase the susceptibility to RV14 infection (data not shown).

**Detection of viral RNA by PCR.** Further evidence of the effects of bafilomycin A<sub>1</sub>, EIPA, and FR-168888 on RV14 infection in human tracheal epithelial cells was provided by PCR analysis. In each of three experiments, RNA extracted from control uninfected cells did not produce any detectable PCR product at 381 bp (0



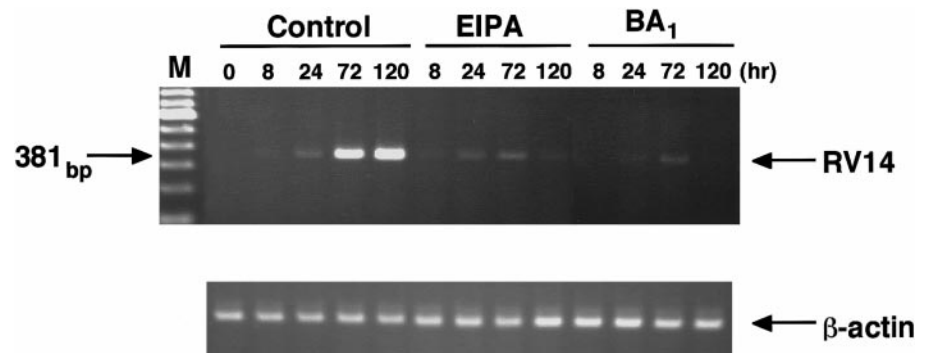
h). A product band was observed in RNA extracted from cells 24 h after infection followed by increases in viral RNA 72 and 120 h after infection (Fig. 3). The time course of the viral titers in the supernatants as detected by the cytopathic effects of viruses on human embryonic fibroblast cells was similar to that of the intensity of the RV RNA product extracted from infected cells detected by PCR analysis. Both bafilomycin A<sub>1</sub> (0.1 μM) and EIPA (10 μM) reduced the intensity of the RV RNA product extracted from infected cells (Fig. 3). Likewise, FR-168888 (10 μM) reduced the intensity of the RV RNA products extracted from infected cells (data not shown).

*Effects of bafilomycin A<sub>1</sub> on cytokine production.* Because both viral infection and bafilomycin A<sub>1</sub> treatment did not alter cell number, all cytokine values are reported in picograms per milliliter of supernatant. Figure 4 shows that secretion of IL-1β, IL-6, IL-8, and TNF-α all increased in response to RV14. However, increases in cytokine production in cells with UV-inactivated RV14 infection were similar to those in cells with a sham infection. Bafilomycin A<sub>1</sub> decreased RV14 infection-induced increases in the production of IL-1β, IL-6, IL-8, and TNF-α as well as the basal secretion of IL-1β and IL-8 (Fig. 4). The inhibitory effect of bafilomycin A<sub>1</sub> on the baseline production of IL-8 was smaller (76% of control value) than that of IL-1β (57% of control value), although bafilomycin A<sub>1</sub> decreased the production of IL-1β and IL-8 after RV14 infection to the baseline levels. In contrast, RV14 infection did not alter IL-1α production (13 ± 2 pg/ml 3 days after RV14 infection vs. 12 ± 2 pg/ml 3 days after sham infection; *P* > 0.20; *n* = 7). Of the cytokines measured, IFN-α, IFN-β, and IFN-γ were under the limit of detection of the assay in the supernatants from cells with RV14 and sham infections throughout the experiments.

IL-1β (200 pg/ml) increased the production of IL-6 (42 ± 3 pg/ml with IL-1β vs. 27 ± 2 pg/ml in control cells; *P* < 0.05; *n* = 7), IL-8 (671 ± 35 pg/ml with IL-1β vs. 592 ± 37 pg/ml in control cells; *P* < 0.05; *n* = 7), and

Fig. 2. A: concentration-response effect of bafilomycin A<sub>1</sub> on RV14 infection in human tracheal epithelial cells. Viral titers were determined in supernatants collected for 1–3 days after RV14 infection (10<sup>5</sup> TCID<sub>50</sub> units/ml). The cells were exposed to bafilomycin A<sub>1</sub> from 60 min before RV14 infection to day 3 after infection. Results are means ± SE of separate experiments with cells from 7 different donors. Significant difference from corresponding control values: \**P* < 0.05, \*\**P* < 0.01. B: effect of incubation time of bafilomycin A<sub>1</sub> (0.1 μM) on viral titer in supernatants collected for 1–3 days after exposure to 10<sup>5</sup> TCID<sub>50</sub> units/ml of RV14. The cells were exposed to bafilomycin A<sub>1</sub> for various times after RV14 infection. The cells were then cultured in the medium containing the vehicle for bafilomycin A<sub>1</sub>. Results are means ± SE of separate experiments with cells from 7 different donors. Significant difference from control value (day 0): \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. C: viral titer in supernatants 1, 3, 5, and 7 days after exposure to 10<sup>5</sup> TCID<sub>50</sub> units/ml of RV14. Cells were exposed to bafilomycin A<sub>1</sub> (0.1 μM) before RV14 infection for 24 h (●) or to bafilomycin A<sub>1</sub> (0.1 μM) during RV14 infection for 1 h (Δ). The cells were then cultured in the medium containing the vehicle for bafilomycin A<sub>1</sub>. ○, Control cells. Results are means ± SE of separate experiments with cells from 7 different donors. Significant difference from corresponding control value: \**P* < 0.05; \*\**P* < 0.01.

Fig. 3. Time course of replication of RV RNA in lysates of human tracheal epithelial cells after RV14 infection as detected by RT-PCR in the presence of either 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA; 10  $\mu$ M), bafilomycin A<sub>1</sub> (BA<sub>1</sub>; 0.1  $\mu$ M), or water as a vehicle for blockers (control). The cells were exposed to blockers or vehicle for blockers from 60 min before RV14 infection to 120 h after infection. A positive signal is demonstrated by a band at 381 bp.  $\beta$ -Actin was used as a housekeeping gene. M,  $\Phi$ X174-*Hinc*II fragment molecular weight marker. Data are representative of 3 different experiments.



TNF- $\alpha$  ( $3.2 \pm 0.2$  pg/ml with IL-1 $\beta$  vs.  $0 \pm 0$  pg/ml in control cells;  $P < 0.05$ ;  $n = 7$ ). However, bafilomycin A<sub>1</sub> failed to inhibit the production of these cytokines induced by IL-1 $\beta$  ( $41 \pm 3$  pg/ml of IL-6;  $659 \pm 36$  pg/ml of IL-8;  $2.9 \pm 0.2$  pg/ml of TNF- $\alpha$ ;  $P > 0.50$ ;  $n = 7$ ).

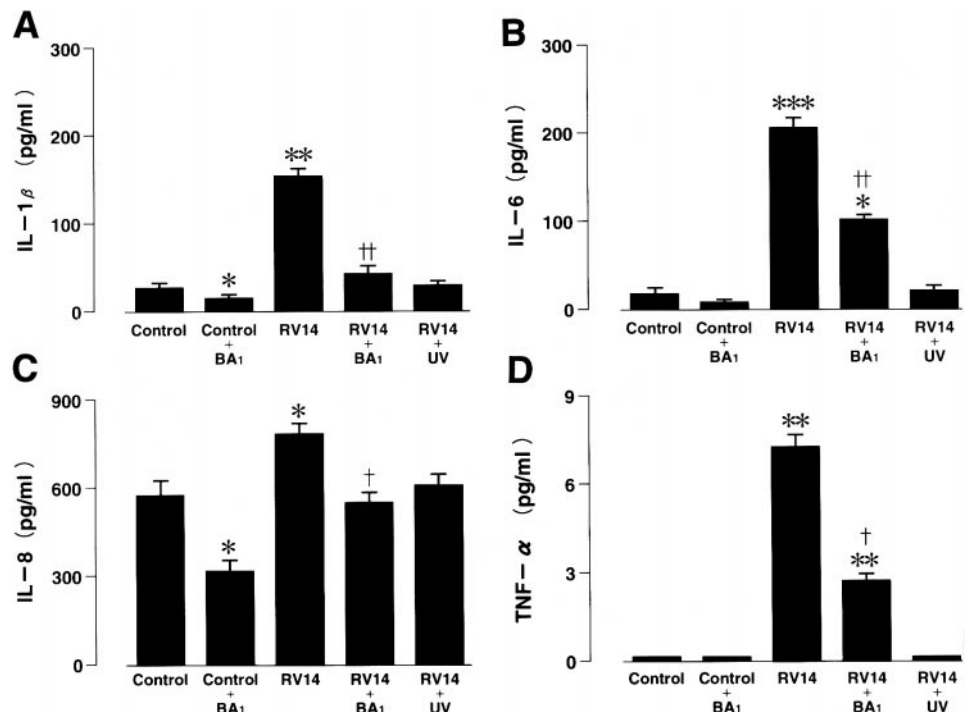
**Effects of bafilomycin A<sub>1</sub> on ICAM-1 mRNA expression.** Exposure of the cells to RV14 caused increases in ICAM-1 mRNA in the absence of bafilomycin A<sub>1</sub> (Fig. 5A). Human tracheal epithelial cells 3 days after RV14 infection were shown to overexpress ICAM-1 mRNA twofold compared with that 3 days after sham exposure (control; Fig. 5B). Bafilomycin A<sub>1</sub> (0.1  $\mu$ M) inhibited increases in ICAM-1 mRNA induced by RV14 infection without effects on  $\beta$ -actin mRNA (Fig. 5A) and significantly decreased ICAM-1 mRNA as well as baseline ICAM-1 mRNA expression 3 days after RV14 infection (Fig. 5B). However, preincubation with bafilomycin A<sub>1</sub> (0.1  $\mu$ M) for 1 h did not change ICAM-1 mRNA expression in the human tracheal epithelial cells ( $0.21 \pm 0.02$  scan units with bafilomycin A<sub>1</sub> vs.  $0.21 \pm 0.02$  scan units in control cells;  $P > 0.50$ ;  $n = 7$ ).

IL-1 $\beta$  (200 pg/ml) increased ICAM-1 mRNA ( $0.35 \pm 0.04$  scan units vs.  $0.20 \pm 0.02$  scan units in control cells;  $P < 0.01$ ;  $n = 7$ ). In contrast, bafilomycin A<sub>1</sub> failed to inhibit IL-1 $\beta$ -induced-ICAM-1 mRNA expression ( $0.34 \pm 0.03$  scan units;  $P > 0.50$ ;  $n = 7$ ).

EIPA (10  $\mu$ M for 72 h) and FR-168888 (10  $\mu$ M for 72 h) inhibited RV14 infection-induced increases in mRNA expression of ICAM-1 ( $0.35 \pm 0.03$  scan units with EIPA and  $0.37 \pm 0.03$  scan units with FR-168888;  $P < 0.05$ ;  $n = 7$ ) compared with that in control cells ( $0.46 \pm 0.03$  scan units;  $n = 7$ ), although the inhibitory effects of EIPA and FR-168888 were smaller than those of bafilomycin A<sub>1</sub> (0.1  $\mu$ M;  $0.24 \pm 0.03$  scan units;  $P < 0.01$ ;  $n = 7$ ).

**NF- $\kappa$ B DNA binding activity in human tracheal epithelial cells.** Nuclear extracts from the human tracheal epithelial cells with RV14 or sham infection contained activated NF- $\kappa$ B as demonstrated by the presence of a complex consisting of a protein bound to a DNA fragment carrying NF- $\kappa$ B (Fig. 6). The baseline intensity of NF- $\kappa$ B binding activity was constant, and

Fig. 4. Effect of BA<sub>1</sub> (0.1  $\mu$ M) on the release of either interleukin (IL)-1 $\beta$  (A), IL-6 (B), IL-8 (C), or tumor necrosis factor (TNF)- $\alpha$  (D) into culture supernatants of human tracheal epithelial cells after RV14, ultraviolet (UV)-inactivated RV14, or sham (control) infection. The effects of BA<sub>1</sub> were examined at maximal production of each cytokine after RV14 infection. The cells were exposed to BA<sub>1</sub> or vehicle for BA<sub>1</sub> from 24 h before RV14 infection to the end of the experiments. Results are means  $\pm$  SE of separate experiments with cells from 7 different donors. Significant difference from corresponding control value: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Significant difference from RV14 infection alone: † $P < 0.05$ ; †† $P < 0.01$ .





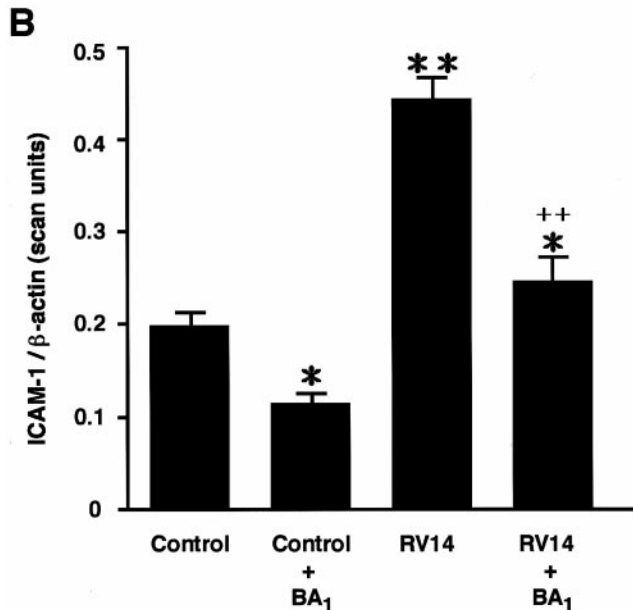
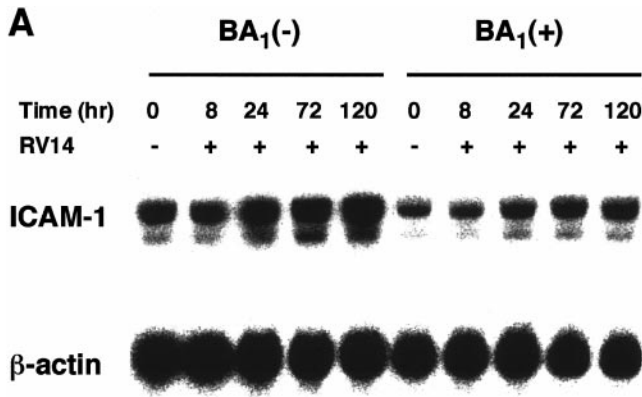


Fig. 5. A: Northern blot analysis for intercellular adhesion molecule (ICAM)-1 mRNA levels in human tracheal epithelial cells before (0) and 8, 24, 72, and 120 h after RV14 infection in the presence (+) and absence (-) of BA<sub>1</sub> (0.1 μM). β-Actin was used as a housekeeping gene. B: effects of BA<sub>1</sub> (0.1 μM) on the expression of ICAM-1 mRNA in human tracheal epithelial cells 3 days after RV14 or sham (control) infection. The cells were exposed to BA<sub>1</sub> or vehicle for BA<sub>1</sub> from 24 h before RV14 infection to the end of the experiments. ICAM-1 mRNA was normalized to constitutive expression of β-actin mRNA. Results are means ± SE of separate experiments with cells from 7 different donors. Significant difference from corresponding control value: \**P* < 0.05; \*\**P* < 0.01. ++ Significant difference from RV14 infection alone, *P* < 0.01.

increased activation of NF-κB was present in cells from 30 min after RV14 infection (Fig. 6). Bafilomycin A<sub>1</sub> (0.1 μM) reduced the increased activation of NF-κB by RV14 infection as well as the baseline intensity of NF-κB binding activity (Fig. 6). Specificity of the NF-κB binding was confirmed by supershift EMSA in which antibodies to the p50 or p65 subunit of NF-κB ablated NF-κB bands (Fig. 7). Supershifting of the NF-κB band with the antibody to the p50 or p65 subunit of NF-κB was constantly observed at any time of cell culture. However, supershifting of the NF-κB band

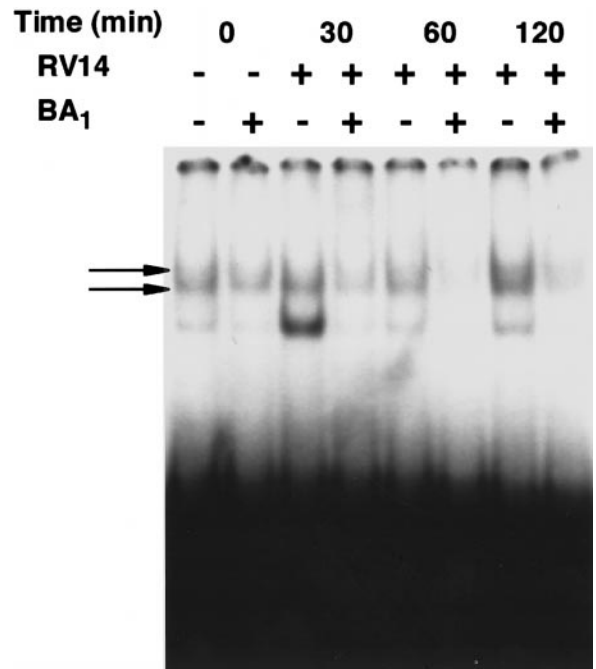


Fig. 6. Electrophoretic mobility shift assay demonstrating the effects of BA<sub>1</sub> (0.1 μM) on the increases in nuclear factor (NF)-κB DNA binding activity of human tracheal epithelial cells before (0) and 30, 60, and 120 min after RV14 infection. The cells were exposed to BA<sub>1</sub> or vehicle for BA<sub>1</sub> from 24 h before RV14 infection to the end of the experiments. Arrows, RV14-induced NF-κB DNA binding activity.

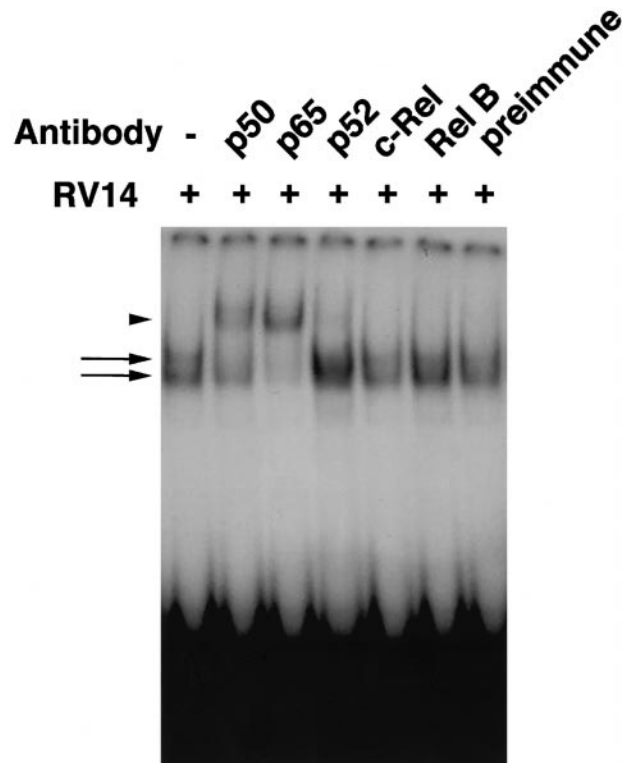


Fig. 7. Identification of RV14-induced NF-κB bands with supershift electrophoretic mobility shift assay (EMSA). EMSAs were performed in the presence and absence of antisera against NF-κB family proteins, and antisera against p50, p65, p52, c-Rel, and Rel B were compared with preimmune serum. Arrows, RV14-induced NF-κB DNA binding activity. Arrowhead, supershifted bands caused by antisera against p50 and p65.

was not observed with the antibody to either p52, c-Rel, Rel B, or preimmune antiserum (Fig. 7).

**Effects of blockers of V-ATPases and NHEs on  $pH_i$ .** Sodium propionate (10  $\mu$ M) induced a transient cytosolic acidification of the epithelial cells followed by a prolonged alkalization (Fig. 8A). Bafilomycin A<sub>1</sub>, EIPA, and FR-168888 each inhibited the alkalization of the epithelial cells after the addition of sodium propionate without changes in the baseline  $pH_i$  (Fig. 8, B–D). The potency order of the inhibitory effects on alkalization after sodium propionate was similar among these three blockers (Fig. 8E). The inhibitory effects of bafilomycin A<sub>1</sub> was concentration dependent, and the maximum effect was obtained at 0.1  $\mu$ M (Fig. 8F).

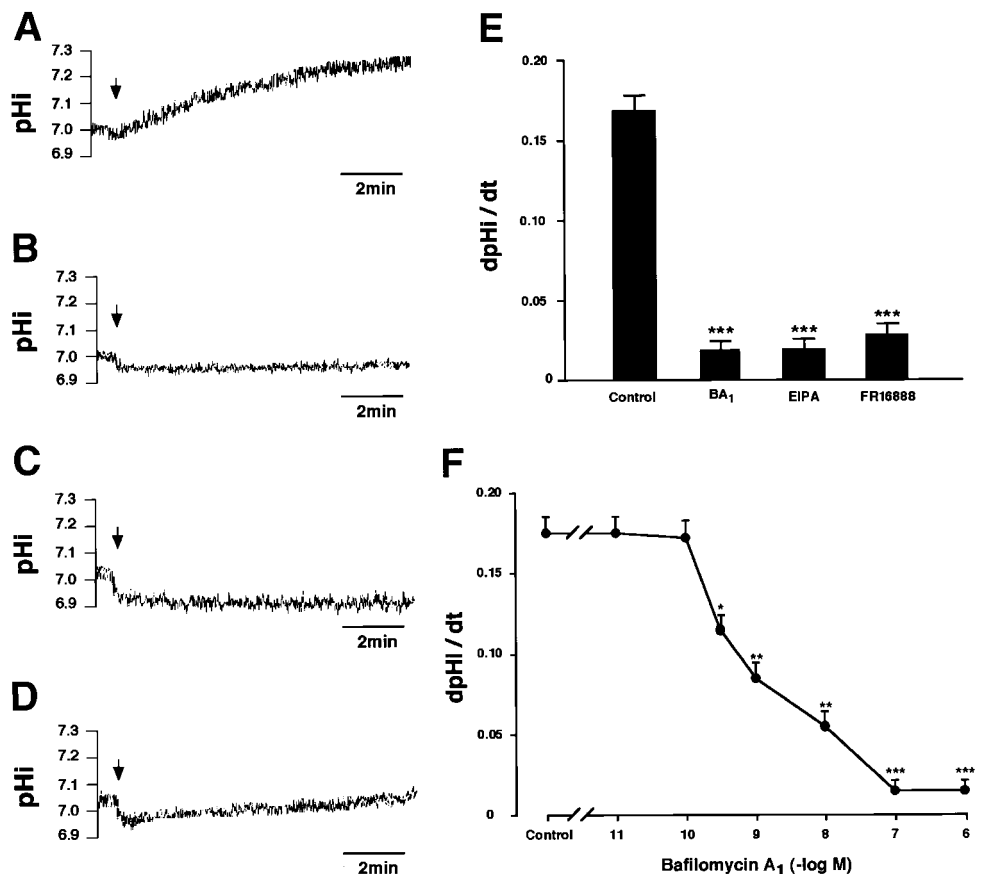
**Effects of bafilomycin A<sub>1</sub> on the acidification of endosomes.** Acidic endosomes in human tracheal epithelial cells were stained green with a dye, LysoSensor Green DND-189. Figure 9 shows the time-course changes in the distribution and the fluorescence intensity of acidic endosomes with green fluorescence in the human tracheal epithelial cells. Green fluorescence was observed in a granular pattern in the cytoplasm. The distribution and intensity of the green fluorescence were constant for at least 300 s in the epithelial cells cultured in PBS alone (Fig. 9, A and C). Bafilomycin A<sub>1</sub> (0.1  $\mu$ M) significantly decreased the number and fluorescence intensity of the acidic endosomes with green fluorescence in the human tracheal epithelial cells at 50 s, and

the fluorescence intensity in the cells was markedly reduced 300 s after the addition of bafilomycin A<sub>1</sub> (Fig. 9, B and C). Likewise, either EIPA (10  $\mu$ M) or FR-168888 (10  $\mu$ M) decreased the green fluorescence intensity of acidic endosomes in the human tracheal epithelial cells (Fig. 9C). The potency order of the inhibitory effects based on fluorescence intensity was bafilomycin A<sub>1</sub> > EIPA > FR-168888 (Fig. 9C).

## DISCUSSION

In the present study, we have shown that a specific inhibitor of V-ATPase, bafilomycin A<sub>1</sub>, reduced the viral titers of a major RV subgroup, RV14, in the supernatants and reduced the cell-associated viral titers and viral RNA in cultured human tracheal epithelial cells. The inhibitory effects on the viral titers of RV14 were observed by incubation with bafilomycin A<sub>1</sub> either during or after RV14 infection. Furthermore, the inhibitory effects of bafilomycin A<sub>1</sub> on RV14 infection were obtained within 30 min after RV14 infection and at a concentration of bafilomycin A<sub>1</sub> > 1 pM. These findings are consistent with previous reports for HeLa cells and MDCK cells (26, 28). Furthermore, bafilomycin A<sub>1</sub> decreased the number of acidic endosomes distributed in a granular pattern in the cytoplasm of human tracheal epithelial cells. Therefore, bafilomycin A<sub>1</sub> may increase the pH of endosomes and inhibit RV14 infection via the inhibitory effects on the entry of RV14

Fig. 8. Typical time courses of intracellular pH ( $pH_i$ ) in response to sodium propionate (10  $\mu$ M) in the absence (A) and presence of BA<sub>1</sub> (0.1  $\mu$ M; B), EIPA (10  $\mu$ M; C), or FR-168888 (10  $\mu$ M; D). Arrows, time of administration of sodium propionate. E: effects of BA<sub>1</sub> (0.1  $\mu$ M), EIPA (10  $\mu$ M), and FR-168888 (10  $\mu$ M) on changes in  $pH_i$  ( $dpH_i/dt$ ) after acidification by sodium propionate (10  $\mu$ M). Results are means  $\pm$  SE of separate experiments with cells from 7 different donors. \*\*\*Significant difference from corresponding control value,  $P < 0.001$ . F: concentration-response effects of BA<sub>1</sub> on  $dpH_i/dt$  after acidification by sodium propionate (10  $\mu$ M). The cells were exposed to blockers or vehicle for blockers from 5 min before the addition of sodium propionate to the end of the experiments. Results are means  $\pm$  SE of separate experiments with cells from 7 different donors. Significant difference from corresponding control value: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



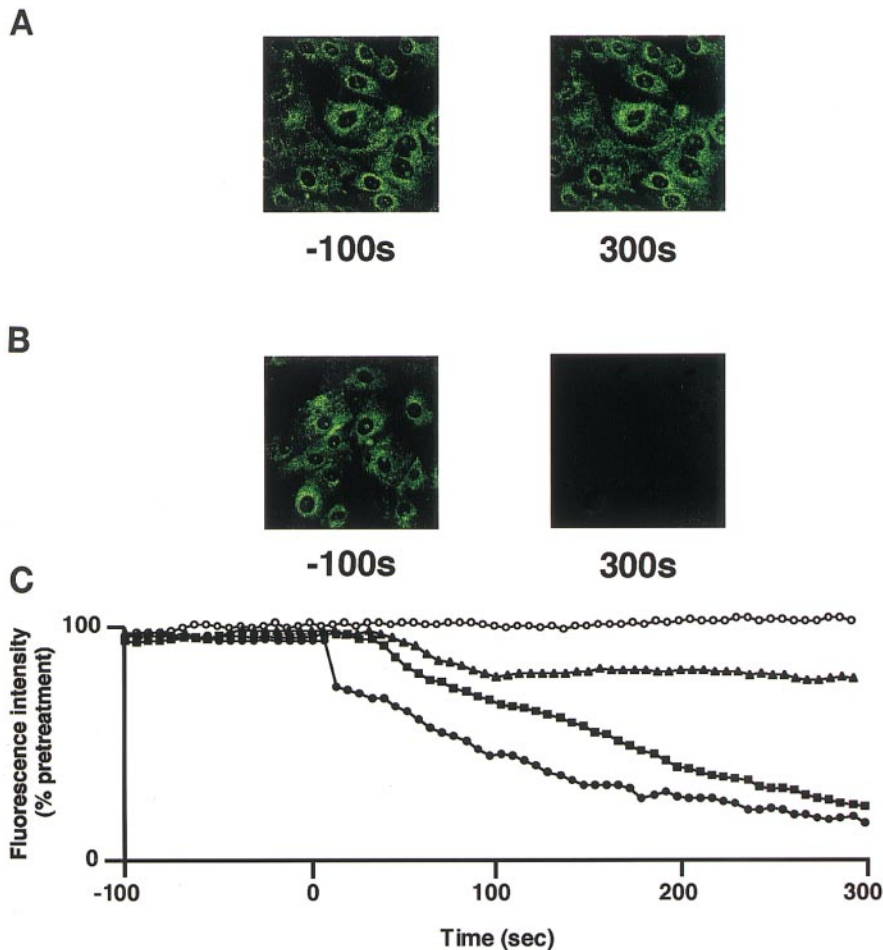


Fig. 9. Changes in the distribution of acidic endosomes with green fluorescence in human tracheal epithelial cells 100 s before (–100s) and 300 s after treatment with BA<sub>1</sub> (0.1 μM; *B*) or vehicle for BA<sub>1</sub> (control; *A*). *C*: time-course changes in the intensity of green fluorescence from acidic endosomes in human tracheal epithelial cells after treatment with either BA<sub>1</sub> (0.1 μM; ●), EIPA (10 μM; ■), FR-168888 (10 μM; ▲), or vehicle for BA<sub>1</sub> (control; ○). Inhibitors were administrated at *time 0*. Acidic endosomes in human tracheal epithelial cells were stained green with a dye, LysoSensor Green DND-189. Data are representative of 3 different experiments.

RNA from acidic endosomes to the cytoplasm in the airway epithelial cells as reported in HeLa cells (2, 28).

Furthermore, pretreatment with bafilomycin A<sub>1</sub> for 24 h also inhibited the viral titers of RV14 and ICAM-1 mRNA expression and reduced the susceptibility to RV14 in the present study. The viral titers of the supernatants recovered 5 days after removal of bafilomycin A<sub>1</sub> when the cells were pretreated with bafilomycin A<sub>1</sub> for 24 h. These findings suggest that bafilomycin A<sub>1</sub> may also inhibit infection of RV14 by reducing its receptor, ICAM-1 (12), in airway epithelial cells. In contrast, 1 h of bafilomycin A<sub>1</sub> pretreatment failed to inhibit RV14 infection, probably due to wash-out of bafilomycin A<sub>1</sub> during 1 h of RV14 infection. Furthermore, 1 h of bafilomycin A<sub>1</sub> pretreatment did not increase the susceptibility to RV14 infection, suggesting that 1 h of bafilomycin A<sub>1</sub> pretreatment might not reduce the production of ICAM-1, the receptor for RV14 (12), in the human tracheal epithelial cells. Brefeldin A, another macrolide antibiotic, is reported to block protein glycosylation and RNA replication of vesicular stomatitis virus (17). Further study is needed to examine the precise mechanisms.

Recent reports (2, 3, 7, 15, 28, 29) revealed the mechanisms of RV entry into the cells. RV14 forms RV-soluble ICAM-1 complexes, and these complexes can release viral RNA (7, 15). Furthermore, RV14

releases RNA after exposure to acidic pH (7), and infection of RV14 in HeLa cells is inhibited by bafilomycin A<sub>1</sub> (28). Therefore, the entry of RV14 RNA into the cells is suggested to be mediated by the destabilization after binding to ICAM-1, by endosomal acidification, or both (7).

pH<sub>i</sub> is suggested to be regulated by ion transport mechanisms across various antiporters and exchangers such as Na<sup>+</sup>/H<sup>+</sup> antiporters (NHEs), H<sup>+</sup>-ATPases (V-ATPase), and Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchangers in the cell membrane and vacuoles (19, 35). Endosomal pH is also suggested to be regulated by ion transport across the endosomal V-ATPase and NHEs (10, 21, 23, 46). In the present study, EIPA and FR-168888, inhibitors of NHEs, and bafilomycin A<sub>1</sub>, an inhibitor of V-ATPase, reduced pH<sub>i</sub> and increased endosomal pH. These findings are consistent with previous reports (19, 21, 39). Therefore, both NHEs and V-ATPase may be distributed in both the cell membrane and the endosomes in human tracheal epithelial cells and may regulate pH<sub>i</sub> and endosomal pH. Furthermore, the potency order of the inhibitory effects of bafilomycin A<sub>1</sub>, EIPA, and FR-168888 on the RV titers was correlated with that of the inhibitory effects on the fluorescence intensity of the acidic endosomes. RV14 may therefore enter human tracheal epithelial cells across the acidic endosome caused by NHEs and V-ATPase.

Bafilomycin A<sub>1</sub> inhibited not only RV14 infection but also the production of cytokines when the cells were treated with bafilomycin A<sub>1</sub> before and after RV14 infection in the present study. In contrast, 1 h of pretreatment with bafilomycin A<sub>1</sub> failed to inhibit the production of cytokines. Short periods of bafilomycin A<sub>1</sub> pretreatment might not be sufficient for the inhibition of cytokine production in the subsequent epithelial cell culture for 24 or 48 h. The role of V-ATPases on the production of cytokines in the airway epithelial cells is uncertain. However, V-ATPases have a number of functions such as providing the acidic environment associated with the dissociation of internalized ligand-receptor complexes within endosomes and activating the secreted lysosomal enzymes and degradation of the bone matrix (10). Furthermore, acidic pH provided by V-ATPases appears to play a role in the delivery of soluble proteins in the cells (42). Therefore, it is possible that bafilomycin A<sub>1</sub> may affect the production of cytokines by, in part, inhibiting the function of intracellular organelles such as Golgi apparatus (10, 22).

The inhibitory effect of bafilomycin A<sub>1</sub> on the baseline production of IL-8 was smaller (76% of control value) than that of IL-1 $\beta$  (57% of control value), although bafilomycin A<sub>1</sub> decreased the production of IL-1 $\beta$  and IL-8 after RV14 infection to the baseline levels. The precise reason is uncertain. However, the inhibitory effects of bafilomycin A<sub>1</sub> on cytokine production may differ among cytokine species because the amount of cytokine production differed among cytokine species in the same cell sheets in the present study.

Nuclear extracts from the human tracheal epithelial cells cultured in medium alone contained activated NF- $\kappa$ B, and bafilomycin A<sub>1</sub> reduced the activated NF- $\kappa$ B before RV14 infection in the present study. Furthermore, increased activation of NF- $\kappa$ B was present in the cells from 0.5 h after RV14 infection. Bafilomycin A<sub>1</sub> also inhibited the increased activation of NF- $\kappa$ B after RV infection. The time course of NF- $\kappa$ B activation is consistent with previous reports (27, 47) in airway epithelial cells caused by RV14 or RV16 infection. RV infection increases the production of proinflammatory cytokines and ICAM-1 in airway epithelial cells (27, 34, 36, 47). The expression of genes for many cytokines and ICAM-1 induced by RV infection are suggested to be mediated by the activation of NF- $\kappa$ B (27, 47). Furthermore, inhibition of NF- $\kappa$ B activation by bafilomycin A<sub>1</sub> in human tracheal epithelial cells is consistent with a previous report (45) in B lymphocytes and monocyte-like cell lines. Therefore, bafilomycin A<sub>1</sub> might reduce production of cytokines and ICAM-1 mRNA before and after RV14 infection via, in part, inhibiting the activation of NF- $\kappa$ B in epithelial cells.

In the present study, we observed that RV14 infection increased production of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  in cultured human tracheal epithelial cells. Of these, IL-1 $\beta$  is suggested to upregulate ICAM-1 expression in cultured human tracheal epithelial cells (36). IL-1 $\beta$  is a potent inflammatory cytokine that induces growth and differentiation of T and B lymphocytes,

other cytokine production, prostaglandin E<sub>2</sub> synthesis, and degranulation from neutrophils (1). IL-1 $\beta$  also causes increases in ICAM-1 expression on both epithelial and vascular endothelial cells (8, 38). The epithelial cells in the human airway express ICAM-1 on their surface, which is the site of attachment for 90% of the ~100 RV serotypes (12, 32). ICAM-1 interacts physiologically with leukocyte function-associated antigen-1, expressed on leukocytes, and thus plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation. Recent studies (27, 31, 36) have shown that RV infection upregulates ICAM-1 expression on the airway epithelial cells, an effect that would facilitate viral cell attachment and entry. Increases in ICAM-1 mRNA expression on human tracheal epithelial cells induced by RV14 infection in the present study are in accord with those in the previous studies.

In summary, the specific V-ATPase blocker bafilomycin A<sub>1</sub> may inhibit RV14 infection in human airway epithelial cells by blocking RNA entry into the endosomes. Furthermore, bafilomycin A<sub>1</sub> may decrease the susceptibility of cultured human tracheal epithelial cells to RV14 infection, probably via inhibition of ICAM-1, and reduce inflammatory cytokine production by human tracheal epithelial cells in RV14 infection. Bafilomycin A<sub>1</sub> failed to inhibit IL-1 $\beta$ -induced increases in the production of IL-6, IL-8, TNF- $\alpha$ , and mRNA of ICAM-1. In contrast, bafilomycin A<sub>1</sub> inhibited RV14 infection-induced production of cytokines and ICAM-1, suggesting that the effects of bafilomycin A<sub>1</sub> were specific for the RV14 infection-induced cell activation. These findings suggest that bafilomycin A<sub>1</sub> may modulate inflammatory responses in airway epithelial cells after RV14 infection.

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