Inhibition of Na⁺ Transport in Lung Epithelial Cells by Respiratory Syncytial Virus Infection

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We investigated the mechanisms by which respiratory syncytial virus (RSV) infection decreases vectorial Na⁺ transport across respiratory epithelial cells. Mouse tracheal epithelial (MTE) cells from either BALB/c or C57BL/6 mice and human airway H441 cells were grown on semipermeable supports under an air-liquid interface. Cells were infected with RSV-A2 and mounted in Ussing chambers for measurements of short-circuit currents (*I*_{sc}). Infection with RSV for 24 hours (multiplicity of infection = 1) resulted in positive immunofluorescence for RSV antigen in less than 10% of MTE or H441 cells. In spite of the limited number of cells infected, RSV reduced both basal and amiloride-sensitive *I*_{sc} in both MTE and H441 cells by approximately 50%, without causing a concomitant reduction in transepithelial resistance. Agents that increased intracellular cAMP (forskolin, cpt-cAMP, and IBMX) increased mainly Cl[−] secretion in MTE cells and Na⁺ absorption in H441 cells. RSV infection for 24 hours blunted both variables. In contrast, ouabain sensitive *I*_{sc} measured across apically permeabilized H441 monolayers, remained unchanged. Western blot analysis of H441 cell lysates demonstrated reductions in α- but not γ-ENaC subunit protein levels at 24 hours after RSV infection. The reduction in amiloride-sensitive *I*_{sc} in H441 cells was prevented by pretreatment with inhibitors of *de novo* pyrimidine or purine synthesis (A77-1726 and 6-MP, respectively, 50 μM). Our results suggest that infection of both murine and human respiratory epithelial cells with RSV inhibits vectorial Na⁺ transport via nucleotide release. These findings are consistent with our previous studies showing reduced alveolar fluid clearance after RSV infection of BALB/c mice.

Keywords: short circuit current; epithelial Na⁺ channels; H441 cells; uridine triphosphate; A77-1726

Respiratory syncytial virus (RSV) is a member of the pneumovirus genus of the *paramyxoviridae*, and has a negative-sense, nonsegmented, single-stranded RNA genome. It is the most common cause of lower respiratory tract disease in infants and children worldwide (1), a frequent initiator of acute asthma exacerbations in young children, and has a disease impact comparable to that of nonpandemic influenza A in the elderly (2). In the United States, 50 to 70% of all infants are infected with RSV in the first year of life, and approximately 2 to 3% of all cases of RSV bronchiolitis result in severe hypoxemia, requiring hospitalization (3). RSV infections are responsible for a signif-

CLINICAL RELEVANCE

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract disease in infants and children worldwide. We have conducted *in vitro* studies to elucidate the mechanisms by which RSV causes fluid accumulation in the lungs.

icant fraction of community-acquired lower respiratory track infections among adults (4) and cause significant morbidity and mortality in immunocompromised adults (5). Murata and Falsey (6) reported that approximately 170,000 hospitalizations and 10,000 deaths associated with RSV occur annually in persons over 65 years old in the United States alone. Importantly, repeated infections are common in all age groups, and previous infection is not protective.

There is no safe and effective RSV vaccine currently available, and specific antiviral pharmacotherapy for RSV is of questionable efficacy (7). In some cases, RSV infections are known to progress to acute respiratory distress syndrome (8). Thus, understanding the basic mechanisms responsible for RSV lung pathology may lead to new modes of treatment.

Respiratory epithelial cells are the primary replication site for RSV (9). Active, vectorial transport of sodium (Na⁺) and chloride (Cl[−]) ions across the airway lumen are seminal functions of bronchoalveolar epithelial cells, and is vital for clearance of fluid from airspaces as well as the proper hydration of mucus (10, 11). Previously we have shown that infection of BALB/c mice with RSV impaired alveolar fluid clearance (AFC, an *in vivo* measure of the ability of the bronchoalveolar epithelium to actively transport Na⁺ ions) at 48 to 96 hours after infection, which results in increased lung water and hypoxemia (12, 13). Nasal potential differences (NPD) in RSV-infected mice also became more positive, reflecting a decreases of either Cl[−] secretion or Na⁺ absorption across nasal epithelial cells (12). Concomitantly, RSV infection also increases levels of uridine and adenosine-5'-triphosphate (UTP and ATP, respectively) in bronchoalveolar lavage fluid of BALB/c mice (12).

Although our *in vivo* studies have provided much useful information regarding the effects of RSV infection on bronchoalveolar epithelial cell Na⁺ transport and lung fluid clearance, measurements of AFC and NPD (and even their amiloride-sensitive components) provide only limited information as to mechanisms by which RSV decreases active epithelial Na⁺ transport. Therefore, we have been unable to fully elucidate whether this effect is due to damage of apical epithelial Na⁺ channels transporters (mainly ENaC) or the basolaterally located Na⁺/K⁺ ATPase. Furthermore, it remains unclear from these *in vivo* studies whether altered AFC after RSV infection is a consequence of viral replication *per se* or results from the inflammatory response to the virus (12).

Herein, we isolated mouse tracheal epithelial cells from either C57BL/6 or BALB/c mice using two different methods

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based on the original report of Clarke and coworkers (14). We then infected both MTE and H441 cells, a human Clara cell line that expresses both ENaC and CFTR (15) with RSV strain A2, and measured both basal and forskolin-stimulated short circuit currents (I_{sc}) and levels of α - and γ -ENaC by immunofluorescence and Western blotting. These measurements were then repeated in H441 cells after inhibition of purine and pyrimidine synthesis. Our findings indicate that replicating RSV virus *per se* decreases vectorial amiloride-sensitive Na⁺ transport by inhibiting ENaC and not Na,K-ATPase via UTP-related mechanisms, in spite of infecting a small fraction of epithelial cells. Furthermore, agents that increase intracellular cAMP increase vectorial Na⁺ and Cl⁻ transport across RSV-infected monolayers, but their effect is considerably blunted compared with mock-infected monolayers. These findings provide new insights as to the mechanisms by which RSV damages vectorial Na⁺ transport *in vivo*.

MATERIALS AND METHODS

Preparation of Viral Inocula

Viral stocks were grown in monolayers of HEP-2 cells. Cells and media were harvested when 80 to 90% cytopathic effect was observed, and centrifuged to remove cell debris. Media was further clarified by ultracentrifugation through 35% sucrose (16). Virions were resuspended in fresh media, aliquoted, and rapidly frozen at -80°C. Viral titers were determined by serial dilution and plaque assay in Vero cells under agar (17). Plaque-forming units (PFU/ml of original sample) were calculated. Virus preparations were checked for mycoplasma contamination by PCR using the Mycoplasma *Plus* PCR primer set (Stratagene, La Jolla, CA) and HotStarTaq DNA polymerase (Qiagen, Valencia, CA), in accordance with manufacturer's instructions. Endotoxin content of viral stocks was determined by a standard *Limulus* amoebocyte assay. Stocks in which mycoplasma or endotoxin contamination were detected were discarded. A mock-infected HEP-2 media stock, prepared in an identical fashion, served as a control to account for possible effects of cellular components in the viral inoculum.

Preparation of Mouse Tracheal Epithelial Cell Monolayers

We used two different protocols to isolate mouse tracheal epithelial cells from both BALB/c and C57BL/6 mice. Both protocols are based on the original methodology of Clarke and colleagues (14) with important modifications. As described below and in RESULTS, MTE cells isolated with method A have low baseline I_{sc} that were partially inhibited by amiloride, while those isolated by method B have higher I_{sc} that were almost completely inhibited by amiloride. These differences are due entirely to composition of the culture media, since similar results were obtained with method B from either species. Furthermore, BALB/c and C57BL/6 mice have very similar levels of AFC and NDP values (13, 18). These two methods are described in detail below:

Method A. C57BL/6 mice (male, 8–12 wk old; 20–25 g body weight [BW]) were killed with intraperitoneal injections of ketamine (8.7 mg/100 g BW; Phoenix Scientific, St. Joseph, MO) and xylazine (1.3 mg/100 g BW; Vedco, St. Joseph, MO). The trachea proximal to the bronchial bifurcation was isolated and removed. The tracheae were then dissected and placed into 50-ml conical tubes and washed twice with 5 ml of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing 500 U/ml penicillin, 500 μ M streptomycin, 500 ng/ml fungizone, and 25 μ g/ml gentamycin. Subsequently they were washed three times with 5 ml of Ca²⁺- and Mg²⁺-free Dulbecco's modified Eagle's medium (DMEM) containing the same antibiotics. While immersed in the second medium, tracheae were dissected further to remove trachealis muscle and extraneous connective tissue and were filleted open.

The tracheae were then immersed in a digestion medium (on ice) consisting of Ca²⁺- and Mg²⁺-free DMEM, containing the aforementioned antibiotics and 1% Protease XIV and 0.05% DNase I and incubated without agitation overnight at 4°C. Fetal bovine serum (FBS) (10%) was added subsequently to quell enzyme activity. Tracheae-containing conical tubes were inverted 22 times to dislodge cells, and the supernatant was collected. An equal volume of DMEM/F12 culture medium (containing in addition to the above reagents, 12% FBS, 5 ml

of 100X L-glutamine stock, 200 U/ml penicillin/200 μ M streptomycin, 250 ng/ml fungizone and 12.5 μ g/ml gentamycin, 2 ml of an insulin-transferrin-selenium stock, 10 μ g/ml hydrocortisone, 3.5 μ g/ml bovine pituitary extract, and 3.5 μ g/ml endothelial cell growth supplement) with 10% FBS was added and the tracheae were washed by inversion 22 times. The supernatant was collected and placed on ice. Fresh and warmed digestion medium (5 ml per trachea) was placed into the conical tubes containing the tracheae. The suspension was incubated for 1 hour at 37°C. FBS (10%) was added subsequently to quell enzyme activity. Tracheae-containing conical tubes were inverted 22 times to dislodge cells and the supernatant was collected. Equal volume of DMEM/F12 culture medium was added and the tracheae were washed again by inversion 22 times. The supernatants from all four stages (two digestions, two washes) were combined, as they all had visible cell pellets.

The resultant pooled cell pellet was resuspended in the residual medium in the bottom of the tube. Additional medium was added to seed approximately 100 μ l of MTE cell suspension per 6.5-mm-diameter Transwell polyester clear filter support (Corning-Costar, Corning, NY). A day before seeding, the filter supports were coated with a proprietary extracellular matrix solution containing collagens, fibronectin, laminin, and bovine serum albumin (BSA). Each trachea generated enough cells to seed three-four filters.

Seeded MTE cells were undisturbed for 3 days to allow attachment and initial growth. After 72 hours, the apical medium was removed and monolayers were cultured with an air-liquid interface. Early indicators of monolayer formation included reduced basolateral to apical fluid leak along with the development of three-dimensional structures, including ridges and domes, by visual inspection. At this time, measurements of transepithelial resistance (R_{TE}) and transepithelial voltage (V_{TE}) were also conducted. Monolayers that developed ridges and domes, and R_{TE} and V_{TE} values higher than 1,000 Ohms and 5 mV, respectively (usually within 5–10 d after seeding), were used for Ussing chamber experiments described below.

Method B. BALB/c and C57BL/6 mice were killed with intraperitoneal injections of ketamine (8.7 mg/100 g BW; Phoenix Scientific) and xylazine (1.3 mg/100 g BW; Vedco). The trachea proximal to the bronchial bifurcation was isolated and removed. The resected section was immediately placed in PBS. Under a dissecting microscope, esophageal remnants and adherent adipose tissue were removed by sharp dissection, and the tracheal sections were opened longitudinally. The tracheal sections were rinsed three times in PBS and then placed in fresh MEM containing 0.1% protease XIV (Sigma, St. Louis, MO), 0.01% DNase (Sigma), and 1% FBS at 4°C for 24 hours. FBS (0.5 vol) was added to the isolation to inhibit the protease, and cells were concentrated by centrifugation (500 $\times$ g, 5 min). The cells were washed twice in fresh MEM containing 5% FBS, then seeded onto 6.5-mm-diameter Transwell filters (3413; Corning-Costar) in a density of 0.8–1.5 $\times 10^5$ cells/cm² (~3–6 filter per mouse). Before seeding, the inserts were collagen-coated by incubating them with a solution containing 0.1 g/L type VI collagen (C-7521; Sigma) and 2 ml/L glacial acetic acid at 37°C for 24 hours. MTE cells were grown in a 1:1 mixture of 3T3 fibroblast preconditioned DMEM (containing 10% FBS, 1% penicillin/streptomycin) and Ham's F-12 medium, supplemented with 10 μ g/ml insulin, 1 μ M hydrocortisone, 3.75 μ g/ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor, 30 nM triiodothyronine, 5 μ g/ml iron saturated transferrin, and 10 ng/ml cholera toxin. The culture media on the basolateral side of the filters was replaced every 48 hours. Starting 4 days after seeding, fluid was removed from the apical side every time the basolateral culture medium was changed, and cells were cultured for 3 to 5 additional days at an air-liquid interface to enhance proper differentiation and polarization (19). Monolayers that remained leaky and did not prevent basolateral culture medium from seeping through to their apical surfaces were discarded.

Preparation of H441 Cell Monolayers

The NCI-H441 cell line (HTB-174; ATCC, Manassas, VA) is derived from a papillary adenocarcinoma of human lung Clara cells. Cells between passages 82 and 97 were grown at 37°C in a humidified atmosphere of 5% CO₂ in air in RPMI 1640 medium with 2 mM L-glutamine, 1 mM sodium pyruvate, 2.0 g/L glucose, 10% FBS, penicillin (100 units/ml), and streptomycin (100 μ g/ml), as previously described (15). Confluent cells were isolated from T-75 tissue culture

flasks (Costar, Cambridge, MA) using 0.05% trypsin and 0.53 mM EDTA, and were seeded at a density of 1×10^6 cells/cm² on 12-mm Millicell PCF filters (Millipore, Bedford, MA), which had been pretreated with human placental collagen. H441 cells were grown at an air-liquid interface for 8 to 16 days in RPMI 1640 medium containing 8.5% FBS and 100 nM dexamethasone, supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenium sulfides (20).

Monolayer Infection Protocol

Confluent MTE or H441 cell monolayers on filters at an air-liquid interface were infected with 10^6 PFU of RSV strain A2, in 100 μ l of media (approximate multiplicity of infection of 1, as assessed by cell counting). RSV was allowed to adhere to cells for 60 minutes, at which time the apical solution was aspirated and discarded. Basolateral media was also replaced at that time. In some cases, the UTP and ATP synthesis inhibitors A77-1726 and 6-MP (50 μ M) were added into the basolateral media of H441 cells, 24 hours before infection with RSV. In this case, the basolateral media was replaced and fresh RPMI medium containing A77-1726 was added at the time of infection with RSV. Control wells were pretreated with an equal volume of DMSO (pH adjusted to 7.9 with 2 M Na₂HPO₄, respectively).

Ussing Chamber Measurements of Monolayer I_{sc} and R_{TE}

MTE or H441 cell monolayers with R_{TE} exceeding $0.3 \text{ k}\Omega \times \text{cm}^2$ were mounted in Ussing chambers (Jim's Instruments, Iowa City, IA) and bathed on both sides with solutions containing: 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.83 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES (Na⁺-free), with 10 mM mannitol (apical compartment) or 10 mM glucose (basolateral compartment), as previously described (21, 22). Osmolarity of all solutions, as measured by a vapor pressure osmometer (Wescor, Inc., Logan, UT) was between 290 and 300 mOsm. Bath solutions were stirred vigorously by bubbling them continuously with 95% O₂, 5% CO₂ at 37°C (pH 7.4). Monolayers were short-circuited to 0 mV and basal and amiloride-sensitive I_{sc} were continuously measured with an epithelial voltage clamp (VCC-600; Physiologic Instruments, San Diego, CA). A 10-mV pulse of 1-

second duration was imposed every 10 seconds to monitor R_{TE} , which was calculated using Ohm's law. Data were collected using the Acquire and Analyze program, version 1.45 (Physiologic Instruments). ΔI_{sc} was calculated from the difference between the initial I_{sc} value (baseline) and the value after adding a drug (amiloride, forskolin, UTP).

RT-PCR for RSV

Total RNA was isolated from H441 cell monolayers using the RNeasy Mini kit (Qiagen, Valencia, CA), according to manufacturer's instructions. cDNAs were generated by reverse transcription, using the Cells-to-cDNA kit (Ambion, Austin, TX). Negative control reactions (for genomic DNA contamination) were performed in the absence of reverse transcriptase. PCR was performed using the HotStarTaq Master Mix PCR kit (Qiagen) in accordance with manufacturer's instructions. After a hot start (3 min at 95°C), 2.5 μ l of each cDNA was subjected to 40 cycles of PCR (30 s each at T_m 95°C, T_a 55°C, T_e 72°C, per cycle) in 25 μ l reaction volume on an Eppendorf Mastercycler (Eppendorf, Westbury, NY), using 100 pmol of primers specific for a 1,200-nt segment of the RSV L gene (a gift from Dr. Tara Cartee, Dept. of Microbiology, UAB) (23). The forward primer, GATTATATAG ATATCATGGGTGGCATCG, corresponded to bases 10,800 to 10,829 and the reverse primer, CCTCATCATCTCAGTGGCTCTAT CAATATCTG, corresponded to the reverse complement of bases 11,976 to 12,007 in the A2 virus genome. Reactions containing RNase-free water in place of cDNA were also performed, as negative controls for contamination with junk DNA. PCR products were visualized by electrophoresis by a standard protocol.

Measurement of Lactate Dehydrogenase Release

Two hundred microliters of normal saline was overlaid onto dry confluent MTE cell monolayers on Transwell filters. After incubation for 2 hours, this overlay fluid was aspirated and stored at -20°C for subsequent analysis. Lactate dehydrogenase (LDH) content of overlay fluid was measured using an LDH colorimetric end-point assay kit (Sigma).

Figure 1. Effect of Respiratory syncytial virus (RSV) infection on mouse tracheal epithelial cells, isolated from C57BL/6 mice and cultured using Method A. (A) Representative I_{sc} records from uninfected (solid black line) and 24 hours after RSV infection (gray line) mouse tracheal epithelial (MTE) monolayers. MTE cells were isolated and cultured using Method A (see MATERIALS AND METHODS for details). Amiloride (Amil, 100 μ M), forskolin (FSK, 10 μ M), a mixture containing cpt-cAMP (100 μ M) plus IBMX (100 μ M), and CFTR_{inh}-172 (CFTR_{inh}, 100 μ M) were added into the apical compartment of the Ussing chambers at the times indicated by arrows. (B) Mean values $\pm$ 1 SEM of I_{sc} for the indicated conditions ($n = 9$ for control and $n = 8$ for RSV). (C) Mean changes of I_{sc} after amiloride, cAMP+IBMX and CFTR_{inh} as compared with the immediately preceding condition; * $P < 0.05$ compared with control.

Western Blotting Protocol

Control and RSV-infected cells were lysed in 500 μ l of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100) supplemented with protease inhibitor cocktail (Complete Mini, EDTA-free; Roche Diagnostics, Mannheim, Germany), then cleared by centrifugation at $16,000 \times g$ for 10 minutes at 4°C. The supernatant was then carefully removed and the protein concentration in all preparations was measured by the BCA method using BSA as a standard. All protein samples were stored at -80°C before use. H441 cell proteins were separated by SDS-PAGE (8%), transferred to PVDF membranes, and probed overnight at 4°C with 1:1,000 dilutions of rabbit primary antibodies to α -ENaC (PA1-920; Affinity Bioreagents, Golden, CO) or γ -ENaC (ab3468; Abcam, Cambridge, MA), stripped, and re-probed for β -actin (Cell Signaling Technology, Danvers, MA). Bound primary antibodies were detected with HRP-conjugated goat anti-rabbit secondary antibodies and enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ), followed by exposure to X-ray film. Band intensity was measured using AlphaEaseFC on a FluorChem imager (Alpha Innotech, San Leandro, CA) and normalized to β -actin levels.

Immunofluorescence

H441 and MTE cell monolayers on filters were rinsed in cold PBS three times, fixed in 3% formaldehyde in PBS for 45 min at room temperature, then post-fixed and permeabilized by addition of 0.5% triton X-100 at room temperature for 3 to 5 minutes. Nonspecific binding was minimized by incubating MTE or H441 cells with blocking buffer (5% BSA in PBS and 1:20 normal goat serum in PBS) for 60 minutes at room temperature. RSV was detected using a mouse monoclonal anti-RSV antibody (MTE cells: 1:50 dilution, for 60 min at 37°C; H441 cells: 1:100 dilution overnight at 4°C; Fitzgerald Industries, Concord, MA:), while α -ENaC was detected with a rabbit polyclonal antibody (Affinity Bioreagents, Golden, CO), diluted 1:100 and incubated overnight at 4°C. Nonimmune mouse or rabbit IgG (both 10 μ g/ml; Sigma) in blocking buffer served as nonimmune controls. Bound primary antibodies were detected with secondary goat anti-mouse IgG conjugated to AlexaFluor 488 (1:500; Molecular Probes,

Eugene, OR) and goat anti-rabbit IgG conjugated to AlexaFluor 594 (1:100; Molecular Probes) for 2 hours at room temperature in the dark. Slides were washed and counterstained with Hoechst 33258 (20 μ g/ml in PBS) for 5 minutes at room temperature to identify nuclei. Filter membranes bearing the cell monolayer were either mounted and sealed on glass slides upside down for *en face* optical sections (XY plane) imaging through the Z axis or folded for side-view (sagittal) sections (XZ plane) imaging through Y axis. Images were taken using an Olympus IX 70 inverted microscope with epifluorescence optics (Olympus, Hamamatsu City, Japan) equipped with a Retiga 1,300 cooled CCD, fire wire, high resolution, monochromatic camera (QImaging, Houston, TX). The fluorescent filters used for visualization of green, red and blue fluorochromes were 83,000 Pinkel filter set (Chroma Technology Corp., Rockingham, VT).

Statistical Analyses

Descriptive statistics were calculated using GraphPad InStat software (Version 3.5; GraphPad Software, San Diego, CA). Differences between multiple group means were analyzed by ANOVA, followed by the with Tukey-Kramer multiple comparison *post hoc* test and considered significant if $P < 0.05$. All data values are presented as mean $\pm$ SEM.

RESULTS

Effects of RSV Infection on I_{sc} in Mouse Tracheal Epithelial Cell Monolayer Cultures

Figure 1A shows a characteristic Ussing tracing from a monolayer of MTE cells isolated with Method A. As described in MATERIALS AND METHODS, this isolation procedure is based in the protocol of Clarke and coworkers (14), but employs much lower concentrations of hormones and growth factors during the culture period. As shown in Figure 1A, baseline currents are considerably lower than previously reported (14, 24, 25). Addition of 100 μ M amiloride into the apical compartment

Figure 2. Effect of RSV infection on forskolin-induced I_{sc} in mouse tracheal epithelial cells, isolated from C57BL/6 mice and cultured using Method A. (A) Representative I_{sc} records from uninfected (solid black line) and 24 hours after RSV infection (gray line) MTE monolayers. MTE cells were isolated and cultured using Method A (see MATERIALS AND METHODS for details). Forskolin (FSK, 10 μ M), a mixture containing cpt-cAMP (100 μ M) plus IBMX (100 μ M), amil (10 μ M), and CFTR_{inh}-172 (CFTR_{inh}, 100 μ M) were added into the apical compartment of the Ussing chambers at the times indicated by arrows. (B) Mean values $\pm$ 1 SEM of I_{sc} for the indicated conditions ($n = 3$ for control and $n = 5$ for RSV). (C) Mean changes of I_{sc} after forskolin, amiloride, cAMP+IBMX, and CFTR_{inh} as compared with the immediately preceding condition; * $P < 0.05$ compared with control.

Figure 3. Immunofluorescent images of mock (A, C, and E)- or RSV (B, D, and F)-infected MTE cells. (A, B) Confocal images of apical plasma membranes of MTE cells, 24 hours after (A) mock and (B) RSV infection stained with anti-RSV antibody followed by AlexaFluor 488-conjugated secondary antibody. Green fluorescence indicates the presence of RSV virus. No staining is seen in mock-infected cells. (C, D) Confocal microscopy of (C) mock- and (D) RSV-infected MTE cells, stained with an antibody to α ENaC, followed by a secondary antibody conjugated to AlexaFluor 594. Cell nuclei are counterstained with Hoechst 33258. Significantly lower levels of α ENaC-associated fluorescence are seen in RSV-infected cells. (E, F) Side-view (sagittal) sections as in C and D showing the apical plasma membranes. Similar results were obtained from three different MTE monolayers.

resulted in a 45% decrease of I_{sc} . Slightly lower levels of I_{sc} inhibition were achieved with lower levels of amiloride (10 μ M; data not shown). The remaining current was totally abolished by addition of a specific inhibitor of CFTR (CFTR_{inh}-172). Addition of 10 μ M forskolin (a stimulator of adenylate cyclase) into the apical bath in the presence of 100 μ M amiloride resulted in significant stimulation of I_{sc} which was inhibited by progressive amounts of CFTR inhibitor added into the apical compartment (Figure 1). Addition of a mixture containing a cell permeable analog of cAMP (CPT-cAMP; 100 μ M) and IBMX (3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor; 100 μ M), known to maximally increase intracellular cAMP

resulted in a small, additional incrementally increase of I_{sc} . Forskolin also stimulated I_{sc} when added into the apical compartment before amiloride (Figure 2). The majority of the forskolin stimulated I_{sc} was inhibited by CFTR_{inh}-172. These data indicate that MTE cells isolated with this method exhibited both Na^+ absorption (most likely through ENaC) and Cl^- secretion (though CFTR) and that agents that increase cAMP stimulate mainly Cl^- secretion.

MTE cells were productively infected with RSV strain A2, as confirmed by direct immunofluorescence, although levels of infection appeared low (< 5%) 24 hours post infection (Figure 3). No effects on basal and amiloride-sensitive I_{sc} were seen in MTE

Figure 4. Effect of RSV infection on mouse tracheal epithelial cells, isolated from BALB/c mice and cultured using Method B. (A) Representative I_{sc} records from uninfected (solid black line) and 24 hours after RSV infection (gray line) MTE monolayers. MTE cells were isolated and cultured using Method B (see MATERIALS AND METHODS for details). Amiloride (Amil, 100 μ M), forskolin (FSK, 10 μ M), a mixture containing cpt-cAMP (100 μ M) plus IBMX (100 μ M) and CFTR_{inh}-172 (CFTR_{inh}, 100 μ M) were added into the apical compartment of the Ussing chambers at the times indicated by arrows. (B) Mean values $\pm$ 1 SEM of I_{sc} for the indicated conditions ($n = 5$ for control and $n = 6$ for RSV). (C) Mean changes of I_{sc} after amiloride, cAMP+IBMX, and CFTR_{inh} as compared with the preceding condition; * $P < 0.05$ compared with control.

Figure 5. Effect of RSV infection on forskolin-induced I_{sc} in mouse tracheal epithelial cells, isolated from BALB/c mice and cultured using Method B. (A) Representative I_{sc} records from uninfected (solid black line) and 24 hours after RSV infection (gray line) MTE monolayers. MTE cells were isolated and cultured using Method B (see MATERIALS AND METHODS for details). Forskolin (FSK, 10 μM), a mixture containing cpt-cAMP (100 μM) plus IBMX (100 μM), CFTR_{inh}-172 (CFTR_{inh}, 100 μM), glybenclamide (0.3 mM), and amil (100 μM) were added into the apical compartment of the Ussing chambers at the times indicated by arrows. (B) Mean values $\pm$ 1 SEM of I_{sc} for the indicated conditions ($n = 6$ for control and $n = 6$ for RSV: [1] Basal, [2] FSK, [3] cAMP+IBMX, [4] CFTR_{inh}, [5] glyb, [6] amil.). (C) Mean changes of I_{sc} after forskolin, amiloride, cAMP+IBMX and CFTR_{inh} as compared with the immediately preceding condition; * $P < 0.05$ compared with control.

cells 8 hours after infection (data not shown). However, infection of MTE cells with RSV A2 for 24 hours caused a significant reduction of both the baseline and of the amiloride-sensitive component of I_{sc} (Figures 1 and 2). Significantly lower values of cAMP stimulated I_{sc} were seen in RSV-infected MTE cells. As seen in Figures 1 and 2, addition of CPT-cAMP+IBMX after forskolin resulted in a marginal and nonsignificant increase of I_{sc} above and beyond what observed with forskolin alone in both mock- and RSV-infected MTE cells. Infection with RSV did not alter transepithelial resistance (data not shown). Moreover, no LDH release into overlaid media was detectable in either mock-infected or RSV-infected MTE cell monolayers at

either 24 or 28 hours after infection (data not shown). These findings indicate that RSV, in spite of its low infectivity, inhibits active ion transport in MTE cells through both ENaC and CFTR without causing cell death or overt cellular injury. Immunofluorescent studies (shown in Figure 3) using anti-RSV and anti- α ENaC antibodies indicate that RSV infection significantly decreased α ENaC levels in MTE cells both at the levels of the cytoplasm and apical plasma membranes. This impression was based on visual examination of a large number of monolayers. Quantitative studies were not attempted.

MTE cells isolated from either BALB/c (Figures 4 and 5) or C57BL/6 mice (data not shown) using Method B (which uses

Figure 6. Characterization of H441 I_{sc} . (A) Representative Ussing chamber I_{sc} traces from a H441 cell monolayer. Amiloride was added in the apical chamber in incremental amounts at times indicated by arrows to achieve steady-state concentrations of 0.01, 0.1, 1, 10, and 100 μM . Subsequent addition of forskolin (10 μM) caused a small increase of I_{sc} (presumably due to anion secretion) that was inhibited by ouabain (1 mM), added into the basolateral compartment. (B) Dose-response inhibition of I_{sc} by apical amiloride. Values are means $\pm$ 1 SEM; $n = 12$ monolayers for each point. Values are expressed as % of I_{sc} before the addition of amiloride. (C) Representative I_{sc} trace of H441 cell monolayer showing the effects of amiloride (amil. 100 μM), forskolin (FSK; 10 μM), and CFTR_{inh}-172 (CFTR_{inh}; 200 μM). As shown in A, in the presence of amiloride, forskolin caused a small increase of I_{sc} that was inhibited by apical addition of CFTR_{inh}. Measurements were reproduced using eight different monolayers with identical results.

Figure 7. Infection of H441 cells with RSV strain A2. (A) Demonstration of RSV replication in H441 cells at 24 hours after infection by RT-PCR using primers specific for an 1,100-nt segment of the RSV L gene (indicated by arrow) in RNA isolated from three RSV-infected H441 cell monolayers (V_1 – V_3). Note absence of PCR product in RNA isolated from three mock-infected monolayers (M_1 – M_3), or in reverse transcriptase control (RT-), and cDNA control monolayer RNA preparations. (B and C) Confirmation of productive RSV infection of H441 cell monolayers (1×10^6 PFU per monolayer) at 24 hours by immunofluorescence using a mouse monoclonal anti-RSV antibody followed by a secondary goat anti-mouse IgG conjugated to AlexaFluor 488. Cell nuclei are counterstained blue with Hoechst 33258. No fluorescence was seen in mock-infected monolayers (data not shown). Representative immunofluorescence images from three repeats per condition are shown.

high concentrations of various hormones and growth factors known to stimulate Na^+ transport) exhibited much higher levels of baseline I_{sc} that were almost completely inhibited by amiloride. Infection of these cells with RSV resulted in significant inhibition of both the baseline and amiloride-sensitive I_{sc} (Figures 4 and 5). Addition of forskolin, or of CPT-cAMP and IBMX, either after (Figure 4) or in the absence of amiloride (Figure 5), resulted in a large increase of I_{sc} that was inhibited by CFTR_{inh} in a dose-dependent fashion, indicating that it was mainly due to the secretion of anions (Cl^- or HCO_3^-). Data shown in Figures 4 and 5 indicate that infection of MTE with RSV resulted in significant decrease of the cAMP-stimulated I_{sc} .

H441 Cells Exhibit Vectorial Active Na^+ Transport

Because of the difficulty and expense of isolating MTE cells in sufficient numbers, additional studies assessing the effects of RSV on vectorial Na^+ transport were performed on H441 cells. We first characterized the transport properties of these cells when grown to confluent monolayers and mounted in Ussing chambers. Addition of 1 to 100 μM amiloride into the apical compartment of the Ussing chamber inhibited more than 95% of I_{sc} , with an IC_{50} of $0.50 \pm 0.50 \mu\text{M}$ ($n = 12$, Figure 6). These findings indicate that the majority of I_{sc} in H441 cell monolayers is due to the movement of Na^+ ions through amiloride-sensitive channels (ENaC). These data indicate that in contrast to MTE cells isolated by Method A, the majority of the I_{sc} in H441 cells

is due to Na^+ reabsorption, as we recently reported for ATI cells. In the presence of 100 μM amiloride, apical addition of 10 μM forskolin induced a small increase in I_{sc} (a fraction of what was seen in MTE cells) which was inhibited either by ouabain (Figure 6A), by CFTR_{inh}-172 (200 μM , Figure 6C) or glibenclamide (data not shown). These data indicate that the resulting currents is caused by anion secretion.

H441 Cells Can Be Infected with RSV Strain A2

Productive infection of H441 cell monolayers with RSV was detected by RT-PCR for RSV L (polymerase) gene mRNA (Figure 7), and confirmed by immunofluorescence using a mouse monoclonal anti-RSV antibody (Figure 7). Approximately 5 to 10% of H441 cells were RSV-positive at 24 hours after infection, similar to what we observed from MTE cells (Figure 3).

Effects of RSV Infection on I_{sc} in H441 Cell Monolayer Cultures

RSV infection caused a significant (50%) reduction in basal and amiloride-sensitive I_{sc} in H441 cell monolayers at 24 hours (Figures 8A and 8B), indicating that, as in MTE cells, RSV inhibits active, amiloride-sensitive Na^+ transport. We did notice a decrease of transepithelial resistance after RSV infection; however, the difference was not significant ($259 \pm 174 \Omega\text{-cm}^2$ in RSV infected monolayers, $n = 23$, versus $347 \pm 170 \Omega\text{-cm}^2$ in uninfected monolayers, $n = 21$, $P = 0.7$) and unlikely to contribute to the observed decrease of amiloride-sensitive I_{sc} .

Figure 8. Effects of RSV infection on I_{sc} of H441 monolayers. (A) Typical I_{sc} traces of control (uninfected; black line) and RSV-infected (1×10^6 PFU/monolayer; gray line) monolayers 24 hours after infection. Amiloride (100 μM) were added into the apical compartment at the indicated time. (D) Mean values ± 1 SEM of base and amiloride-sensitive I_{sc} ($n = 21$ for control and $n = 23$ for RSV). (B) Representative I_{sc} responses to forskolin (10 μM) of mock (black line)- and RSV (gray line)-infected monolayers followed by addition of 100 μM amiloride. Subsequently, the apical membrane was permeabilized by the addition of amphotericin-B (20 μM) in the apical compartment, followed by the addition of ouabain (1 mM). The difference current before and after addition of ouabain represents the maximum activity of the Na,K-ATPase. (E) Mean values ± 1 SEM of baseline, forskolin, and amiloride-sensitive (ΔA_{mil}) I_{sc} ($n = 3$ for control and $n = 5$ for RSV). (C, F) Same as in C and D for a group of monolayers with lower baseline I_{sc} values. Notice that in this case, addition of forskolin (10 μM) returned I_{sc} to the control baseline ($n = 4$ for both control and RSV). * $P < 0.05$ as compared with the corresponding control uninfected monolayers.

Figure 9. The effects of terbutaline on I_{sc} of MTE and H441 monolayers. Typical I_{sc} traces showing time courses of (A, C) mock- or (B, D) RSV (1×10^6 PFU)-treated MTE (A, B) and H441 (C, D) monolayers 24 hours after infection, after treatment of terbutaline into both compartments of the Ussing chamber. In (A) control and (B) RSV-treated MTE cell monolayers, terbutaline (0, 1, 2.5, 5, and 10 μM) caused a dose-dependent increase of I_{sc} that was significantly inhibited by amiloride and CFTR_{inh}. This effect was prevented by pretreatment with propranolol (10 μM), an inhibitor of β_1 - and β_2 -adrenergic receptors (gray lines). Terbutaline did not cause significant increase of I_{sc} in either control or RSV-infected H441 cells (C, D). Typical records which were reproduced at least five times (see RESULTS for mean values).

RSV infection for 24 hours also significantly reduced (by 32%) the forskolin (10 μ M)-stimulated I_{sc} (Figures 8C and 8D). Subsequent addition of 10 μ M amiloride completely abrogated this forskolin-induced increase in I_{sc} in both uninfected and infected monolayers, indicating that forskolin stimulates mainly active Na^+ transport in H441 cells, and that this stimulation is impaired by RSV infection. Measurement of I_{sc} in RSV-infected H441 cell monolayers after apical membrane permeabilization with 20 μ M amphotericin B (thereby loading the cytosol with Na^+ to elicit maximal Na^+ transport by the basolateral Na^+/K^+ ATPase), followed by addition of 1 mM ouabain to inhibit Na^+/K^+ ATPase activity, indicated that RSV infection has no effect on the ouabain-inhibitable Na^+/K^+ ATPase I_{sc} (mean ΔI_{sc} after amphotericin B followed by ouabain was $4.98 \pm 1.1 \mu\text{A}/\text{cm}^2$ in RSV-infected monolayers, $n = 11$, versus $5.04 \pm 0.6 \mu\text{A}/\text{cm}^2$ in uninfected monolayers, $n = 8$; $P > 0.1$).

Previously we reported that RSV infection decreases Na^+ -dependent alveolar fluid clearance (AFC) by approximately 25 to 30% and that intratracheal instillation of forskolin restores AFC to its normal levels (12, 26, 27). In contrast, as shown in Figure 8B RSV infection of H441 cells decreased their I_{sc} by more than 50% and although forskolin increased I_{sc} , it did not restore it to its control, noninfected value. However, in another group of H441 cells that were cultured for shorter periods of time and had lower baseline I_{sc} values, infection with RSV decreased their baseline values by only about 25%, and addition of forskolin returned the current to the noninfected baseline levels (Figures 8E and 8F). Subsequent addition of 8-Br-cGMP and IBMX (which greatly increase cAMP levels) had only a minor effect on I_{sc} (indicating that forskolin generated the maximum response). Similar results were obtained with MTE cells cultured with Method A, which results in smaller baseline I_{sc} values as compared with Method B. Terbutaline (up to 10 μ M) as well as a number of other β -adrenergic receptor agonists (data not shown) increased I_{sc} across mock- and (to a lesser extent) RSV-infected MTE cells (Figures 9A and 9B; Mean ± 1 SEM for 0, 1, 2.5, 5, and 10 μ M terbutaline: 8 ± 1.4 , 8.6 ± 2.0 , 9.2 ± 1.9 , 9.9 ± 2.0 , and $10.2 \pm 2.0 \mu\text{A}/\text{cm}^2$ [$n = 6$] while for RSV-infected monolayers: 5.3 ± 2.1 , 6.3 ± 3.0 , 7.0 ± 3.5 , 7.6 ± 3.7 , and $8.6 \pm 4.6 \mu\text{A}/\text{cm}^2$ [$n = 5$]). In both cases, pretreatment with propranolol (10 μ M) totally abolished the effects of terbutaline. In contrast, no effect of terbutaline was seen on I_{sc} of either mock- or RSV-infected H441 cells (Figures 9C and 9D). These results suggest that H441 cells, cultured under our experimental conditions, lack either β_2 -adrenergic receptors at their plasma membrane or Gs proteins, which transduce signals from these receptors, resulting in increases of cAMP. *In vivo* findings indicate while forskolin increased AFC in RSV-infected mice, short- and long-term β_2 -agonists had no effect on this variable (27).

Inhibitors of *De Novo* Pyrimidine or Purine Synthesis Block RSV-Induced Inhibition of I_{sc} in H441 Cells

We have reported that RSV infection inhibited AFC in BABL/c mice by increasing UTP levels in their BAL (12). Therefore, we tested whether RSV infection inhibits I_{sc} of H441 cells through the same mechanism. First, H441 cells were tested for their response to UTP. As shown in Figure 10, addition of UTP decreased I_{sc} in H441 cells in a dose-dependent manner. Second, when H441 cells were pretreated by *de novo* pyrimidine or purine synthesis inhibitor A77-1726 or 6-MP (50 μ M), inhibition of amiloride-sensitive I_{sc} at 24 hours after RSV infection was reversed (Figure 11). A77-1726 had no effect on basal I_{sc} and amiloride sensitivity in uninfected H441 cell monolayers (Figure 11C). Thus, these data are consistent with our *in vivo* data and suggested that RSV infection inhibits active Na^+ transport in respiratory epithelium by up-regulation of UTP.

Figure 10. UTP decreases amiloride-sensitive I_{sc} in H441 cells. (A) Representative I_{sc} traces showing the effect of increasing concentrations of UTP (1–1,000 μM), or equal volumes of vehicle (water) added into the apical compartments of Ussing chambers containing monolayers of H441 cells followed by amiloride (100 μM). (B) UTP (or vehicle)-sensitive currents for the indicated concentrations. Mean values ± 1 SEM ($n = 4$ for vehicle-treated monolayers; $n = 5$ for UTP-treated monolayers). * $P < 0.05$, compared with vehicle-treated monolayers.

Infection with RSV Reduces H441 cell ENaC Subunit Protein Expression

Immunofluorescent analysis demonstrated that reduced amiloride-sensitive I_{sc} after infection of H441 cells with RSV for 24 hours was accompanied by a reduction in α -ENaC subunit protein expression level (Figure 12). Likewise, Western blot analysis of whole-cell lysates also indicated that α - but not γ -ENaC protein level decreased in RSV-infected H441 cells after 24 hours (Figure 13).

DISCUSSION

Herein we show that infection of both mouse tracheal epithelial cells, derived from the lungs of two C57BL/6 and BALB/c mice, as well as H441 cells, a human Clara cell-like line, with RSV A2 decrease baseline amiloride-sensitive I_{sc} and modulate the increase of I_{sc} by cAMP. In contrast, Na,K -ATPase function was not affected. Importantly, despite the different baseline I_{sc} values and proportion of amiloride-sensitive components within the cell systems, we observed similar extent of inhibition of amiloride-sensitive Na^+ currents by RSV infection, which suggests that a common mechanism is involved. Moreover, our data indicate that the inhibition of ENaC requires active, replicating viruses and is independent of the RSV-induced inflammatory response.

untreated monolayers; $n = 3$ for mock-infected, 6-MP-treated monolayers; $n = 25$ for RSV-infected, untreated monolayers; $n = 4$ for RSV-infected, DMSO-treated monolayers; $n = 9$ for RSV-infected, A77-1726-treated monolayers). (B) Effect of pretreatment of 50 μM A77-1726 on amiloride sensitive I_{sc} (ΔI_{amil}) 24 h after infection for the groups mentions in the previous panel. (C). Effect on basal I_{sc} of treatment of mock-infected monolayers for 24 h with 50 μM A77-1726 ($n = 24$ for untreated monolayers; $n = 3$ for DMSO-treated monolayers; $n = 7$ for A77-1726-treated monolayers). (D) Effect of pretreatment of monolayers with 50 μM of the *de novo* purine synthesis inhibitor 6-MP on I_{sc} in mock-infected monolayers and at 24 hours after RSV infection ($n = 24$ for mock-infected, RSV-infected monolayers), and A77 plus RSV monolayers. * $P < 0.005$, compared with mock-infected, untreated monolayers.

We previously demonstrated that RSV-induced inhibition of AFC in BALB/c mice is mediated by the action of *de novo* synthesized UTP on bronchoalveolar epithelial P2Y purinergic receptors (12). In contrast, Kunzelmann and colleagues reported that nucleotide-mediated P2Y receptor activation plays no role in

inhibition of active Na⁺ transport in mouse tracheal tissue after exposure to RSV for 60 minutes (28). Nevertheless, we found in the current study that the inhibitory effects of RSV infection for 24 hours on active Na⁺ transport (amiloride-sensitive I_{sc}) in H441 cells appear to be nucleotide mediated: H441 cell basal I_{sc} is

Figure 12. Immunofluorescent staining for α ENaC in H441 cell monolayer. Mock (top panel)- or RSV (bottom panel)-infected H441 cells (1×10^6 PFU for 24 h) were immunostained with either anti-RSV antibody (A and B; see Figure 3 for details of RSV staining) or an antibody to α ENaC (C and D, see Figure 3 for details of α ENaC staining). The secondary antibodies for RSV and α ENaC staining were goat anti-mouse IgG conjugated to AlexaFluor 488 (green fluorescence) and goat anti-rabbit IgG conjugated to AlexaFluor 594 (red fluorescence), respectively. Only background immunofluorescence levels were seen in monolayers stained with an equivalent amount of nonimmune rabbit IgG, followed by secondary goat anti-rabbit IgG conjugated to AlexaFluor 594 (picture not shown). In all cases nuclei were counterstained blue with Hoechst 33258 dye. Representative immunofluorescence images from five repeats per condition are shown.

Figure 13. RSV decreases α -ENaC levels in H441 cells. (A) Sample Western blots demonstrating immunodetection of α - and γ -ENaC subunit proteins in total cellular lysates of mock-infected H441 cells (V) or H441 cells infected with RSV strain A2 for 24 h (R). Representative blots from three repeats are shown. (B) Effect of infection of H441 cells with RSV for 24 hours on α - and γ -ENaC subunit protein expression level. Values are means $\pm$ 1 SEM ($n = 3$ per group; relative density normalized to β -actin). * $P < 0.05$ compared with the corresponding vehicle value.

inhibited by exogenous UTP (which suggests that these cells express functional P2Y receptors) and RSV-mediated inhibition of active Na^+ transport in H441 cells can be reversed by inhibition of *de novo* pyrimidine or purine synthesis. This finding suggests that elevated *de novo* nucleotide synthesis and release is induced by RSV replication and gene expression within infected respiratory epithelial, but not by mere exposure to viral antigens.

Interestingly, the inhibitory effect of Sendai virus on active Na^+ transport reported by Kunzelmann and coworkers (28) is also not mediated by pyrimidines, but is instead induced by ATP, acting on P2Y receptors in a similar manner to that we have described here and elsewhere for RSV (12, 13, 26, 27). However, it is not clear from the above study whether this ATP is derived from *de novo* purine synthesis. Again, it should be noted that, *in vivo*, RSV induces elevated release of both ATP and UTP into the bronchoalveolar space, although only UTP appears to mediate impaired AFC after RSV infection (26). ATP may be inactive *in vivo* because RSV infection has been shown to enhance ecto-nucleotide pyrophosphatase phosphohydrolase-mediated extracellular ATP hydrolysis (29). It is possible that in our H441 cell culture system, this effect does not occur, and so both ATP and UTP can mediate inhibition of active Na^+ transport. Alternatively, the comparable effects of A77-1726 and 6-MP may instead reflect the co-ordinate regulation of cellular *de novo* nucleotide synthesis, which we have found to result in concomitant suppression of both pathways by inhibitors. Finally, the findings reported herein indicate that the respiratory epithelium itself is at least one of the sites of

elevated *de novo* nucleotide synthesis and release after RSV infection, since the *in vivo* effects of RSV on AFC and nucleotide synthesis/release could be recapitulated *in vitro* in the absence of any other cell type. Moreover, they demonstrate that RSV inhibition of Na^+ transport can occur in the absence of immune cell activity.

We found that RSV reduced forskolin-stimulated I_{sc} in both MTE and H441 cell monolayers at 24 hours after infection. Forskolin-stimulated I_{sc} was reduced by RSV when forskolin was added in both the absence and presence of amiloride. In the former case, RSV reduced cAMP-mediated stimulation of amiloride-sensitive active Na^+ transport, while in the latter case, RSV most likely blocked cAMP-mediated activation of anion secretion via CFTR. An inhibitory effect of RSV on anion secretion by respiratory epithelial cells has not previously been described, although it was suggested in a recent report by Tarran and colleagues (29). This group demonstrated that in well-differentiated, ciliated, airway epithelial cell cultures from normal donors, the majority (50%) of periciliary liquid (PCL) secretion under phasic motion occurs via CFTR (with a further 20% occurring via Ca^{2+} -activated Cl^- channels). However, they also showed that after RSV infection, PCL height falls by 44%, which is highly suggestive of a significant effect of RSV on anion secretion via CFTR (29). Similarly, Kunzelmann and coworkers (28) reported that, after an initial transient stimulatory effect on Ca^{2+} -activated Cl^- channels, acute exposure to Sendai virus caused a reduction in forskolin-stimulated I_{sc} in mouse tracheal tissue. However, these studies were not performed in the presence of amiloride, so it is unclear whether this reduction in forskolin-stimulated I_{sc} was due to a reduction in both Cl^- secretion and Na^+ absorption, or solely the latter (as was also observed in our studies). Interestingly, this group did not find any effect of RSV on either Ca^{2+} -activated or forskolin-stimulated I_{sc} responses after exposure of mouse tracheal tissue to virus for 1 hour (30), which clearly differs from our findings: again, this may reflect a difference between exposure to RSV viral antigen and viral infection and replication/gene expression. Activity of CFTR has been proposed as a necessary component of the normal AFC mechanism (31, 32). Moreover, RSV has been proposed as a candidate vector system for airway gene therapy in cystic fibrosis (9). This aspect of the effect of RSV on respiratory epithelial ion transport therefore deserves further study.

It should be noted that inhibition of active ion transport in RSV-infected MTE or H441 cell monolayers is not a consequence of cell death, in agreement with earlier *in vitro* studies that have demonstrated that RSV does not cause cytopathology or syncytium formation in well-differentiated, polarized, primary human airway epithelial cells (9, 33). In addition, the fact that Na^+ transport inhibition by RSV in H441 cell monolayers could be prevented by pretreatment with inhibitors of *de novo* nucleotide synthesis also indicates that the defect in ion transport is functional (as we found in RSV-infected mice [26]), rather than being a consequence of cell death. Finally, the absence of effect of RSV on function of the basolateral Na^+/K^+ ATPase in H441 cells also indicates that the inhibitory effects of RSV on amiloride-sensitive and forskolin-stimulated ion transport are selective and not a result of nonspecific cytopathic effects on respiratory epithelial cells. This observation also extends our previous *in vitro* findings—while we found that the predominant inhibitory effect of RSV is on the amiloride-sensitive component of AFC (which suggests that RSV has little or no effect on bronchoalveolar epithelial cell Na^+/K^+ ATPase function), we have been unable to directly measure the effects of RSV on ouabain-sensitive AFC *in vivo* because of the extreme cardiotoxicity of this ATPase inhibitor (34).

The exact mechanisms by which the RSV mediated increased UTP levels decrease ENaC have not been elucidated. In a recent study, we infected H441 cells with RSV expressing GFP. Cells expressing GFP (indicating that they had been infected with RSV) had lower amiloride-sensitive currents and lower levels of α ENaC mRNA as compared with uninfected cells (35). Incubation with A77 increased the values of the amiloride-sensitive currents but did not alter α ENaC mRNA. In the present study we report that infection with RSV decreased α ENaC protein levels both by immunofluorescence and Western blotting. However, the overall decrease of α ENaC was small (about 30%) and unlikely to account for the large decrease of amiloride-sensitive currents. However, it is still unclear as to how RSV infection up-regulates UTP production and how elevated UTP mediates the inhibition of Na⁺ channel currents. P2Y receptors (the main targets of UTP) are G protein-coupled and act via the inositol phosphate pathway to stimulate Ca²⁺ release from intracellular stores, but can also act via multiple secondary signal transduction pathways, including protein kinase C (PKC) (36). Activation of PKC has been shown to reduce ENaC activity and modify its subunit composition (37, 38). Our previous data indicate that infection of mice with RSV results in activation of PKC ζ as indicated by its phosphorylation and translocation from the cytoplasm to the plasma membrane (27).

Conflict of Interest Statement: E.S.'s company DiscoveryBioMed did receive compensation under a "fee-for-service" agreement for primary mouse lung cells prepared and used by Dr. Matalon's group in this paper. I.C.D., W.M.S., and S.M. have been granted US Provisional Patent Application # 60/573558 (Filed May 21, 2004; Inventors: Dr. Ian C Davis, Dr. Wayne Sullender and Dr. Sadis Matalon), which converted to International PCT application (#PCT/US2005/017939); May 2005. I.C.D. has received \$500 in consultancy fees from Inspire Pharmaceuticals for advising on licensing issues related to this patent. I.C.D. is the principal investigator for a grant from Inspire Pharmaceuticals (11/01/2007–10/31/2008; \$130,000, indirect + direct costs). S.M. is the principal investigator for a grant from Inspire Pharmaceuticals, from 7/15/06–7/15/07 for \$130,000 for direct costs; as a principal investigator for a grant from Talecris Biotherapeutics, Inc from 7/01/07–12/31/07 for \$38,000 for direct costs; and as a principal investigator for a grant from Sepracor, Inc for \$164,196 (date pending). W.M.S. served as a co-investigator for the grant from Inspire Pharmaceuticals, from 7/15/06–7/15/07 for \$130,000 for direct costs. None of the experiments and results presented in this article were funded from these grants. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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