

Inhibition of Airway Na^+ Transport by Respiratory Syncytial Virus[†]

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In previous studies, we have shown that two major respiratory pathogens, influenza virus and parainfluenza virus, produce acute alterations in ion transport upon contacting the apical membrane of the respiratory epithelium. In the present study, we examine the effects on ion transport by the mouse tracheal epithelium of a third major respiratory pathogen, respiratory syncytial virus (RSV). RSV infections are associated with fluid accumulation in the respiratory tract and cause illnesses that range in severity from rhinitis, sinusitis, otitis media, and bronchitis to bronchiolitis and pneumonia. We find that within minutes of RSV contacting the apical membrane; it inhibits amiloride-sensitive Na^+ transport by the epithelium. This effect is mediated by protein kinase C and is reproduced by recombinant viral F (fusion) protein. Since this inhibition is not accompanied by any alteration in the epithelial responses to carbachol or to forskolin plus 3-isobutyl-1-methylxanthine (IBMX), it is not due to a nonspecific toxic action of the virus. The inhibition also appears to require Toll-like receptor 4 and the presence of asialogangliosides in the apical membrane. Since the concentration range over which this inhibition is observed (10^2 to 10^5 PFU/ml) is comparable to the viral concentrations observed in clinical and experimental RSV infections, it seems likely that direct inhibition by the virus of epithelial Na^+ transport may contribute to the fluid accumulation that is observed in RSV infections.

The thickness of the fluid layer covering the respiratory surfaces is determined by the ion transport activity of the respiratory epithelium (5). In particular, it is regulated by the balance between the rate of Na^+ absorption through epithelial Na^+ channels and the rate of Cl^- secretion through cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+} -activated Cl^- channels. This is well illustrated by the clearance of the lung fluid at birth, which is due to the activation of the epithelial Na^+ channels in the respiratory epithelium (4). Similarly, the dehydration of the respiratory surface which is observed in cystic fibrosis appears to be due to the increased activity of the epithelial Na^+ channels consequent on the loss of the normal inhibitory effects of CFTR (2, 29). On the other hand, inactivating mutations of the epithelial Na^+ channels, as occur in pseudohypoaldosteronism type I, lead to fluid accumulation in the respiratory tract (19). A number of acquired conditions characterized by increased amounts of respiratory surface fluid have also been found to be associated with abnormal respiratory ion transport. These conditions include high-altitude pulmonary edema (32), hypoxic pulmonary edema (36), and neonatal respiratory distress syndrome (3).

Viral respiratory infections are associated with fluid accumulation in the respiratory tract which can range in severity from rhinitis, sinusitis, otitis media, or bronchitis to pneumonia. Given the role of ion transport by the respiratory epithelium in controlling the amount of respiratory surface fluid, this association suggested to us that respiratory viruses alter

epithelial ion transport. When we tested this hypothesis for influenza virus, we found that the virus inhibited epithelial transport of Na^+ (20). It did so as a consequence of the hemagglutinin in the viral coat binding to a neuraminidase-sensitive glycoprotein in the apical membrane of the epithelial cells. This, in turn, led to the activation of phospholipase C and protein kinase C. These effects were evident within minutes of the virus contacting the epithelium and did not require infection of the cells (8). Recently, Lan and coworkers extended these findings by showing that influenza virus inhibits the clearance of respiratory surface fluid by a mechanism mediated by phospholipase C, protein kinase C, and Src (26).

Subsequently, we examined the effects of another major respiratory pathogen, parainfluenza virus, on epithelial ion transport. We found that contact of the model parainfluenza virus, Sendai virus, with the epithelium also changes epithelial ion transport so as to promote an increase in respiratory fluid (21). These changes were not, however, limited to the inhibition of epithelial Na^+ absorption. The virus also stimulated Cl^- secretion through Ca^{2+} -activated Cl^- channels. The mechanism by which Sendai virus acted on the epithelium also differed from the mechanism used by influenza virus. Sendai virus triggered the release of ATP from the epithelium, which then acted in an autocrine fashion on apical purinergic receptors to activate phospholipase C, leading to an increase in intracellular Ca^{2+} and the activation of protein kinase C (21). A very similar mechanism was also recently described for *Pseudomonas* flagellin-induced effects on airway transport (22).

In the present study, we examine the acute effects of a third major respiratory virus, respiratory syncytial virus (RSV). RSV is the major respiratory pathogen among infants and children

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less than 5 years of age and causes 20% of childhood admissions to hospitals for respiratory infections (15). It also causes repeated reinfections in both children and adults. Less serious infections lead to nasal congestion, conjunctivitis, and otitis media, while more serious infections include bronchiolitis in children and potentially life-threatening pneumonia in adults (15). Moreover, RSV infection alters the sensitivity of laryngeal chemoreceptors, which may result in prolonged apnea (27). The clinical characteristics of the illness thus suggest that the virus may alter epithelial ion transport. Furthermore, it was recently reported that mice infected with RSV have decreased alveolar fluid clearance and a loss of sensitivity of the alveolar fluid clearance to amiloride, consistent with the virus directly, or indirectly, inhibiting the rate of epithelial Na⁺ transport (8). We thus examined whether exposure to RSV produces rapid alterations in the rate of ion transport by the respiratory epithelium. We find that it does and that the mechanism involved resembles that used by influenza virus rather than the mechanism used by parainfluenza virus.

MATERIALS AND METHODS

Viruses. RSV (strain A2), a kind gift from P. Young (Sir Albert Sakzewski Virus Research Centre, Brisbane, Australia), was propagated in HEp-2 cells (ATCC), which were derived via HeLa contamination and maintained in medium 199 supplemented with 5% fetal calf serum and antibiotics. Semiconfluent (80%) monolayers were infected with RSV at a multiplicity of infection of 1. Supernatants from RSV-infected cultures were collected 4 to 5 days after infection, frozen and thawed twice to disrupt cells, vortexed for 1 min, and clarified by centrifugation at $1,200 \times g$ for 15 min. The virus was titrated prior to use. RSV protein F was purified from virions, a generous gift from D. Spielman (Lederle-Praxis Biologicals, Rochester, NY). RSV protein F preparations may contain a certain fraction of G protein due to interprotein disulfide bonding (1).

Cell culture. M1 mouse cortical collecting duct cells, provided by C. Korbacher (Oxford University, United Kingdom), were grown to confluence for 3 days on permeable supports (Transwell-Coll; Costar, Cambridge, MA) in Dulbecco modified Eagle medium-F12 containing 10% fetal calf serum, glutamine (2 mmol/liter), penicillin (100,000 U/liter), streptomycin (100,000 U/liter), and dexamethasone (0.1 μ mol/liter).

Ussing chamber experiments. QS and C57BL mice were killed by cervical dislocation. The trachea was then removed, freed of connective tissue, and divided into small pieces. These pieces were then stored in a chilled solution containing the following (mmol/liter): NaCl (145), KCl (3.8), D-glucose (5), MgCl₂ (1), HEPES (5), and Ca²⁺ gluconate (1.3), pH 7.4. Tissues were mounted in an Ussing chamber with a circular aperture of 0.95-mm² area. The apical and basolateral surfaces of the epithelium were perfused continuously at a rate of 10 to 20 ml/min (chamber volume, 2 ml) at 37°C. The bath solution contained the following (mmol/liter): NaCl (145), KH₂PO₄ (0.4), K₂HPO₄ (1.6), D-glucose (5), MgCl₂ (1), and Ca²⁺ gluconate (1.3), pH 7.4. All experiments were carried out under open-circuit conditions. The transepithelial potential difference (V_{te}) was recorded relative to the luminal side of the epithelium, and the current was regarded as positive when conventional current flowed from the apical to the serosal side of the epithelium. The transepithelial resistance (R_{te}) was determined by applying short (1-s) current pulses ($\Delta I = 0.5 \mu A$) across the mucosa. Voltage deflections recorded in the absence of the mucosa were subtracted from those obtained in its presence, and R_{te} was calculated according to Ohm's law ($R_{te} = \Delta V_{te} / \Delta I$). Tissues were accepted only if the R_{te} exceeded that obtained for an empty chamber by at least a factor of 3. Under these conditions, the recordings were stable for 3 to 4 h.

Compounds and statistics. All compounds used were of the highest available grade of purity. 3-Isobutyl-1-methylxanthine (IBMX), forskolin, amiloride, bisindolylmaleimide 1 (BIM1), carbachol, apyrase, and suramin were obtained from Sigma (Deisenhofen, Germany). 1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) was obtained from Adalab Scientific (Norwood, South Australia), and neuraminidase was obtained from Boehringer (Mannheim, Germany). Results are presented as means \pm standard errors of the means (SEM) (n = the number of preparations tested). Statistical significance was assessed using paired or unpaired Student's *t* tests at a *P* value of <0.05 .

RESULTS

Baseline electrical properties of the mouse tracheal epithelium. Under open-circuit conditions, V_{te} was 9.1 ± 0.5 mV ($n = 32$) and R_{te} was $67.9 \pm 5.1 \Omega cm^2$ ($n = 32$), resulting in an equivalent short-circuit current (I_{scEq}) of $137.3 \pm 11.5 \mu A/cm^2$ ($n = 32$). The addition of the inhibitor of epithelial Na⁺ channels, amiloride (10 μ mol/liter), to the solution bathing the apical membrane of the epithelium reduced the equivalent short-circuit current to $24.3 \pm 1.9 \mu A/cm^2$ ($n = 32$). This indicates that electrogenic ion transport by the tracheal epithelium was dominated by the transport of Na⁺ through epithelial Na⁺ channels, consistent with other studies of this tissue (23).

Effects of RSV on the mouse tracheal epithelium. We found that exposure of the apical membrane of the mouse tracheal epithelium to 2×10^4 PFU/ml of RSV for 1 h reduced V_{te} from 13.1 ± 1.5 mV ($n = 7$) to 8.1 ± 1.1 mV ($n = 7$) (Fig. 1A) and increased R_{te} from $74.1 \pm 7.1 \Omega cm^2$ ($n = 7$) to $87.8 \pm 8.6 \Omega cm^2$ ($n = 7$). It should be noted that RSV did not cause an immediate transient increase in V_{te} coupled with a reduction in R_{te} (Fig. 1A) of the type that we have previously found to accompany the exposure of the tracheal epithelium to Sendai virus (21). Incubation for 1 h in the absence of RSV did not significantly change the electrical properties of the epithelium (Fig. 1B). V_{te} prior to incubation in the control medium, $13.8 \text{ mV} \pm 1.9 \text{ mV}$ ($n = 7$), did not differ significantly from that observed after 1 h of incubation, $12.1 \pm 1.7 \text{ mV}$ ($n = 7$). Similarly, there was also no change in R_{te} during control incubation. At time zero, R_{te} was $78.8 \pm 7.1 \Omega cm^2$ ($n = 7$), which was not significantly different from V_{te} observed after 1 h of incubation in control medium, $81.8 \pm 8.6 \Omega cm^2$ ($n = 7$). Moreover, incubation of the tissues with supernatants isolated from noninfected HEp-2 cells had no effect on amiloride-sensitive transport ($20.2 \pm 3.0 \mu A/cm^2$ versus $21.7 \pm 1.9 \mu A/cm^2$; $n = 6$).

As is evident from Fig. 1A and B, exposure to RSV for 1 h reduced the magnitude of the response of V_{te} to amiloride, suggesting that RSV had inhibited the amiloride-sensitive Na⁺ transport. This is confirmed in Fig. 1C, which shows that exposure to RSV reduced the amiloride-sensitive component of the short-circuit current by approximately 50%. Incubation for 1 h without RSV, however, had no effect (Fig. 1C). We further found that the inhibition of the amiloride-sensitive short-circuit current produced by apical exposure to RSV for 1 h was dependent on the concentration of the virus over the range of 100 to 100,000 PFU/ml (Fig. 2).

We then investigated whether exposure to RSV affected any other transport properties of the epithelium in the absence of amiloride. We first examined the effects of RSV on the Ca²⁺-activated secretory pathway (Fig. 3). We found that the serosal addition of 100 μ mol/liter carbachol produced an increase in I_{scEq} of $173 \pm 32 \mu A/cm^2$ ($n = 7$) in tissues exposed apically for 1 h to RSV. This response did not differ significantly from their response to serosal carbachol prior to RSV exposure, $165 \pm 36 \mu A/cm^2$ ($n = 7$). In additional control experiments, we confirmed that the increase in the short-circuit current evoked by carbachol in tissues incubated in control medium for 1 h, $208 \pm 24 \mu A/cm^2$ ($n = 7$), did not differ significantly from the response observed prior to incubation, $199 \pm 16 \mu A/cm^2$ ($n = 7$).

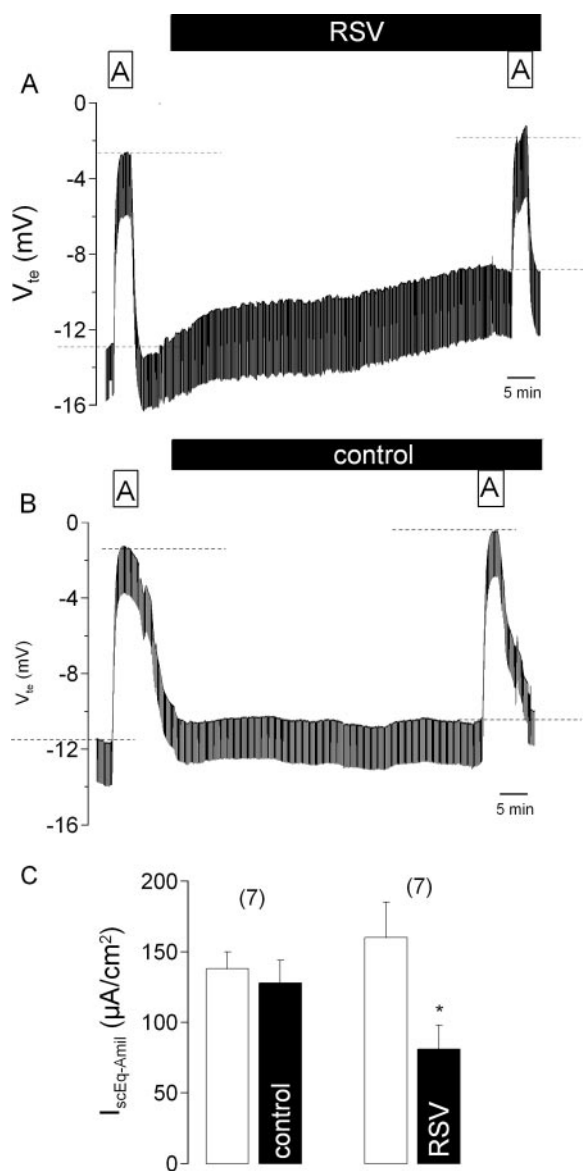


FIG. 1. Effect of RSV on amiloride ($10 \mu\text{mol/liter}$)-sensitive Na^+ absorption in the mouse trachea. Original Ussing chamber recordings of V_{te} before and during apical exposure for 1 h to 2×10^4 PFU/ml RSV (A) or control buffer (B). Dashed lines in panels A and B indicate the effects of amiloride. Panel C summarizes the measurements of the amiloride-sensitive Na^+ current ($I_{scEq-Amil}$) obtained under control conditions and after incubation with either control buffer solution or RSV. The data are presented as means \pm SEM (with the number of experiments in parentheses). The asterisk indicates a statistically significant difference compared to the control.

Hence, exposure to RSV did not affect the Ca^{2+} -activated secretory pathway.

We also examined whether RSV affected the cyclic AMP-activated secretory pathway (Fig. 4). We found that in tissues exposed to RSV for 1 h, the serosal addition of a combination of $2 \mu\text{mol/liter}$ forskolin and $10 \mu\text{mol/liter}$ IBMX produced an increase in the short-circuit current, $89 \pm 12 \mu\text{A/cm}^2$ ($n = 7$), which was not significantly different from the response observed prior to RSV exposure, $97 \pm 14 \mu\text{A/cm}^2$ ($n = 7$). We

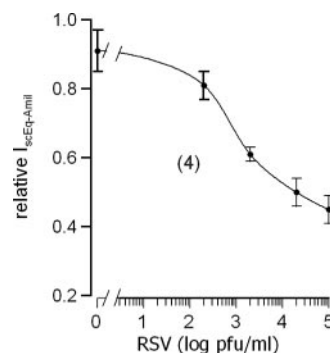


FIG. 2. Concentration-response curve for the inhibitory effect of application for 1 h to the apical membrane of RSV on the amiloride-sensitive short-circuit current ($I_{scEq-Amil}$) in the mouse trachea. In this figure, the $I_{scEq-Amil}$ measured 1 h after exposure to RSV has been normalized to the $I_{scEq-Amil}$ measured prior to exposure to give the relative $I_{scEq-Amil}$. The data are presented as means \pm SEM (with the number of experiments in parentheses).

further found that the short-circuit current response to forskolin plus IBMX, when added to tissues incubated in control medium for 1 h ($91 \pm 9 \mu\text{A/cm}^2$; $n = 7$), was not different to their response prior to incubation ($99 \pm 11 \mu\text{A/cm}^2$; $n = 7$). Hence, exposure to RSV does not affect the cyclic AMP-activated secretory pathway. Considering this finding with our finding that RSV does not affect the response to carbachol (see above), we conclude that the inhibition of amiloride-sensitive

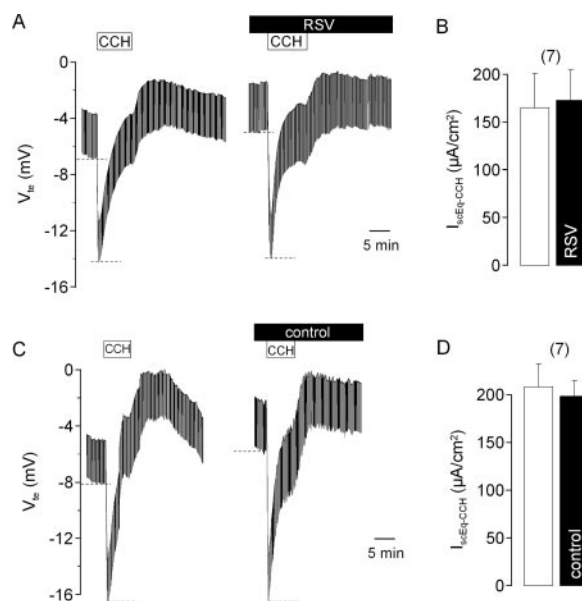


FIG. 3. Effect of RSV on carbachol (CCH) ($100 \mu\text{mol/liter}$)-induced ion transport in the mouse trachea. Original Ussing chamber recordings of V_{te} and the CCH responses prior to and following apical exposure for 1 h to RSV (1×10^4 PFU/ml) (A) or to control buffer (C). Panel B summarizes the effects of 1 h of exposure to RSV on the CCH-induced change in the short-circuit current ($I_{scEq-CCH}$). Dashed lines in panels A and C indicate the effects of CCH. Panel D summarizes the effects of 1 h of exposure to control buffer on the CCH-induced change in $I_{scEq-CCH}$. The data are presented as means \pm SEM (with the number of experiments in parentheses).

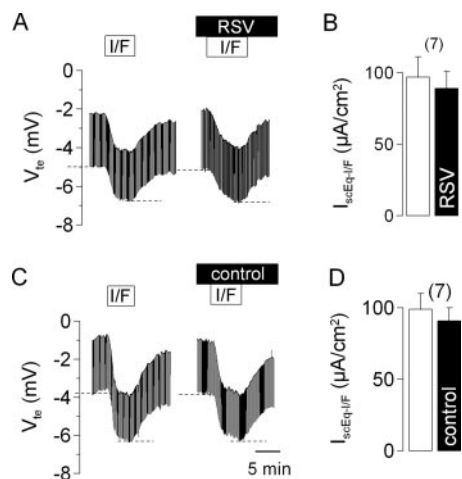


FIG. 4. Effect of RSV on IBMX (100 $\mu\text{mol/liter}$) and forskolin (2 $\mu\text{mol/liter}$)-induced ion transport in the mouse trachea. Original Ussing chamber recordings of V_{te} and the responses to IBMX plus forskolin (I/F) prior to and following apical exposure for 1 h to RSV (2×10^4 PFU/ml) (A) or control buffer (C). Panel B summarizes the effects of 1 h of exposure to RSV on the IBMX-plus-forskolin-induced change in the short-circuit current ($I_{scEq-I/F}$). Dashed lines in panels A and C indicate the effects of IBMX and forskolin. Panel D summarizes the effects of 1 h of exposure to control buffer on the IBMX-plus-forskolin-induced change in $I_{scEq-I/F}$. The data are presented as means \pm SEM (with the number of experiments in parentheses).

Na^+ absorption by RSV is selective and is not due to a non-specific toxic effect on the tracheal epithelium.

Effects of the RSV F protein. The RSV F, or fusion, protein mediates the fusion of viral particles with the membrane of uninfected cells and has been shown to trigger many of the cellular responses to infection with RSV (13). Thus, we investigated whether the effect of intact RSV on the amiloride-sensitive Na^+ current could be reproduced by the F protein. As shown in Fig. 5A, we found that the amiloride-sensitive short-circuit current is significantly reduced by exposure of the apical membrane for 1 h to 2.5 $\mu\text{g/ml}$ of recombinant F protein. Since the actions of influenza virus are mediated by protein kinase C (20), we then investigated whether the actions of the F protein could be blocked by the potent inhibitor of protein kinase C, BIM-I (100 nmol/liter). We found that they were. We further found that incubation in BIM-I prevented the inhibitory effect of the intact virus (Fig. 5A).

RSV inhibits amiloride-sensitive Na^+ absorption in cultured epithelia. We then examined whether substromal cells are required for the inhibitory effects of RSV on amiloride-sensitive Na^+ absorption. For this purpose, we used cultured monolayers of the M1 mouse collecting duct cell line, which we have previously shown to have the same responses to influenza virus and parainfluenza virus as does the tracheal epithelium (20,21). As is shown in Fig. 5B, we found that amiloride-sensitive Na^+ absorption in M1 cell monolayers is significantly inhibited by RSV. Furthermore, this inhibition can be prevented by treating the monolayer with 100 nmol/liter BIM1, in agreement with our findings for the mouse tracheal epithelium (see above).

Nature of the apical receptor by which RSV alters epithelial ion transport. When UV light-inactivated RSV was applied to

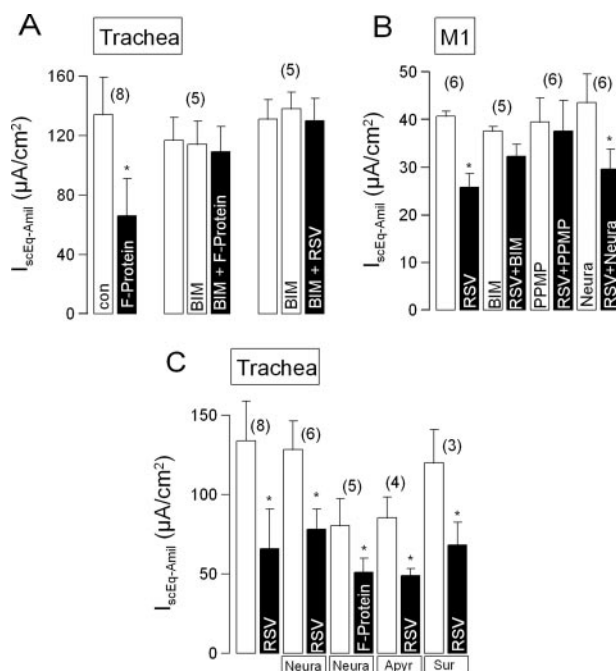


FIG. 5. (A) Summary of the effects on the amiloride-sensitive short-circuit current ($I_{scEq-Amil}$) across the mouse tracheal epithelium of apical application for 1 h of the RSV F protein (2.5 $\mu\text{g/ml}$), the RSV F protein (2.5 $\mu\text{g/ml}$) in the presence of the protein kinase C inhibitor BIM-I (100 nmol/liter), and intact RSV (10^5 PFU/ml) in the presence of BIM-I (100 nmol/liter). (B) Summary of the effects on $I_{scEq-Amil}$ across cultured mouse collecting duct (M1) cell monolayers of application for 1 h of RSV (2×10^5 PFU/ml) and RSV (2×10^5 PFU/ml) in the presence of BIM1 (100 nmol/liter). Also shown are the effects on the inhibition of I_{scEq} by RSV (2×10^5 PFU/ml) of preincubation of the monolayers in PPMP (40 $\mu\text{mol/liter}$) for 24 h or preincubation in neuraminidase (0.5 U/ml) for 30 min. (C) Summary of the effects on $I_{scEq-Amil}$ across the mouse tracheal epithelium of 1 h of apical exposure to RSV (2×10^5 PFU/ml) or the F protein (2.5 $\mu\text{g/ml}$) following pretreatment of the apical surface of the epithelium with neuraminidase (Neura) (0.5 U/ml) for 30 min or when apyrase (Apyr) (2 U/ml) or suramin (Sur) (100 $\mu\text{mol/liter}$) have been included in the apical bathing solution. The data are presented as means \pm SEM (with the number of experiments in parentheses). The asterisks indicate statistically significant differences compared to the corresponding controls. con, control.

mouse airways, 65% ($n = 5$) of the amiloride-sensitive transport was inhibited, further suggesting that RSV inhibits transport through binding to the cell membrane. We investigated whether the actions of RSV on cultured M1 cells are mediated by a glycoprotein or a glycolipid. Our previous studies have shown that influenza virus acts via a neuraminidase-sensitive glycoprotein in the apical membrane of the epithelial cells (20), whereas the action of parainfluenza virus requires an apical membrane glycolipid, the synthesis of which can be inhibited by incubation of the cells in PPMP, an inhibitor of the enzyme glucosylceramide synthase (12, 21). In the present experiments, we found that preincubation of the apical surface of M1 cell monolayers in neuraminidase (0.5 U/ml) for 30 min had no impact on the inhibition of the amiloride-sensitive current produced by the subsequent addition of RSV (Fig. 5B). Similarly, preincubation of the apical surface of the tracheal epithelium with neuraminidase (1 U/ml) for 30 min did not

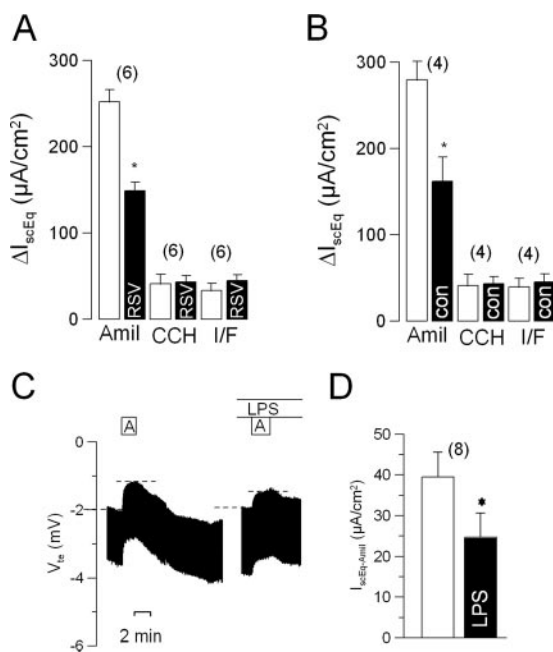


FIG. 6. Summaries of the effects of RSV (A) or control (con) (B) incubation on short-circuit current responses (ΔI_{scEq}) to amiloride (Amil), carbachol (CCH), and forskolin plus IBMX (I/F). Dashed lines in panel C indicate the effects of amiloride. The effects of lipopolysaccharide (LPS) (10 μ M) on transepithelial voltage and amiloride-sensitive transport (C) and amiloride-sensitive I_{scEq} (D) are shown. The data are presented as means \pm SEM (with the number of experiments in parentheses). The asterisks indicate statistically significant differences compared to the corresponding controls.

prevent the reduction of the amiloride-sensitive current produced by intact RSV or recombinant F protein (Fig. 5C). Preincubation of M1 cell monolayers for 24 h in 40 μ Mol/liter PPMP did, however, abolish the inhibitory action of RSV F protein on amiloride-sensitive Na^+ absorption (Fig. 5B).

Potential role for Toll-like receptor type 4. Some effects of RSV on cellular behavior have been reported to be mediated by Toll-like receptor 4 (14, 16); hence, we examined the effects of RSV on the tracheal epithelium of C3H/HeJ mice, a mouse strain in which Toll-like receptor 4 has been rendered unable to activate intracellular signaling pathways by a natural mutation in its TIR domain (31). When measured under baseline conditions, the transport properties of the tracheal epithelium of C3H/HeJ mice were quite different from those of the C57BL and QS strains. In particular, the short-circuit current activated by the serosal addition of 100 μ Mol/liter carbachol, $41 \pm 13 \mu\text{A}/\text{cm}^2$ ($n = 4$) (Fig. 6), was approximately 25% of that observed in the C57BL and QS strains, consistent with the reported reduction in bronchoconstriction provoked by methacholine and acetylcholine in the C3H/HeJ strain (9). Similarly, the addition of 2 μ Mol/liter forskolin plus 10 μ Mol/liter IBMX to the serosal solution induced an increase in the short-circuit current of only $39 \pm 10 \mu\text{A}/\text{cm}^2$ ($n = 4$), which was roughly 50% of the response in the C57BL and QS strains. In contrast, the amiloride-sensitive short-circuit current, $280 \pm 21 \mu\text{A}/\text{cm}^2$ ($n = 4$), was approximately twice that observed in the C57BL and QS strains but then declined to $162 \pm 28 \mu\text{A}/\text{cm}^2$ during 1 h of perfusion under control conditions, while the responses

to carbachol and to forskolin plus IBMX remained unchanged (Fig. 6B). Incubation of the tracheal epithelium from C3H/HeJ mice with RSV for 1 h was accompanied by a decline in the amiloride-sensitive short-circuit current from $280 \pm 21 \mu\text{A}/\text{cm}^2$ ($n = 8$) at time zero to $148 \pm 9 \mu\text{A}/\text{cm}^2$ after 1 h. The responses to carbachol and to forskolin plus IBMX did not change significantly during incubation with RSV. The decline in the amiloride-sensitive short-circuit current observed during 1 h of incubation with RSV, 42%, did not differ from the decline we observed under control conditions, 43%. Hence, it appears that RSV may require functional type 4 Toll-like receptors to exert its effects on epithelial ion transport. This is further supported by the finding that lipopolysaccharides (10 $\mu\text{g}/\text{ml}$) also inhibited amiloride-sensitive transport (Fig. 6C and D).

Are the effects of RSV mediated by the autocrine action of UTP? It was recently reported that RSV infection in mice leads to a decrease in the amiloride-sensitive component of alveolar fluid clearance, apparently by a mechanism involving the activation of suramin-sensitive purinergic receptors by UTP (8). Furthermore, our own studies on parainfluenza virus indicated that this virus inhibits amiloride-sensitive Na^+ absorption by the autocrine action of ATP on apical purinergic receptors (21). Hence, we examined whether the effects of RSV on the tracheal epithelium are mediated by the activation of purinergic receptors or the release of UTP or ATP. We found that the inclusion of 2 U/ml apyrase, an enzyme which degrades both ATP and UTP, in the solution bathing the apical membrane did not prevent the RSV F protein from reducing the amiloride-sensitive short-circuit current from $85.4 \pm 13.1 \mu\text{A}/\text{cm}^2$ ($n = 4$) to $49.0 \pm 4.4 \mu\text{A}/\text{cm}^2$ ($n = 4$). Furthermore, the inclusion of 100 $\mu\text{Mol/liter}$ suramin, a blocker of P2Y receptors, in the solution bathing the apical membrane did not prevent the RSV F protein from reducing the amiloride-sensitive short-circuit current from $120.4 \pm 21.1 \mu\text{A}/\text{cm}^2$ ($n = 3$) to $68.1 \pm 14.5 \mu\text{A}/\text{cm}^2$ ($n = 3$). Hence, the actions of the RSV F protein are not mediated by the action of ATP or UTP on apical purinergic receptors.

DISCUSSION

We have found that exposure of the apical membrane of the tracheal epithelium to RSV produces a rapid inhibition of amiloride-sensitive Na^+ absorption. This inhibition is mediated by protein kinase C, can be reproduced by the F protein in the viral coat, and appears to be dependent on the presence of asialogangliosides in the apical membrane. Given that the responses of the epithelium to stimulation with carbachol or with forskolin plus IBMX are not affected by RSV, the effect of the virus on epithelial Na^+ transport cannot be regarded as the consequence of a “nonspecific” toxic effect of the pathogen on the epithelium.

The inhibition of amiloride-sensitive Na^+ transport produced by RSV appears to require functional type 4 Toll-like receptors. This is consistent with the evidence that these receptors mediate many cellular responses to RSV (6, 14, 24). A role for type 4 Toll-like receptors in mediating the effects of RSV on epithelial ion transport is also supported by the ability of these receptors to respond to the RSV F protein (24) and by the known role of asialogangliosides in Toll-like receptor sig-

naling (33). Whatever the exact nature of the receptor is by which RSV exerts these effects, it must be intrinsic to the epithelial cells and not mediated by the immune system, as we found that RSV could inhibit amiloride-sensitive Na⁺ transport across monolayers of the M1 collecting duct cell line, which are free of immune cells. The inhibitory effects of RSV on ion channels could also be related to the changes in sensitivity of laryngeal chemoreceptors, which may even lead to fatal apnea. In this respect, the results may be relevant to the pathogenesis of the sudden infant death syndrome associated with RSV infection (27).

The concentration range over which the inhibition is observed, 10² to 10⁵ PFU/ml, is comparable to the levels observed during RSV infections both in animal models and in humans. For example, Davis and coworkers found that RSV levels in lung tissue following nasal inoculation of mice reached 10⁴ PFU/g (8). Similarly, levels ranging from 10⁴ to 10⁵ PFU/g lung tissue have been reported in guinea pigs (7) and cotton rats (18), while concentrations of RSV in nasal and tracheal secretions in children with RSV infections average 10⁵ PFU/ml and can be as high as 10⁷ PFU/ml (30). Furthermore, the available information indicates that the concentrations of the F protein in respiratory secretions are substantially greater than would be expected from the concentration of viable RSV (17).

While our finding that RSV inhibits amiloride-sensitive Na⁺ transport in isolated epithelia agrees with the recently published report that the amiloride-sensitive component of lung fluid absorption is abolished in mice infected with RSV (8), there are differences between the findings of the two studies. In particular, Davis and coworkers found that the inhibitory effect of RSV infection on the amiloride-sensitive component of airway fluid clearance could be overcome by the instillation into the lungs of agents, such as suramin, which block apical P2Y receptors (8). They further found that instillation of the enzyme apyrase, which degrades both UTP and ATP, blocked the inhibitory effect of RSV infection whereas instillation of the enzyme hexokinase, which degrades ATP but not UTP, did not (8). That Davis et al. identified a role for the autocrine action of UTP in the effects of RSV, which we have not observed, is likely to be due to the marked differences between the two experimental situations; however, we have no mechanistic explanation for it. It is noteworthy that the mechanism by which RSV produces acute alterations in the rate of epithelial Na⁺ absorption differs from the mechanisms used by influenza virus and parainfluenza virus. As described in the introduction, influenza virus inhibits amiloride-sensitive Na⁺ transport as a consequence of the hemagglutinin in the viral coat binding a neuraminidase-sensitive glycoprotein in the apical membrane of the epithelium, leading to the activation of phospholipase C and protein kinase C and the inhibition of Na⁺ absorption (20). Sendai virus, a parainfluenza virus, on the other hand, affects epithelial transport as a consequence of triggering ATP release from the epithelial cells by a glycolipid-dependent mechanism. The ATP then stimulates apical purinergic receptors, leading to the activation of the Ca²⁺-activated Cl⁻ current and the inhibition of the amiloride-sensitive Na⁺ current (21). RSV, in inhibiting amiloride-sensitive Na⁺ but leaving Cl⁻ secretion unaffected and in using a glycolipid-dependent signaling pathway, thus appears to be using elements of both

the mechanism used by influenza virus and the mechanism used by parainfluenza virus.

RSV thus joins the growing list of respiratory pathogens that have been reported to directly affect epithelial ion transport. In addition to the respiratory viruses mentioned in the previous paragraph, bacterial pathogens have been found to alter ion transport by the respiratory epithelium so as to promote fluid accumulation in the respiratory tract. *Pseudomonas aeruginosa*, for example, has been reported to acutely inhibit Na⁺ absorption by respiratory epithelia in a variety of species (10, 11, 22, 34). This inhibition has variously been reported to require adhesion of the bacterium to the epithelium (10) or to involve products secreted by the bacterium into the extracellular medium, such as rhamnolipids (11) and heat-stable hemolysin (34). Lipopolysaccharide from *Klebsiella pneumoniae* has been reported to inhibit Na⁺ absorption by the canine tracheal epithelium (35), while *Mycobacterium tuberculosis* (37) and *Mycoplasma pulmonis* (25) have been reported to inhibit Na⁺ transport across cultured respiratory epithelia. In a previous paper on this subject (21), we suggested, on the basis of the mechanistic similarities between the epithelial responses to parainfluenza virus, to *Pseudomonas aeruginosa*, and to mechanical trauma, that these responses were all examples of a stereotypic epithelial defense mechanism that operates to sweep noxious agents away from the epithelial surface. The marked differences among the mechanisms by which influenza virus, parainfluenza virus, and RSV alter epithelial electrolyte transport are not really compatible, however, with this proposal. Rather, it would appear that these viruses have independently developed mechanisms for regulating epithelial ion transport so as to increase fluid accumulation in the respiratory tract, presumably in order to foster their spread throughout the host and from host to host. Finally, the actions of respiratory viruses on epithelial transport are an interesting example of the capacity of viruses to harness host systems to their own benefit, as has previously been observed in the dependency of viral replication on the activity of the host cells' own signaling systems (28).

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