Influenza Virus Inhibits ENaC and Lung Fluid Clearance

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A fluid-free alveolar space is critical for normal gas exchange. Influenza virus alters fluid transport across respiratory epithelia producing rhinorrhea, middle ear effusions, and alveolar flooding. However, the mechanism of fluid retention remains unclear. We investigated the effects of influenza virus strain A/PR/8/34, which can attach and enter mammalian cells but is incapable of viral replication and productive infection in mammalian epithelia, on epithelial Na channels (ENaC) in alveolar type II (ATII) cells isolated from rat lungs and grown on permeable supports with an air interface and steroids to promote ENaC expression. In parallel, we determined the effects of the virus on amiloride-sensitive (i.e., ENaC-mediated) fluid clearance in rat lungs in vivo. Although influenza virus did not change the inulin permeability of ATII monolayers, it rapidly (within 1 hr) reduced the net volume transport from the lumenal to serosal surface of the monolayers. When single channel activity was recorded from cell-attached patches on the apical membranes of ATII cells, virus exposure resulted in a reduction in the open probability (Po) of apical ENaC. U-73122, a phospholipase C inhibitor, and PP2, a Src inhibitor, blocked the effect of virus on ENaC function. GF-109203X, a protein kinase C (PKC) inhibitor, also blocked the effect, suggesting a PKC-mediated mechanism. In parallel, intratracheal administration of influenza virus produced a rapid (within 1 hr) inhibition of amiloride-sensitive (i.e., ENaC-dependent) lung fluid transport. Taken together, these results show that influenza virus rapidly inhibits ENaC in the apical membranes of ATII cells via a PLC- and Src-mediated activation of PKC, but does not increase epithelial permeability with this same rapid time course. We speculate that this rapid inhibition of ENaC and formation of alveolar edema when the virus first attaches to the alveolar epithelium might facilitate subsequent influenza infection of the epithelium, and at the very least may exacerbate influenza-mediated alveolar flooding that can
lead to acute respiratory failure and death in selected patients.
INTRODUCTION

Several respiratory pathogens, including influenza virus, are known to alter respiratory epithelial fluid transport resulting in an increased propensity for rhinorrhea, middle ear effusions, and alveolar edema (3; 7; 9; 10). The public health impact of influenza virus infection is considerable, given its high annual attack rate worldwide and significant number of hospitalizations and deaths (1; 8). The majority of infected individuals develop a self-limited acute respiratory illness characterized by rhinorrhea, cough, and fever. However, a significant number of individuals, especially those in certain high-risk groups, can develop severe lower respiratory tract complications including pneumonia, alveolar flooding, and acute respiratory distress syndrome (8; 14). In these patients, the pathogenesis of alveolar edema formation remains unclear (2).

The ability of the lungs to prevent edema formation is regulated by a delicate balance between secretion of fluid and electrolytes mediated by chloride channels, and absorption of fluid and electrolytes mediated by amiloride-sensitive Na channels (22; 26; 27). Fluid absorption is achieved through a two step process involving movement of Na from the lumen into the cell interior through Na channels located on the apical surface of epithelial cells, followed by active extrusion of Na into the serosal space by Na pump located on the basolateral surface of the cell (11; 22; 26; 27). A significant portion of the net Na absorption can be inhibited by amiloride, and since molecular biological studies have confirmed the presence of amiloride-sensitive epithelial Na$^+$ channel $\alpha$, $\beta$, and $\gamma$-subunit protein ($\alpha$, $\beta$, and $\gamma$-ENaC) in lung epithelia, it is generally believed that this portion of the Na$^+$ transport is mediated by some form of ENaC (16; 17; 32). ENaC loss of function mutations as well as ENaC knock-out mice provide additional evidence to
show that disruption of this process can lead to altered fluid balance in the lung (13; 18).

It has recently been reported that influenza virus can inhibit amiloride-sensitive transport in tracheal epithelium (19). In the present study, we have extended these observations to distal alveolar epithelial cells and performed single channel studies to evaluate the acute effects of influenza virus on ion transport by rat alveolar type II cells. Our findings show that exposure to influenza virus causes rapid inhibition of apical Na$^+$ channels in type II cells and that this effect is mediated through the cytosolic, non-receptor tyrosine kinase, Src, phospholipase C, and protein kinase C. Consistent with an inhibition of ENaC, we have also shown that instillation of influenza into the lungs in vivo reduces the rate of lung fluid clearance. These findings provide an explanation for the acute edematous injury and ARDS that can complicate the course of influenza viral infection of the lower respiratory tract, and suggest potential targets for therapeutic intervention.
Methods and Procedures

Influenza Virus. The pneumotropic influenza virus A/PR/8/34 (PR8; H1N1) was grown at 33°C for 2 days in the allantoic cavity of 10-day embryonated hen eggs after injection of a 1:10 diluted virus. The eggs were further incubated on ice overnight and the allantoic fluid was then harvested and clarified by centrifugation at 3000g for 15 minutes. Aliquots of allantoic fluid containing the virus were then stored at -80°C. This strain is capable of infecting mammalian cells in that it can attach, enter cells, and induce transcription of certain early genes such as hemagglutinin. However, it is incapable of replication within mammalian cells and therefore cannot produce cell lysis or the subsequent inflammatory response that characterizes clinically relevant influenza pneumonia. Therefore, this strain served as an ideal tool to distinguish the immediate effects of influenza attachment and internalization in a target epithelium from any later effects that could be secondary to epithelial cell lysis rather than a direct effect of the virus per se. We confirmed that the virus we harvested could indeed infect cells by exposing monolayers of freshly isolated rat alveolar epithelial type II (ATII) cells in 24-well plates to the isolated virus (10^6 PFU/ml) for 1 hr. The supernatants were then removed at either 0, 3, 12, or 14 hrs after virus exposure and assayed for virally-induced hemagglutinin activity. To measure the hemagglutinin titers, 100 l of PBS was added to wells of a 96-well round bottom microtiter plate after which 100 l of the supernatant to be tested was added to the first well, and the first well was serially diluted in two-fold steps. Then 100 l 0.5% Chick Red Blood cells (washed thoroughly) were added to each well and allowed to settle for 60 min at 4°C. The hemagglutinin titers were determined based on whether the wells showed a diffuse appearance (negative) or a button appearance (positive) at the bottom of the wells for a given serial dilution.
**Cell Culture.** ATII cells were isolated by enzymatic digestion of lung tissue from adult Sprague-Dawley rats (200-250g) using published techniques (17). Briefly, the rats were anesthetized with pentobarbital and heparinized (100 units/kg). ATII cells were digested by tracheal instillation of elastase (0.4 mg/cc). Lung tissue purification was based on differential adherence of cells to dishes coated with rat IgG. Non-adherent ATII cells were collected, centrifuged, and seeded onto permeable supports in a highly enriched medium (3 parts Coon's modification of Hamm's F-12 and 7 parts Liebovitz's L-15 with 1 µM dexamethasone). Cells were grown on a specialized culture support (17), which is optimized for patch clamp recording and which allows the cells to grow on a permeable support (Millipore CM) while they are submerged in medium. After the cells had attached to the culture surface (this usually required 2-4 hours), medium was drained from the apical surface and cells were allowed to grow with medium on the basolateral surface and air on the apical side. Cells were incubated in 95% air and 5% CO₂ and used for patch-clamp studies between 24-96 hours after plating.

Using this method, we were able to obtain an ATII cell population with 95% viability (confirmed by LIVE/DEAD Eukolight Viability/Cytotoxicity Kit (1-3224), Molecular probes Inc., Eugene, OR) and 95% purity (confirmed by staining for surfactant protein A and B). Contamination by macrophages and fibroblasts was <5%. Cells were used for patch clamp experiments during the first 24-96 hours in culture while they maintained ATII cell phenotype (light microscopy) and function (surfactant production using radiolabeled choline incorporation) as we have previously reported (17).
Measurement of the inulin permeability and volume flux of ATII monolayers. ATII cells were plated at a density of 1 X 10^6 cells/cm^2 on polycarbonate transwell membranes with a 0.4 m pore size and 0.3 cm^2 surface area (Corning, NY). The culture medium in the upper and lower chambers was DMEM/F12 containing 10% fetal bovine serum. After 72 h, the medium in the upper chamber was gently removed by aspiration and culture was continued with an air-liquid interface. At 96 h, the medium in the lower chamber was replaced with fresh medium and 150 l of medium (with or without 10^6 pfu/ml influenza virus) was added to the apical surface. The transwells were then returned to the incubator maintained at 37°C, 100% humidity and 10% CO2 for 1 h. In parallel experiments, fresh medium containing virus (10^6 pfu/ml) was added to the lower chamber and 150 l of fresh medium containing no virus was added to the apical surface. In either experiment, after 1 h of incubation the cells were moved to a humidified tent and the medium in the lower chamber was removed and fresh medium with no virus was added. The medium on the apical surface was removed and replaced with fresh medium containing 1.0 Ci/ml of ^3H-inulin. After 5 min, 20 l of the medium on the apical surface of the cells was removed and designated as the zero time point. The transwells were then returned to the incubator for an additional 2 h. After this incubation period, the media from the apical surface was removed and centrifuged to remove non-adherent cells. A 20 l sample was removed from the supernatant to determine inulin concentration and another 20 l was removed to determine specific activity. A 20 l sample of the medium in the lower chamber was removed to determine radioactivity. To avoid a hydrostatic pressure gradient, fluid in the upper and lower chambers were maintained at the same level during the experimental period. Each experiment was performed in duplicate using type II cells isolated from six different animals.
**Chemicals.** Bath and pipette solutions used in the cell-attached mode contained (in mM): NaCl (140), MgCl₂ (1), CaCl₂ (1), KCl (5), HEPES (10), pH 7.4 with 2N NaOH. The contents of bathing and pipette solutions were varied as appropriate for specific protocols. All chemicals were obtained from Sigma Chemicals (St. Louis, MO).

**Patch clamp experiments.** Patch-clamp experiments were carried out at room temperature. The pipettes were pulled from filamented borosilicate glass capillaries (TW-150, World Precision Instruments, FL) with a two-stage vertical puller (Narishige, Tokyo, Japan). The pipettes were coated with Sylgard (Dow Corning) and fire polished (Narishige, Tokyo, Japan). The resistance of these pipettes was 5-8 Mohms when filled with pipette solution. We used the cell-attached configuration for our studies since in this configuration the cytoplasmic constituents remain intact, thus allowing us to study the role of cytoplasmic second messengers in regulation of ion-channel activity. After formation of a high-resistance seal (>50 Gohms) between the pipette and cell membrane, channel currents were sampled at 5 kHz with patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) and filtered at 1 kHz with a low-pass Bessel filter. Data were recorded by a computer with pCLAMP 6 software (Axon Instruments, Foster City, CA). The open probability (Pₒ) of the channels was calculated using FETCHAN in pCLAMP 6. Single-channel conductance was determined using a linear regression of unitary current amplitudes over the range of applied pipette potentials.

For cell-attached patches, voltages are given as the negative of the patch pipette potential (−Vpipette). This potential is the displacement of the patch potential from the resting potential (about -40 mV for ATII cells) and positive potentials represent depolarizations and negative
potentials represent hyperpolarizations of the cell membrane away from the resting potential. For a highly selective Na channel with a Na concentration gradient of 10 to 1 (outside to inside), the reversal potential would be +60 mV. Therefore, it would require a 100mV positive voltage displacement (-V_{\text{pipette}} = +100mV) from the resting potential to reach the reversal potential.

**Single channel analysis.** We used the product of the number of channels (N) times the single channel open probability (P_o) as a measure of channel activity within a patch. This product was calculated without making any assumptions about the total numbers of channels in a patch or the P_o of a single channel:

\[ NP_o = \sum_{n=1}^{N} \frac{nt_n}{T} \]

where T is the total recording time, n is the numbers of channels opening, and t_n is the open time for the n channels. The total number of functional channels (N) in a patch was estimated by observing the number of peaks in current-amplitude histogram over the entire duration of the recording after which P_o could be calculated from NP_o and N.

**Intratracheal administration of virus and determination of lung liquid clearance:** Young adult male Sprague-Dawley rats (300 – 25 gm; Charles River Laboratory, Wilmington, MA) were anesthetized with pentobarbital (100 mg/kg intraperitoneally) and a tracheostomy tube was placed. In the untreated control group, the lungs were removed immediately after anesthesia was induced with pentobarbital. In the experimental groups, 0.5 cc of sterile saline (0.9%) containing 10^6 PFU per cc (5 x 10^5 PFU total) of the virus or 0.5 cc of sterile saline alone (vehicle) was then
administered intratracheally and the rats were ventilated with room air using a Harvard rodent ventilator with the following settings: tidal volume of 2.5 cc, positive end-expiratory pressure (PEEP) of 2.5 cm H$_2$O, and a rate of 60 breaths/min. Ventilation was maintained for 1 hr at which time either 1 cc of sterile saline alone or 1 cc of saline containing amiloride (1 mM) was administered intratracheally in each rat and ventilation was continued for an additional 1 hr. Therefore, each rat was challenged with a total of 1.5 cc of saline during the 2 hr experimental protocol. This volume was chosen based on our empiric observations with this model. This is a significant alveolar liquid challenge for rats of this size (~ 5 cc/kg) and is similar to the volume of 2 cc we used with this same method to evaluate the effects of chronic ethanol ingestion on lung liquid clearance in rats (28). At the end of the experimental period, the rats were euthanized by en bloc excision of the heart and lungs. The lungs were dissected from the large airways, heart and mediastinal structures, and excess fluid was removed by gently blotting the external surface with filter paper. Lungs were weighed (wet weight), and then re-weighed after desiccation by overnight incubation at 70$^\circ$C (dry weight). Wet/dry ratios were then calculated and used as an index of lung liquid clearance. Specifically, any saline instilled into the airway that is not cleared from the lung by sequential clearance from the alveolar space and the interstitial space increases the wet/dry ratio of the lung, and the wet/dry ratios correlate inversely with lung liquid clearance. In this closed chest model lung lymphatic function is intact. Further, although general anesthesia could depress cardiac function, thereby raising the pulmonary capillary wedge pressure and increasing interstitial edema, this should be equivalent in both virus-treated and vehicle-treated rats. Therefore, any significant differences in the virus-treated group are consistent with a significant effect of the influenza virus on the ability of the lung to clear sodium and water from the alveolar and/or interstitial space.
**Statistical Analysis.** Statistical analysis for the changes in open probability of channels, and the biochemical estimations were performed using SigmaStat (Jandel, CA). Statistical significance between two groups was determined by paired or unpaired tests, as appropriate. When the comparison among three or more groups was required, statistical significance was determined by one-way analysis of variance (ANOVA) followed by pair-wise comparisons with Student-Neumann-Keul’s test to determine significant differences among groups.
Results

*Influenza virus A/PR/8/34 strain infects rat alveolar type II cells in vitro.* Before we could evaluate the potential effects of influenza virus on rat alveolar epithelial ENaC function, we had to confirm that the virus we harvested (see Methods) could infect rat ATII cells. Although the influenza virus A/PR/8/34 strain is not capable of replication in mammalian cells and therefore cannot produce influenza pneumonia *per se*, it is capable of attachment and endocytosis (i.e., infection) of mammalian cells. To confirm that our harvested virus could infect rat alveolar epithelial cells, freshly isolated rat ATII cells were cultured in 24-well plates and exposed to the virus ($10^6$ PFU/ml) for 1 h at $37^\circ$ C, and the supernatants were analyzed for hemagglutinin activity (reflecting cellular transcription of early viral genes). Formalin-inactivated virus served as a negative control. As shown in Table 1, the A/PR/8/34 strain of influenza virus yielded a titer of 64 HAU/ml at 3 h post exposure and an increased titer of 256 HAU/ml at 12 h and 24 h post exposure compared to the inactivated virus that did not produce detectable viral infection even at 24 h post exposure.

*Influenza virus reduces ENaC activity in alveolar type II cells.* We have previously shown that when ATII cells are grown on permeable supports in the presence of steroids and an apical air interface, the predominant channel is a 6-ps highly selective cation (HSC) channel with a Na$^+$-to-K$^+$ selectivity of $>80:1$, moderate inward rectification, and a reversal potential near $+100$ mV (17). This channel is sensitive to submicromolar concentrations of amiloride, with 37 nM amiloride producing a $50\%$ decrease in channel open probability (17).

Treatment of ATII cells with $10^6$ PFU/ml of influenza virus for 1 hour resulted in a significant
reduction in the activity (measured as open probability, Po) of apical membrane HSC channels compared to untreated cells (mean ± SEM, control 0.15 ± 0.02 vs influenza 0.05 ± 0.01, n=6, p<0.05) (Fig. 1A and B). Exposure to the virus had no effect on other single channel characteristics. Similarly, exposure of the cells to virus-free saline had no effect on channel open probability.

**Neuraminidase treatment blocks the viral effect.** Since one of the first steps in the viral infection process is attachment and endocytosis of the virus into the cell, we evaluated the effect of neuraminidase that can prevent the binding of hemagglutinin in the viral envelope to sialic acid residues on receptors in the apical membrane. Neuraminidase (0.1 unit/ml) alone had no effect on channel activity. However, pretreatment of cells with neuraminidase followed by the virus blocked the inhibitory effect of virus on channel activity, suggesting that viral attachment to the cell is essential for its effect on Na\(^+\) channels (Fig. 1B). However, 10 M cytochalasin D, an inhibitor of some endocytotic processes, did not block the viral effect (Fig. 2). This suggests that in the time frame studied (60 min), viral attachment is necessary, but cytochalasin-D-sensitive endocytosis may not be necessary to produce inhibition of ion channels.

**Inhibiting a cytosolic tyrosine kinase, Src, blocks the effect of influenza virus.** Although cytochalasin D did not block the effects of the virus on ENaC function, we reasoned that some of the initial events associated with endocytosis might still be required for the action of influenza on ENaC. One of the early events in clathrin-mediated endocytosis is activation of the cytosolic tyrosine kinase, Src; therefore, we examined whether exposure to influenza virus could activate Src. We determined that inhibition of Src by PP2 blocked the influenza virus inhibition of HSC
channels (Fig. 3), indicating that indeed early cellular events associated with viral endocytosis are linked to the decrease in ENaC function.

**Phospholipase C and protein kinase C activity is necessary for the viral effects.** We next examined the intracellular mechanism by which influenza virus acted on HSC channels. Src activation modulates PLC by phosphorylation of PLC-γ1 and a previous study by Kunzelmann et al (19) on tracheal epithelium had suggested a protein kinase C-mediated action of influenza virus. Therefore, we examined whether blocking this kinase and its upstream activating pathway reduced the effect of influenza virus. Inhibitors of phospholipase C (U-73122) and protein kinase C (GF 109203X) also blocked the viral inhibition of HSC (Fig. 3). In addition, pretreatment of ATII cells with the protein kinase C inhibitor, GF 109203X, without any viral exposure resulted in increased open probability of channels compared to untreated controls, suggesting a baseline tonic inhibition of HSC channels by protein kinase C.

**The inulin permeability of ATII monolayers exposed to influenza virus is unchanged, but net fluid flux is reduced.** Another critical component of alveolar epithelial fluid clearance is the integrity of the intercellular junctions that form the normally tight alveolar epithelial barrier. Therefore, viral attachment could also increase intercellular permeability that would exacerbate the inhibition of ENaC function and further increase pulmonary edema. To test this possibility, we determined the flux of $^3$H-inulin from the apical to the basolateral surface as an indicator of epithelial barrier integrity. For twelve untreated monolayers, $8.3 \pm 1.2\% (4.2 \pm 0.6\%/h)$ of total luminal inulin counts appeared in the basolateral medium after a 2 hr incubation period (Table 2). For monolayers treated with either apical or basolateral virus, the percentage of inulin counts
that appeared in the basolateral medium (5.4 ± 0.5%/h for apical application of virus to twelve monolayers and 5.3 ± 0.6%/h for basolateral application to twelve monolayers) was not significantly different from untreated monolayers (Table 2). Therefore, the addition of the virus to either the apical surface or the basolateral surface had no apparent effect on epithelial monolayer integrity. In contrast, although there were no differences in intercellular integrity as assessed by inulin permeability, the addition of the virus to the apical surface of the cells resulted in a significant decrease (~30%) in the inulin concentration of the medium when compared to the control (1.038 ± 0.004 g/ml and 0.733 ± 0.006 g/ml for control and apical virus application to twelve monolayers, respectively, p < 0.001) (Table 2). In contrast, addition of the virus to the basolateral surface did not significantly alter the concentration of inulin in the medium on the apical surface when compared to the control (1.055 ± 0.017 g/ml). Since there was no difference between groups in the concentration of inulin on the apical surface at the initially and no difference in leak, the change in concentration suggested that, in the control cells, there was a significant net bulk flow of fluid from the lumenal to the serosal compartments that was reduced by the application of the virus to the apical surface (i.e., there was accumulation of fluid on the apical surface of the cells).

*The lungs of rats exposed to influenza virus are unable to clear fluid as well as untreated lungs following intratracheal saline challenge.* Our findings in the *in vitro* studies indicated that the influenza virus produced a rapid (i.e., within 60 min) inhibition of ENaC function and, in parallel, decreased transepithelial sodium and water transport. In contrast, the virus had no effect on epithelial monolayer permeability in this time frame. Therefore, the prediction from the *in vitro* studies was that influenza virus would produce a rapid inhibition of ENaC function *in*
vivo and impair clearance of a saline challenge from the airways. To test this prediction, we
determined the amiloride-sensitive clearance of an intratracheal saline challenge in rats with and
without exposure of their airways to influenza virus \textit{in vivo}. We first determined that rat lungs
challenged with either saline alone or saline plus influenza virus had increased (P<0.05) wet/dry
ratios compared to unchallenged rat lungs, indicating that the 1.5 cc saline challenge was
sufficient to exceed the fluid clearance capacity of the normal rat lung in 2 hrs (Figure 4 and
Table 3). However, rat lungs treated with influenza virus and challenged with a total of 1.5 cc of
saline had significantly decreased (P<0.05) lung liquid clearance, as reflected by increased lung
wet/dry ratios, compared to rats not treated with virus but challenged equivalently with a total of
1.5 cc of saline (Figure 4 and Table 3), indicating that influenza virus decreased lung fluid
clearance. Further, treatment with amiloride significantly (P<0.05) impaired lung liquid
clearance in the rat lungs that were not exposed to influenza virus (Figure 4 and Table 3),
indicating that ENaC function was intact in these lungs. In contradistinction, amiloride
treatment did not further decrease (P>0.05) lung liquid clearance in the virus-treated rat lungs
(Figure 4 and Table 3). If influenza virus had produced an epithelial permeability defect \textit{in vivo}
(which one would not predict based on the \textit{in vitro} studies), then one would have expected that
the combination of virus exposure and amiloride treatment would produce greater lung edema
than amiloride treatment alone. In fact, rat lungs exposed to influenza virus and challenged with
saline (with or without amiloride) had the same (P>0.05) wet/dry ratios as rat lungs not exposed
to virus but challenged with saline + amiloride (Figure 4 and Table 3). In a recent study on the
effects of chronic ethanol ingestion on alveolar epithelial permeability using this same model of
lung liquid clearance, lung weight/dry ratios following a 2 cc saline challenge were as high as
10-12 (Pelaez et. al.), illustrating that in this study lung edema was not maximized and therefore
we would have been able to detect a significant permeability defect superimposed on ENaC inhibition. In summary, these studies indicate that influenza virus inhibited amiloride-sensitive clearance of saline from the airways \textit{in vivo}, consistent with its inhibition of ENaC function \textit{in vitro}. 
Discussion

This study provides novel evidence for a mechanism by which influenza virus acutely inhibits alveolar epithelial ENaC function and thereby reduces transepithelial fluid clearance. Using an influenza strain that is capable of epithelial cell attachment and internalization but is incapable of inducing viral replication and consequent cell lysis, we were able to identify that during influenza virus attachment and endocytosis there is a rapid and significant decrease in ENaC function. This ENaC inhibition is mediated via a phospholipase C-induced activation of protein kinase C subsequent to the attachment of the virus to the cell surface via hemagglutinin. In contrast, although influenza virus rapidly inhibits ENaC function, the attachment and endocytosis of the virus produced no measurable effects on epithelial permeability. In parallel, we determined that these effects on ENaC function and transepithelial fluid transport are relevant \textit{in vivo}, since treatment of rat lungs with influenza virus produced a similarly rapid decrease in amiloride-sensitive (i.e., ENaC-dependent) lung fluid clearance. These early effects of influenza viral infection on airway epithelial fluid clearance appeared to be solely due to an inhibition of amiloride-sensitive Na$^+$ channels in the apical membranes of distal alveolar cells and not due to a virus-induced change in epithelial permeability. In fact, as these effects occur within 1 hr and the viral strain used in this study is not capable of replication in mammalian cells, these results argue that influenza virus-mediated inhibition of ENaC can be induced by cell attachment and internalization, early events in viral infection that precede the viral replication and epithelial cell lysis that characterize influenza pneumonia clinically. Therefore, this study provides additional evidence that influenza virus can affect lung fluid clearance independently of epithelial barrier disruption caused by viral infection \textit{per se}, and that influenza virus-mediated inhibition of ENaC provides a potential cellular mechanism for alveolar edema formation that frequently
accompanies influenza infection (1; 19; 20). In this study, we examined single channel activity and inulin permeability \textit{in vitro}, and amiloride-sensitive fluid clearance \textit{in vivo}, to evaluate the effects of influenza virus on ion transport by the distal alveolar epithelium. To our knowledge, this is the first study to investigate the consequences of immediate early influenza infection (prior to viral replication and cell lysis) on ion transport by distal lung epithelial cells. The only previous study of this nature performed short circuit current measurements using tracheal epithelial cells (19). Our study confirms the findings of Kunzelmann et al (19) who showed that Na\textsuperscript{+} channel inhibition was caused by hemagglutinin in the viral coat and appears to not require endocytosis of the virus into the cell. We have also shown that viral attachment mediates Src activation, and the downstream activation of phospholipase C and protein kinase C. This is also the mechanism by which influenza virus suppresses neutrophil function (4; 12), and leads to proliferation of B lymphocytes (29).

We have focused only on the acute effects of the virus on ion transport and, in line with the findings of Kunzelmann et al (19), we found that maximum suppression of the HSC channels was achieved at 60 minutes of exposure. There are unresolved issues about what types of Na\textsuperscript{+} channels are normally present in the apical membranes of ATII cells (22). We have previously shown that, based on conditions prevailing in the lung microenvironment, at least three types of Na\textsuperscript{+}-transporting channels may be expressed (15-17). Under ideal conditions, which include the presence of adequate oxygen concentration, an air interface, and corticosteroid stimulation, ATII cells predominantly express HSC channels (17). In studies that rely on short circuit current measurements, only the effects of an agent on net ion transport can be measured, with no specific
information about the effects on individual ion channels. Our single channel studies unambiguously identify effects of influenza virus on Na\(^+\) channels in the ATII cells.

The mechanism of action of influenza virus on Na\(^+\) transport is supported by other studies looking at viral effects in the lung (19), and other organ systems (4-6; 29; 30). The time course of the inhibitory effect on Na\(^+\) channels pointed to one of the earliest steps in viral infection, namely attachment of the virus to the cell, or its endocytosis into the cell (23; 25). Influenza A and B viruses carry two surface glycoproteins, the hemagglutinin and the neuraminidase (33). It is now well established that hemagglutinin in the viral coat binds to the sialic acid residues on a receptor present in the cell membrane of the target cell, whereas neuraminidase cleaves sialic acids from cellular receptors and extracellular inhibitors to facilitate progeny virus release, thus promoting spread of infection to neighboring cells (33). Pretreatment of cells with neuraminidase can thus inhibit the attachment of virus to the cells through loss of sialic acid receptors (24). In our studies, neuraminidase successfully blocked the viral effect, suggesting that viral attachment to the cell was an essential step in producing its effect on ion channels, and that a product released by the virus did not mediate it.

To examine the intracellular mechanism of inhibition of HSC channels by influenza virus (Fig. 5), we relied on previously published reports showing that one of the ways by which influenza virus produces its effects is through phospholipase C mediated activation of protein kinase C (4; 19; 29). Indeed, we found that inhibition of phospholipase C by U-73122 blocked the inhibitory effect of the virus on HSC channels. When cells were pretreated with the protein kinase C inhibitor, GF-109203X, there was an increase in open probability of HSC channels, suggesting a tonic inhibition of channel activity by this kinase in the basal state. Further addition
of influenza virus to cells pretreated with GF-109203X produced no effect on channel activity confirming the role of protein kinase C. There are previous reports of inhibition of amiloride-sensitive channels in renal epithelial cells by protein kinase C (20; 21); however, there are no previous reports of its effect in distal alveolar epithelium. It is also not clear whether protein kinase C regulates Na$^+$ channel activity by direct phosphorylation of the channel or by some other mechanism. Aldosterone and insulin have recently been shown to induce phosphorylation of ENaC- and -subunits in MDCK cells in which subunits are over expressed (31). However, examining subunit phosphorylation in over expressing cells may be a problem since subunits appear at high concentrations in cellular compartments in which they are not normally found or only found in low concentrations. However, in native epithelial cells, there is no evidence of for PKC-induced phosphorylation (34).

Pulmonary edema is a common and often devastating effect of several types of diverse biological insults including sepsis, trauma, and aspiration. The trigger for edema formation is often a multitude of pro-inflammatory signals including cytokines, complement, and endotoxins released by the infectious agent that can cause a breakdown in the endothelial and epithelial barriers leading to excessive leakage of fluid into the interstitial and alveolar spaces. This problem can be acutely exacerbated if the normal alveolar fluid clearing mechanisms are concomitantly affected. Although we did not specifically evaluate endothelial and/or epithelial permeability in virus-exposed rat lungs in vivo, our findings are consistent with the results of the studies in vitro in which influenza virus inhibited ENaC function and transepithelial fluid clearance but had no effect on epithelial permeability. In the intact rat lung model, influenza virus inhibited lung fluid clearance to the same degree as amiloride, consistent with an inhibition of ENaC as predicted by
the in vitro studies. Further, there was no additive effect on lung fluid clearance by amiloride and influenza virus together. If the virus had somehow induced a rapid epithelial permeability defect that was not observed in vitro, then lung edema should have increased in lungs treated with both the virus and the amiloride. Therefore, although we did not specifically evaluate radiolabeled albumin flux or other indices of permeability, our findings nevertheless argue that influenza virus inhibits amiloride-sensitive (i.e., ENaC-dependent) fluid transport both in vitro and in vivo and does not appear to have comparable effects on epithelial permeability in the immediate early stages of infection. There is little doubt that during the later stages of severe influenza pneumonia, alveolar epithelial cell lysis and disruption of the epithelial barrier leads to a pathological state in which permeability is increased in addition to inhibition of active sodium transport. What remains unknown is whether this almost immediate inhibition of ENaC function during influenza viral infection offers a virulence advantage to the virus or is merely an epiphenomenon with unfortunate consequences to the infected host.

In this study, we have provided new evidence that influenza virus, via rapid and direct interactions with alveolar epithelial cells, alters ENaC function as determined by patch clamp studies on isolated cells in vitro, and inhibits alveolar liquid clearance as reflected by increased edema following a saline challenge via the airway in vivo. These findings indicate that the influenza virus has potent affects on alveolar fluid balance independently, and perhaps even prior to, actual viral invasion of the alveolar epithelium. These effects on sodium transport may well contribute to the dramatic pulmonary edema pattern that many patients exhibit. Further, this study also suggests that therapeutic strategies that have been touted for the treatment of other types of non-cardiogenic pulmonary edema, such as the use of -adrenergic agonists to increase
vectorial sodium transport by promoting ENaC function within the alveolar epithelium, may be ineffective in severe influenza pneumonia because the virus rapidly inactivates these channels. Unfortunately, if these effects are shared by other respiratory viral pathogens such as respiratory syncytial virus (RSV), this suggests a general mechanism by which these viral infections can rapidly lead to refractory alveolar edema and the consequential severe gas exchange abnormalities and respiratory failure that continue to produce significant morbidity and mortality in our society.

Acknowledgements

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Figure Legends

Figure 1. Influenza reduces ENaC activity in a neuraminidase-dependent fashion. Panel A shows representative single channel records from cell attached patch clamp. Pretreatment of ATII cells with influenza A virus for 1 hour significantly reduced the ENaC single channel activity. Downward deflections indicate inward current. Panel B shows that the sialic acid of glycoproteins on the cell surface membrane mediates the influenza-virus-induced decrease in ENaC activity. Pretreatment of ATII cells with influenza virus significantly reduced the $P_0$ value. After neuraminidase treatment (0.1 unit/ml) of cells, influenza virus no longer affected ENaC activity. Cleavage of sialic acid by neuraminidase can prevent the binding of influenza virus to ATII cells. Thus, the results in this figure indicate that viral attachment to ATII cells plays an important role in inhibition of ENaC activity. Mean ± SE, *:P<0.05 compared with control.

Figure 2. Influenza-virus-mediated inhibition of ENaC depends upon Src activation, but may not require endocytosis. 10 µM PP2, a Src inhibitor, prevents the effect of influenza A virus on ENaC activity while 10 M cytochalasin B, an endocytosis inhibitor, does not. Src is a cytosolic tyrosine kinase that plays an important role in intracellular signaling and is involved in clathrin-mediated endocytosis after interaction with -arrestin. Mean ± SE, *:P<0.05 compared with control.

Figure 3. Influenza virus-mediated inhibition of ENaC depends upon PLC and PKC activation. Src modulates phospholipase C activity by phosphorylating PLCγ-1 and PLC-produced diacylglycerol and calcium activates PKC. Pretreatment of ATII cells with 10 µM U73122, a
phospholipase C inhibitor, prevents the inhibition of open probability by subsequent treatment with influenza virus. Pretreatment of ATII cells with 1 μM GF109203x, a protein kinase C inhibitor, prevents the inhibition of open probability by subsequent treatment with influenza virus. These results suggest that the effect of influenza virus A on ENaC activity is mediated by phospholipase C and protein kinase C. Mean ± SE, *:P<0.05 compared with control.

Figure 4. Rat lung tissue wet/dry ratios as an index of lung fluid clearance in vivo, in untreated lungs (none), in lungs treated intratracheally with either 0.5 cc of saline vehicle (gray bars) or 0.5 cc of saline vehicle containing 5 x 10^5 PFU of influenza virus (black bars) followed by a challenge with 1 cc of saline amiloride. Treated rats were anesthetized and mechanically ventilated as described in the text. Saline vehicle or virus suspended in saline vehicle was instilled into the airways and mechanical ventilation continued for 1 hr at which time the saline challenge amiloride was instilled and the lungs ventilated for an additional hr. After a total of 2 hrs of ventilation, the lungs were removed and the tissue wet/dry ratios were determined as described in the text. Each value represents the mean SEM of 5 or more determinations. * P<0.05 greater compared to untreated rat lungs. **P<0.05 greater compared to vehicle-treated, saline challenged rat lungs.

Figure 5. A putative intracellular signaling pathway for influenza virus-induced inhibition of ENaC. The sequence is initiated (at ①) by influenza A virus interaction with sialic acid residues on the surface membrane (at ②) followed by recruitment and activation of the adaptor protein AP1 (at ③). AP1 promotes clathrin recruitment (at ④) and subsequent activation of -arrestin and Src (steps ⑤ and ⑥). Src is known to activate PLC (at ⑦) and PLC can activate PKC (at ⑧).
Activation of PKC is known to inhibit ENaC (at ®).
Table 1. Ability of influenza virus to attach and enter rat alveolar type II cells. ATII cells in a 24-well plate were exposed to either the intact influenza virus PR/8 strain (10^6 PFU/ml) or to formalin-inactivated virus for 1 h at 37°C. The supernatants were removed at 0h, 3 h, 12 h and 24 h after virus exposure and the virus titers in each sample were determined by performing hemagglutinin assays as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Hours after virus exposure</th>
<th>Intact virus (HA titer)</th>
<th>Formalin-inactivated virus (HA titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>256</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>256</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2. Effects of influenza virus on epithelial permeability and fluid flux *in vitro*. 150 l of medium (with or without 10^6 pfu/ml influenza virus) was added to either the apical or basolateral surface of monolayers of ATII cells on transwell supports. After 1 h incubation the virus containing medium was replaced with medium containing no virus and the medium on the apical surface was removed and replaced with medium containing 1.0 Ci/ml of ^3^H-inulin. After 5 min, 20 l of the medium on the apical surface of the cells was removed and designated as the zero time point. After a 2 h period, a 20 l sample was removed from the apical medium to determine inulin concentration. A 20 l sample of the medium from the basolateral compartment was removed to determine inulin flux. Values represent duplicate measurements from six different animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apical-to-Basolateral Inulin Flux (% total inulin counts/hour)</th>
<th>Final Apical Inulin Concentration (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4.2 ± 0.6</td>
<td>1.038 ± 0.004</td>
</tr>
<tr>
<td>Apical virus application</td>
<td>5.4 ± 0.5*</td>
<td>0.733 ± 0.006**</td>
</tr>
<tr>
<td>Basolateral virus application</td>
<td>5.3 ± 0.6*</td>
<td>1.055 ± 0.017*</td>
</tr>
</tbody>
</table>

* Not significantly different from untreated. ** Significantly different from untreated (p<0.01).
Table 3. Instilled influenza virus reduces the ability of lungs to clear a saline challenge. Wet/dry ratios were determined and used as an index of lung liquid clearance (see Methods). In the experimental groups, 0.5 cc of sterile saline (0.9%) with or without $5 \times 10^5$ PFU influenza virus was administered intratracheally. After 1 hr either 1 cc of sterile saline alone or 1 cc of saline containing amiloride (1 mM) was administered intratracheally. After an additional 1 hr., lungs were removed to measure wet and (after overnight drying) dry weights.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wet/Dry Weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>No saline challenge</td>
<td>4.8 ± 0.05</td>
</tr>
<tr>
<td>Saline challenge</td>
<td>7.4 ± 0.18*</td>
</tr>
<tr>
<td>Saline challenge + amiloride</td>
<td>8.4 ± 0.16**</td>
</tr>
<tr>
<td>Saline challenge + influenza virus</td>
<td>8.5 ± 0.27**</td>
</tr>
<tr>
<td>Saline challenge + amiloride + influenza virus</td>
<td>8.2 ± 0.17**</td>
</tr>
</tbody>
</table>

* P<0.05 greater compared to untreated rat lungs. **P<0.05 greater compared to vehicle-treated, saline challenged rat lungs. Each value represents the mean SEM of 5 or more determinations.
References


23. **Matlin KS.** The sorting of proteins to the plasma membrane in epithelial cells. *J Cell Biol*


31. **Shimkets RA, Lifton R and Canessa CM.** In vivo phosphorylation of the epithelial


A

Untreated

10^6 pfu influenza A virus

closed

closed

B

Untreated
Influenza A virus
Neuraminidase
Neuraminidase + influenza A

Chen, Seth, et al., Fig. 1
Untreated
Influenza
PP2
PP2 + Influenza
Cytochalasin B
Cytochalasin B + Influenza

Chen, Seth, et al., Fig. 2
Untreated, GF109203X, GF109203X + virus, U-73122, U-73122 + virus 

Chen, Seth, et al., Fig. 3
Lung Wet/Dry Ratio

**Treatment**

- None
- Saline
- Saline + Amiloride
- Saline
- Saline + Amiloride

Chen, Seth, et al., Fig. 4