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Respiratory Syncytial Virus Infection Results in Activation of Multiple Protein Kinase C Isoforms Leading to Activation of Mitogen-Activated Protein Kinase[1]

Martha M. Monick,[2] Janice M. Staber, Karl W. Thomas, and Gary W. Hunninghake

Respiratory syncytial virus (RSV) is an important respiratory pathogen that preferentially infects epithelial cells in the airway and causes a local inflammatory response. Very little is known about the second messenger pathways involved in this response. To characterize some of the acute response pathways involved in RSV infection, we used cultured human epithelial cells (A549) and optimal tissue culture-infective doses ($TCID_{50}$) of RSV. We have previously shown that RSV-induced IL-8 release is linked to activation of the extracellular signal-related kinase (ERK) mitogen-activated protein kinase pathway. In this study, we evaluated the upstream events involved in ERK activation by RSV. RSV activated ERK at two time points, an early time point consistent with viral binding and a later sustained activation consistent with viral replication. We next evaluated the role of protein kinase C (PKC) isoforms in RSV-induced ERK kinase activity. We found that A549 cells contain the Ca^{2+}-dependent isoforms α and β1, and the Ca^{2+}-independent isoforms δ, ϵ, η, μ, θ, and ζ. Western analysis showed that RSV caused no change in the amounts of these isoforms. However, kinase activity assays demonstrated activation of isoform ζ within 10 min of infection, followed by a sustained activation of isoforms β1, δ, ϵ, and μ 24–48 h postinfection. A cell-permeable peptide inhibitor specific for the ζ isoform decreased early ERK kinase activation by RSV. Down-regulation of the other PKC isoforms with PMA blocked the late sustained activation of ERK by RSV. These studies suggest that RSV activates multiple PKC isoforms with subsequent downstream activation of ERK kinase. ***The Journal of Immunology,*** **2001, 166: 2681–2687.**

Human respiratory syncytial virus (RSV)[3] infection is a major cause of serious lower respiratory disease in young children (<2 years of age). A large number of children hospitalized with bronchiolitis (40–60%) and pneumonia (10–25%) are infected with RSV (1–4). In addition, a significant percentage of children develop chronic pulmonary function abnormality after RSV infection (5–7). RSV is associated with significant morbidity in children with chronic lung diseases such as cystic fibrosis, bronchopulmonary dysplasia, and congenital heart disease (8–10). Recent evidence has suggested that early RSV infection contributes to the development of asthma (10–13).

RSV is a negative-stranded RNA virus in the genus *Pneumovirus*, family *Paramyxoviridae* (14). RSV infection targets the respiratory epithelium and elicits a local inflammatory response. This is characterized by inflammatory cellular infiltration and release of inflammatory mediators from both epithelial and activated immune cells (14–18). Release of IL-8 by respiratory epithelial cells is an important consequence of RSV infection. Although significant work has been done on RSV-induced transcription of the IL-8 gene, not much is understood about the second messenger pathways that generate this response. Previous work in this laboratory has described the role of the transcription factors NF-κB and AP-1 in the production of IL-8 by RSV-infected epithelial cells (16, 17). We have also established a link between activation of extracellular signal-related kinase (ERK), a mitogen-activated protein kinase (MAPK), and release of IL-8 after RSV infection (15). In this study, we extend these observations by showing that RSV produces a biphasic activation of ERK. An early ERK activation after infection appears consistent with viral binding and entry, the later activation consistent with active viral replication. Activation of one or more protein kinase C (PKC) isoforms is a likely candidate for upstream events leading to ERK activation by RSV.

The MAPK family of signaling molecules is involved in multiple cell functions, including differentiation, division, and death (19). Five distinguishable MAPK subfamilies have been identified in mammalian systems; the best described of these are the ERK (p42/p44), p38, and c-Jun N-terminal kinase pathways (20–24). Downstream events linked to ERK activation include phosphorylation of cytoplasmic (p90RSK, cytosolic phospholipase A_2, and epidermal growth factor receptor) and nuclear (Elk-1, c-*fos*, c-Myc, Sap-1, and c-Jun) substrates (25–27). Additionally, we have established a link between ERK kinase activation and RSV-induced IL-8 release (15). Our present study focuses on the upstream events leading to activation of ERK during RSV infection. ERK is activated by phosphorylation of both threonine and tyrosine by the upstream kinase MAP/ERK kinase (MEK). Potential candidates for the upstream activators of MEK and ERK include PKC ζ and Raf-1 (20, 21, 28).

The PKC family of proteins phosphorylates serine or threonine residues on multiple protein substrates. These kinases modulate

Department of Medicine, University of Iowa College of Medicine and Veterans Administration Medical Center, Iowa City, IA 52242

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[2] Address correspondence and reprint requests to Martha M. Monick, Division of Pulmonary, Critical Care, and Occupational Medicine, Room 100, Ekstein Medical Research Building, University of Iowa Hospitals and Clinic, Iowa City, IA 52242. E-mail address: martha-monick@uiowa.edu

[3] Abbreviations used in this paper: RSV, respiratory syncytial virus; DAG, diacylglycerol(s); ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP/ERK kinase; PKC, protein kinase C; $TCID_{50}$, tissue culture-infective dose.

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membrane structure events, mediate immune responses, and regulate gene transcription and cell growth (29). The PKC enzyme family includes multiple isoforms that display different activities in the presence or absence of cofactors, including calcium, diacylglycerols (DAG), and phospholipids (29, 30). The PKC isoforms can be categorized into three classes based on these differences: the conventional isoforms (designated α, $\beta 1$, $\beta 2$, and γ) are dependent on both Ca^{2+} and DAG, the novel isoforms (δ, ϵ, η, μ, and θ) are Ca^{2+} independent but DAG dependent, and the atypical isoforms (λ and ζ) are neither Ca^{2+} nor DAG dependent (31, 32). As a part of this study, we have determined the PKC isoforms present in A549 cells and evaluated their activation by RSV.

We found that RSV infection activated ERK kinase in epithelial cells in a biphasic pattern, both immediately following viral binding and later during active viral replication. In evaluating the upstream events responsible for ERK activation, we found that activation of the atypical isoform, PKC ζ, was involved in early ERK activation. We also found that multiple Ca^{2+}-dependent PKC isoforms and Raf-1 were associated with the long-term activation of ERK by RSV.

Materials and Methods

Reagents

CL-4B beads were purchased from Sigma (St. Louis, MO). Myelin basic protein (MBP) was purchased from Upstate Biotechnology (Lake Placid, NY). GammaBind Sepharose beads were purchased from Pharmacia Biotech (Piscataway, NJ). Abs (ERK 2, sc-4024, Raf-1, sc-133, PKC α, sc-208, PKC $\beta 1$, sc-209, PKC $\beta 2$, sc-210, PKC δ, sc-937, PKC ϵ, sc-214, PKC η, sc-215, PKC μ, sc-935, and PKC ζ, sc-726) were purchased from Santa Cruz Biotechnology (Beverly, MA). Phosphorylation-specific Abs were purchased from Cell Signaling (phosphoERK, threonine 202/tyrosine 204) and Upstate Biotechnology (phosphoRaf-1, serine 338). Anti-rabbit, anti-mouse, anti-rat, and anti-goat HRP-linked Abs and nitrocellulose paper were purchased from Jackson ImmunoResearch (West Grove, PA) and Amersham Life Science (Arlington Heights, IL), respectively. Goat anti-RSV antiserum was purchased from Biodesign International (Kennebunk, ME; B65860G). [α-^{32}P]ATP was purchased from DuPont-NEN Life Science Products (BLU 502Z; Boston, MA).

Cell culture

A549 cells, a tumor cell line with properties of alveolar epithelial cells (33, 34), were obtained from American Type Culture Collection (ATCC, Manassas, VA). We have previously shown that the response of A549 cells to RSV is similar to that of normal airway epithelial cells (16). The cells were incubated at 37°C in 5% CO_2. The tissue culture medium was Eagle's MEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), and 40 mg/ml gentamicin. The A549 cells used in our studies had been subcultured by trypsinization no more than 25 times from stock originally designated at pass 70.

Virus

RSV, strain A2, lot 995594, was obtained from ATCC, where it was harvested at a concentration of 10^6 tissue culture-infective dose ($TCID_{50}$)/ml, as measured in confluent HEP-2 cells. $TCID_{50}$ refers to the quantity of virus that will produce obvious cytopathic effects in 50% of the tissue culture plates infected and was calculated using the method of Reed and Munch (16). Sterile vials were kept frozen at −135°C. For all experiments, the virus was rapidly thawed at 37°C and used immediately.

Western analysis

Western analysis was used to evaluate PKC isoform presence and viral replication. For these assays, whole cell proteins were obtained from A549 cell controls and cells infected with RSV at a final dilution of 2×10^4 $TCID_{50}$/ml, a concentration previously shown to produce maximal IL-8 release by A549 cells (16). At sequential time points following exposure to RSV, cells were harvested in lysis buffer that contained 0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate (all protease inhibitors were obtained from Boehringer Mannheim, Indianapolis, IN). The cells were then sonicated for 20 s to ensure complete lysis. The lysate solution was then centrifuged at $15{,}000 \times g$ for 10 min, and the supernatant was saved. Protein concentration in the cell lysates was measured by Bradford assay, mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris, pH 6.8, all chemicals were obtained from Sigma), and separated by SDS-PAGE. Cell proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was then blocked with 5% nonfat milk in TTBS (Tris-buffered saline with 0.1% Tween 20) for 1 h, then incubated in with the desired primary Ab diluted in 5% milk TTBS for an additional hour (in the case of the phosphorylation-specific Abs, the blots were incubated with the primary Ab overnight at 4°C). The blots were then washed four times with TTBS and incubated for 1 h with HRP-conjugated secondary Ab. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus; Amersham) and visualized by autoradiography.

Isolation of cytoplasmic and membrane PKC isoforms

Negative controls and A549 cells exposed to RSV at a dilution of 2×10^4 $TCID_{50}$/ml were harvested by scraping in lysis buffer (see Western protocol) without Tween 20 (400 μl). The isolates were then sonicated for 10 s and fractionated by centrifuging at $100{,}000 \times g$ for 10 min. The supernatant containing the cytoplasmic fraction was immediately frozen at −70°C for later analysis. The membrane pellet was resuspended in lysis buffer with 1% Tween 20 and sonicated for 5 s. Following a 20-min incubation on ice, cell debris was removed by centrifugation, and the supernatant containing membrane-associated proteins was frozen at −70°C. Western analysis was performed as described above.

PKC isoform depletion or inhibition

A549 cells were exposed to PMA (100 ng/ml) for 24 h, followed by infection with RSV at 2×10^4 $TCID_{50}$/ml for an additional 30 min or 24 h. PKC protein depletion was evaluated by Western analysis for specific PKC isoforms. To examine the effect of PKC isoform inhibition on ERK 2 kinase activity, specific inhibitory peptides (PKC ζ, myr-SIYRRGARRWRKL-OH; PKC $\alpha\beta$, myr-RFARKGALRQKNV-OH; nonsense, myr-LRISRAGRYRANWYRKR-OH; the myristate on the N terminus of these peptides allows for membrane permeability) were added to the culture medium 1 h before RSV infection. Whole cell protein from treated cells and appropriate controls was then harvested and assayed for ERK 2 kinase activity (see below).

Immunoprecipitation

Cells were harvested by rinsing with PBS, followed by scraping into lysis buffer (see Western blot protocol), and incubated on ice for 20 min. These lysates were then sonicated for 20 s and centrifuged at $15{,}000 \times g$ for 10 min, and the supernatant was saved. Protein concentration was measured by Bradford assay. A quantity amounting to 200–600 μg of total protein from each sample was cleared by incubating for 2 h with nonspecific rabbit IgG (1 μg/sample), and unconjugated GammaBind Sepharose (10 μl/sample). After centrifuging, the supernatants were transferred to a tube containing 3 μg/sample of Ab bound to GammaBind Sepharose and rotated at

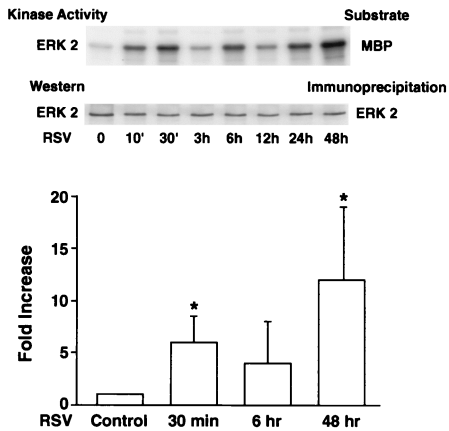

FIGURE 1. RSV activates ERK kinase in A549 cells. A549 cells were treated with RSV for 0 h, 10 min, 30 min, 3 h, 6 h, 12 h, 24 h, and 48 h. ERK-2 was immunoprecipitated from whole cell lysates, and a kinase activity assay was performed using MBP as a substrate. *A*, An autoradiogram of phosphorylated MBP following a kinase activity assay. *B*, A graph showing densitometry data from four separate experiments. *, $p < 0.05$.

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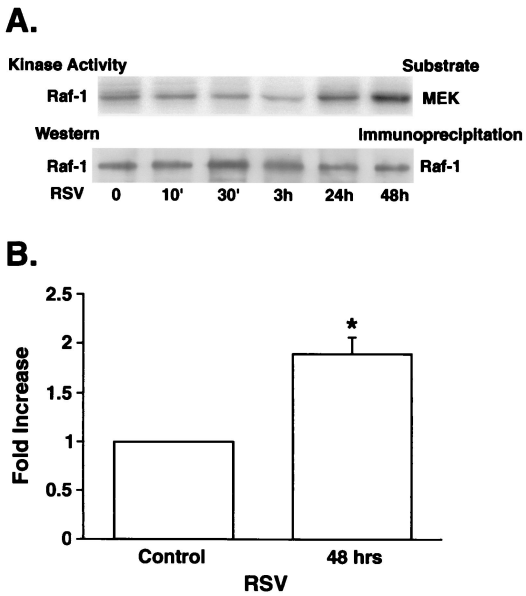

FIGURE 2. RSV activates Raf-1 in A549 cells. A549 cells were treated with RSV for 0 h, 10 min, 30 min, 3 h, 24 h, and 48 h. Raf-1 was immunoprecipitated from whole cell lysates, and a kinase activity assay was performed using MEK as a substrate. *A*, An autoradiogram of phosphorylated MEK following a kinase activity assay. *B*, A graph showing densitometry data from four separate experiments. $*$, $p < 0.05$.

4°C overnight. The beads with the immunoprecipitated protein were subsequently washed three times with high salt buffer (0.5 M Tris, pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer. The immunoprecipitated complexes were either released with 2× sample buffer for Western analysis or used to determine kinase activity.

Kinase activity assay

The solution containing the immunoprecipitate was centrifuged to pellet the Sepharose and attached Ab/kinase complex. The protein-containing pellet was washed twice with kinase buffer (20 mM $MgCl_2$, 25 mM HEPES, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 20 mM sodium orthovanadate, and 2 mM DTT). The pellet was suspended in 20 μl kinase buffer, to which 20 μM ATP and 5 μCi γATP (32) were added. Substrates, 10 μg MBP (dephosphorylated MBP) or 5 μg MEK-1 or 5 μg ERK (polyhistidine-tagged fusion proteins from Santa Cruz Biotechnology, Santa Cruz, CA), were added last to bring the total assay volume to 25 μl. The reaction was continued at 25°C for 15 min to 1 h, depending on the kinase, and stopped by the addition of 25 μl/sample of 2× sample buffer (see Western protocol). The samples were incubated for 5 min at 95°C and run on a 12% SDS-PAGE gel. The gel was dried, and autoradiography was performed to visualize the ^{32}P-labeled MBP, MEK-1, or ERK. Densitometry measurements of individual bands were obtained, and fold increase was calculated.

Statistical analysis

Statistical analysis of the densitometric data was performed by determining the fold increase of all the samples as they relate to the control. Statistical comparisons were performed using a paired *t* test, with a probability value of $p < 0.05$ considered to be significant.

Results

RSV activates ERK 2

We initially determined whether RSV infection of A549 cells activated the MAPK, ERK 2. To do this, we seeded A549 cells at a subconfluent density, allowed them to grow for 1 day, then added RSV. At sequential time points, whole cell protein was harvested and in vitro kinase activity assays were performed (Fig. 1). In all experiments, we found both an early and a later, more pronounced activation of ERK by RSV. In some experiments, we also found a peak of activity that occurred at 6 h; however, this was not a consistent finding. For this reason, all additional experiments were done at the early (10–30 min) and late (24–48 h) time points. The early activation (10–30 min) coincides with viral binding; the late activation accompanies viral replication. It is of interest that we have previously shown that there also is a biphasic response of IL-8 mRNA to RSV (16, 17). These data were confirmed using Western analysis for activated ERK (phosphorylated on threonine 202 and tyrosine 204) (Fig. 2). The Western for activated ERK showed a significant increase in the amounts of activated ERK at both early and late time points after RSV infection.

RSV activates Raf-1 at time points consistent with viral replication

We next evaluated the activation of Raf-1 by RSV. Raf-1 is a well-described activator of MEK, the kinase directly upstream of ERK (35–37). Raf-1 was immunoprecipitated from RSV-treated whole cell lysates and in vitro kinase activity assays performed. Fig. 2 demonstrates that RSV activates Raf-1 and consequently MEK at 24 and 48 h, but not at earlier time points. Because MEK is the kinase directly upstream of ERK, the data suggest that the early activation of ERK seen in Fig. 1 cannot be a consequence of Raf-1 activity. This is confirmed in a Western for activated Raf-1 (serine 338 phosphorylation) (Fig. 3) (38).

Detectable RSV replication occurs in the same time frame as sustained RSV-induced ERK activity

To demonstrate a temporal association of RSV replication with the late activation of ERK, we evaluated the time course of viral protein synthesis in A549 cells by Western analysis. Whole cell proteins from RSV-infected A549 cells were separated by SDS-PAGE, transferred to membranes, and immunoblotted for viral proteins. In Fig. 4, we show that detectable viral protein first appears at 24 h postinfection. Ongoing viral replication continues through the 48-h time point. Beyond 48 h, infected A549 cells rapidly progress toward cell death (data not shown). These studies show that significant RSV replication is temporally associated with the late sustained activation of ERK by RSV.

A549 cells contain multiple PKC isoforms

Multiple studies in other systems have demonstrated that PKC or specific PKC isoforms are involved in Raf-1 and subsequent ERK activation (39–42). To investigate this possibility in RSV-induced

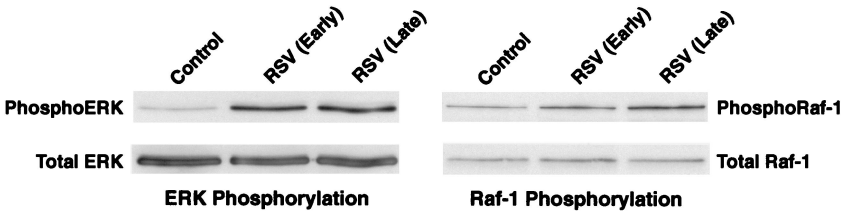

FIGURE 3. RSV increases the phosphorylation of ERK and Raf-1 in A549 cells. A549 cells were treated with RSV for 30 min or 24 h. Whole cell lysates were obtained, and Western analysis was performed for phosphorylated ERK (Thr^{202} and Tyr^{204}) and phosphorylated Raf-1 (Ser^{338}). Primary Ab concentrations of 1:500 and secondary Ab concentrations of 1:10,000 were used. Immunoreactive bands were visualized using ECL Plus. Blots were stripped and reprobed for total protein (ERK and Raf-1).

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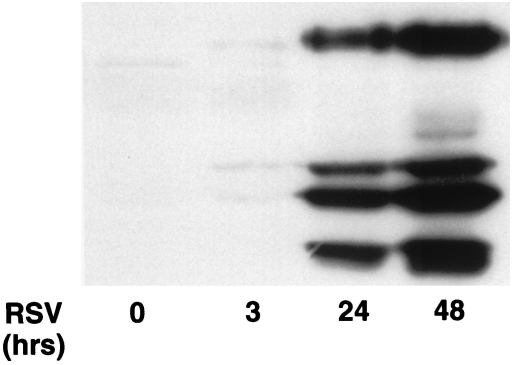

FIGURE 4. RSV proteins increase over time. A549 cells were treated with RSV and then harvested at 0, 3, 24, and 48 h. Western blots of whole cell lysates were probed with goat anti-RSV antiserum. Primary Ab concentrations of 1:1000 and secondary Ab concentrations of 1:50,000 were used. Immunoreactive bands were visualized with ECL. This is a representative of three experiments.

ERK activation, we initially determined the profile of PKC isoforms present in A549 cells. Western analysis for each of the11 described PKC isoforms showed that only six (α, β1, δ, ϵ, μ, and ζ) are present in A549 cells (Fig. 5). The total amounts of each specific isoform did not appear to change over the time course of RSV infection, as shown by analysis of both control cells and of cells 48 h postinfection (Fig. 5).

RSV activates multiple PKC isoforms

The effect of RSV on PKC activation was determined by performing in vitro kinase activity assays at sequential time points following infection. The activity was determined for the individual PKC isoforms by using specific Abs to separately immunoprecipitate each isoform from whole cell lysates before the activity assay. Fig. 6 shows that RSV activates the isoforms PKC δ and ζ at early time points (<3 h), and β1, δ, ϵ, and μ at later time points (>24 h). PKC activation has been associated with translocation of the enzyme to the cell membrane (29, 32). To confirm PKC isoform activation by RSV infection, we also evaluated the translocation of these enzymes from the cytoplasm to the membrane. We harvested cytosol and membrane protein fractions from cells infected with RSV for 48 h and noninfected controls. Western analysis for individual PKC isoforms was then performed on the separate fractions. Fig. 7 shows an increase in the membrane-associated quantities of PKC isoforms β1, δ, ϵ, and μ at 48 h postinfection. These are the same isoforms with increased activity at later time points by in vitro kinase assay. Combined, these results provide substantial evidence that RSV activates the PKC isoforms δ and ζ shortly after binding, and isoforms β1, δ, ϵ, and μ after initiation of viral protein synthesis.

Activation of PKC ζ is necessary for RSV-induced ERK activation at early time points

We have previously shown in alveolar macrophages that LPS activates ERK in a PKC ζ-dependent manner (28). Based on this information and the increase in PKC ζ activity within minutes of RSV infection, we next evaluated the role of PKC ζ in RSV activation of ERK. To do this, we made use of pseudosubstrate-specific peptides. PKC isoforms are maintained in an inactive form by the binding of a pseudosubstrate region on their N-terminal to the substrate-binding domain in the catalytic region (32, 43). Pseudosubstrate-specific peptides inhibit the catalytic activity of PKC isoforms by binding to the substrate-binding domain (Fig. 8*C*). Myristolation on the N terminus of the peptides makes them membrane permeable. In these experiments, A549 cells were treated with myristolated inhibitory peptides (PKC ζ pseudosubstrate specific, PKC $\alpha\beta$ pseudosubstrate specific, or nonsense) for 1 h before addition of RSV. Whole cell proteins were obtained and an ERK kinase activity assay was performed. Fig. 8*A* shows that blocking PKC ζ prevents RSV-induced ERK 2 activation. Control experiments with a nonsense peptide or an $\alpha\beta$ isoform-specific

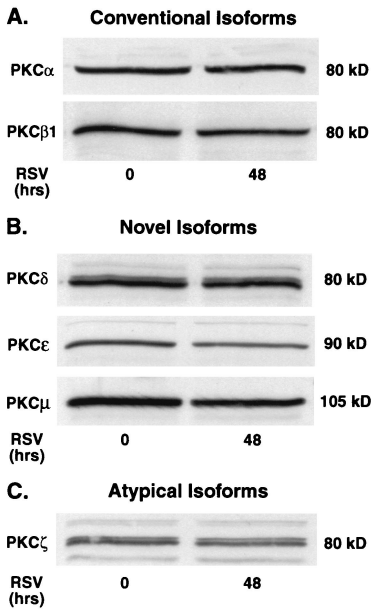

FIGURE 5. PKC isoforms α, βI, δ, ϵ, μ, and ζ were detected in A549 cells. A549 cells were treated with RSV for 0 and 48 h. Western analysis was performed on whole cell lysates, and immunoreactive bands were visualized with chemiluminescence (ECL). Primary Ab concentrations of 1:1000 and secondary Ab concentrations of 1:10,000 were used. Shown in this figure are the PKC isoforms found in A549 cells both constitutively and after 48 h of RSV infection.

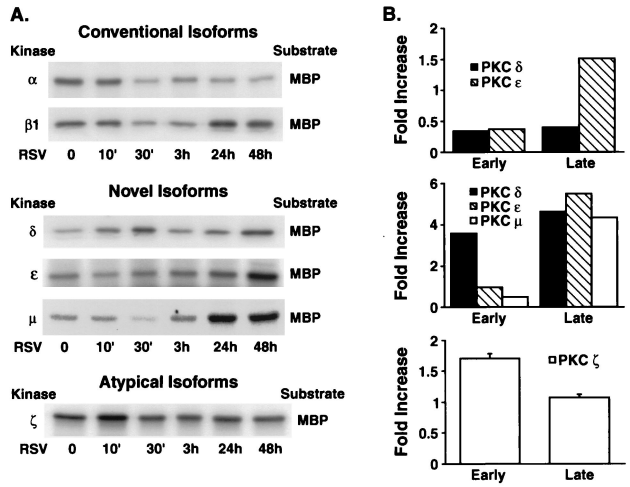

FIGURE 6. PKC δ and ζ are activated at early time points, and PKC βI, δ, ϵ, and μ are activated at late time points. A549 cells were treated with RSV for 0 h, 10 min, 30 min, 3 h, 24 h, and 48 h. PKC isoforms (α, βI, δ, ϵ, μ, or ζ) were immunoprecipitated from whole cell lysates, and a kinase activity assay was performed using MBP as a substrate. *A*, Autoradiograms of the phosphorylated MBP found after kinase activity assays. This blot is representative of three separate experiments. *B*, Densitometry data from the kinase activity assays.

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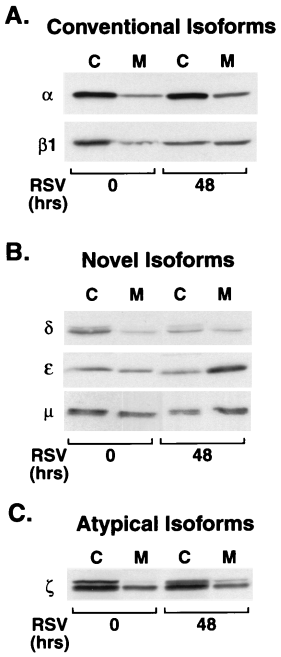

FIGURE 7. RSV causes membrane translocation of multiple PKC isoforms. A549 cells were treated with RSV for 0 and 48 h. Cytosol (C) and membrane (M) fractions were obtained, and Western analysis for the specific PKC isoforms was performed. The figure shows the immunoreactive bands visualized by chemiluminescence (ECL). Primary Ab concentrations of 1:1000 and secondary Ab concentrations of 1:10,000 were used. This blot is representative of three separate experiments. This figure demonstrates that RSV infection causes the membrane translocation of all the isoforms (except ζ) that demonstrated activity in Fig. 6.

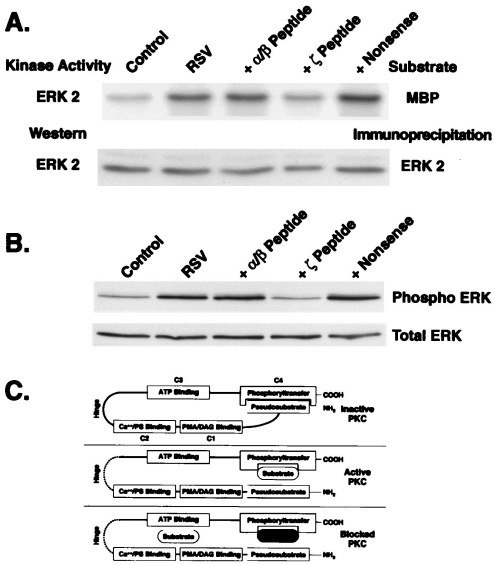

FIGURE 8. Blocking PKC ζ activity blocks the early activation of ERK 2 by RSV. A549 cells were treated with RSV (30 min) with and without an inhibitory peptide (added 30 min before the RSV). ERK 2 was immunoprecipitated from whole cell lysates, and a kinase activity assay was performed using MBP as a substrate. *A*, An autoradiogram of the phosphorylated MBP found after a kinase activity assay. Also included is a Western analysis for ERK 2 demonstrating equal loading of the kinase in the activity assay. This blot is representative of three separate experiments. *B*, A Western analysis of whole cell lysates demonstrating ERK phosphorylation matching the kinase activity data. Primary Ab concentrations of 1:500 and secondary Ab concentrations of 1:10,000 were used. Immunoreactive bands were visualized using ECL Plus. Blots were stripped and reprobed for total protein (ERK). *C*, A diagram demonstrating how an isoform-specific peptide would block activity of a particular PKC isoform.

inhibitory peptide showed no effect on ERK 2 activity. These data were confirmed using Western analysis and Abs to activated ERK (pThr^{202} and pTyr^{204}) (Fig. 8*B*).

Blocking of multiple PKC isoforms inhibits late RSV-induced ERK activation

To examine the role of other PKC enzymes in ERK activation, RSV-stimulated ERK 2 activity was studied in cells depleted of the conventional and novel PKC isoforms. PMA has been shown to result in activation and eventual depletion of all PKC isoforms except the atypical (ζ) isoform (44–46). Following 24-h pretreatment with PMA, A549 cells were infected with RSV and subsequently harvested at 30 min and 48 h. Western blotting for individual isoforms and ERK 2 in vitro kinase assays was subsequently performed on the isolated whole cell protein. Fig. 9*A* shows a striking decrease in the levels of all PKC isoforms except ζ in the PMA-treated cells. Fig. 9, *B* and *C*, shows that PMA pretreatment abolished the late ERK 2 activation, but had no substantial effect on the early activation of ERK 2 by RSV. These studies suggest that multiple Ca^{2+}-dependent PKC isoforms are involved in RSV replication-dependent ERK activation.

Discussion

The goal of the current study was to further define signaling events in the induction of inflammatory mediators after RSV infection. This study evaluated the role of various PKC isoforms in the activation of ERK kinase by RSV. We found that in A549 cells, RSV caused two separate peaks of ERK activity, an early (10–30 min) and a late (24–48 h) activation of ERK (Fig. 10). The early time point, consistent with viral binding, was matched by activation of PKC ζ. Furthermore, inhibiting PKC ζ blocked the early activation of ERK by RSV. Other studies (insulin signaling) have demonstrated that PKC ζ can activate MEK, the dual kinase that phosphorylates the threonine and tyrosine responsible for ERK activation (47). The late activation of ERK was temporally related to viral protein synthesis and replication. This late activation did not correlate with the activity of PKC ζ, but rather with the activities of Raf-1 and the PKC isoforms β1, δ, ϵ, and μ. Depleting these isoforms by PMA pretreatment did not inhibit early activation, but did block the late, sustained ERK activation. Therefore, late activation of ERK may result from PKC-induced activation of Raf-1, leading to activation of MEK and subsequently ERK. Taken together, these studies suggest that RSV-induced ERK activation and subsequent inflammation depend on the differential activation of multiple PKC isoforms in a time-dependent manner.

Previous studies have shown that viral infection can result in the activation of MAPK (48–51). Kujime et al. (52) showed that infection of cells with influenza virus leads to the activation of p38, ERK, and c-Jun N-terminal kinase. Infection with the EBV results in the activation of the MAPK (ERK) (49), as does infection with HIV type-1 (50, 51). Rodems et al. (48) found that CMV causes an early, sustained activation of ERK kinase. Johnson et al. (53) demonstrated that human CMV infection produced activation of ERK at both an early time point (5–15 min) and a late time point (4–8 h) after infection. This study also showed activation of p38, but only at late time points (8–48 h). Our results demonstrating a biphasic activation of ERK in response to RSV are consistent with the effects reported for these other viruses.

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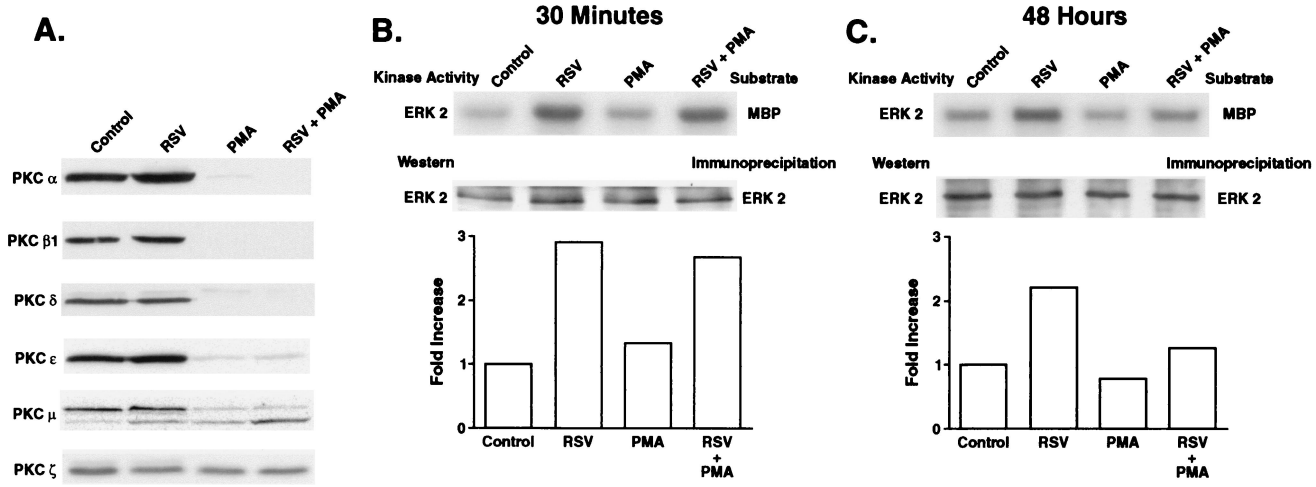

FIGURE 9. Depletion of multiple PKC isoforms blocks ERK 2 activation by RSV at replication-consistent time points. A549 cells were treated with PMA (100 ng/ml) for 24 h to deplete all of the PKC isoforms except PKC ζ. Control and PMA-treated cells were then infected with RSV for 30 min or for 48 h, and ERK kinase activity was determined. *A*, Demonstrates isoform depletion of all the isoforms, except for ζ, by the PMA treatment. Western blots of whole cell lysates were probed with anti-PKC isoforms (α, βI, δ, ϵ, μ, and ζ). *B*, An ERK kinase activity assay performed on control and RSV-treated cells both in normal and PMA-treated cells at 30 min. MBP was used as a substrate. Also shown is a Western analysis of the immunoprecipitates showing equal loading of the kinase in the activity assay. *C*, An ERK kinase activity assay performed on control and RSV-treated cells both in normal and PMA-treated cells at 48 h. ERK 2 was immunoprecipitated from whole cell lysates, and a kinase activity assay was performed using MBP as a substrate. Also shown is a Western analysis of the immunoprecipitates showing equal loading of the kinase in the activity assay. Both *B* and *C* are representative of two separate experiments.

We have previously shown that RSV-induced IL-8 mRNA increases occur in a biphasic manner, an early activation that accompanies viral binding and a later peak that requires viral replication (15, 17). We have also demonstrated that IL-8 production is linked to ERK activation (15). Fiedler et al. (54) have demonstrated that blocking viral replication with ribavirin also blocks RSV-induced IL-8 release and NF-κB activation. These studies confirm the importance of replication in the inflammatory response of epithelial cells to RSV. They suggest that viral protein synthesis or the accumulation of viral proteins is coincident with activation of signaling pathways upstream of cytokine production. These patterns of intracellular signaling appear to be distinct from cellular responses to viral binding and internalization at the earlier time points.

A number of studies have suggested the possibility that activation of the ERK MAPK is linked not only to cytokine production, but also to the effectiveness of viral replication or infection. Yang et al. (55) reported that that ERK was involved in activation of HIV-1 gene expression in latently infected cells. The induction of HIV-1 protein synthesis by cytokines and phorbol esters was inhibited by PD98059, a specific inhibitor of ERK. In a second study, they found that the HIV-1 proteins, Rev, Tat, p17 (Gag), and Nef, could all be directly phosphorylated in vitro by activated ERK (56). In another HIV-1 study, Jacque et al. (57) identified ERK as a virion-associated protein that increased HIV-1 infectivity. In this system also, PD98059 had a major effect, decreasing viral infectivity (57).

The data presented in this study suggest that activation of the ERK MAPK by RSV is regulated in part by activation of a number of PKC isoforms. There is an early and a late sustained activation of ERK. Our data suggest that the upstream activators involved in the two time points are different. As with insulin and LPS, PKC ζ appears to be the upstream activator of ERK at the early time point with RSV infection (28, 47). Later, the sustained activation of ERK (24–48 h) is linked to activation of Raf-1, and this in turn is linked to a number of conventional and novel PKC isoforms. The biological relevance of these findings relates in part to our prior observations, which have shown that ERK activation is linked to the production of inflammatory mediators by RSV.

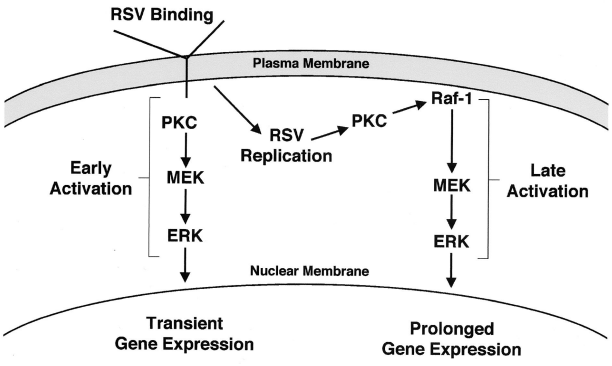

FIGURE 10. RSV activates ERK kinase via activation of PKC isoforms. This is a diagrammatic representation of our model for ERK kinase activation by RSV. Viral binding induces a transient activation of ERK through PKC ζ activation of MEK. Viral replication causes a more sustained activation of ERK through Raf-1 activation of MEK.

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