Pertussis Toxin–sensitive G Proteins as Mediators of the Signal Transduction Pathways Activated by Cytomegalovirus Infection of Smooth Muscle Cells

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Abstract

We demonstrated recently that the arachidonic acid (AA) cascade is involved in cytomegalovirus (CMV)-induced generation of reactive oxygen species (ROS) and the activation of nuclear factor (NF)-κB in human smooth muscle cells (SMCs). Since AA release from neutrophils is mediated by pertussis toxin (PTx)-sensitive guanine nucleotide–binding (G) proteins, we hypothesized by analogy that CMV stimulates ROS generation in SMCs and ultimately activates NF-κB via a PTx-sensitive G protein–coupled pathway. Our first test of this hypothesis demonstrated that PTx blocked AA release induced by CMV infection of SMCs, as well as blocked the terminal products of this reaction, ROS generation and NF-κB activation. More proximal components of the pathway were then examined. CMV infection increased phosphorylation and activity of cytosolic phospholipase A_2 ($cPLA_2$), an enzyme causing AA release; these effects were inhibited by PTx. CMV infection activated mitogen-activated protein (MAP) kinase, a key enzyme for $cPLA_2$ phosphorylation, an effect also inhibited by PTx. Finally, inhibition of MAP kinase kinase (MAPKK), which phosphorylates and thereby activates MAP kinase, inhibited CMV-induced ROS generation. These data demonstrate that a PTx-sensitive G protein–dependent signaling pathway mediates cellular effects of CMV infection of SMCs. The downstream events include phosphorylation and activation of MAP kinase by MAPKK and subsequent phosphorylation and activation of $cPLA_2$ (with its translocation to cell membranes), followed by stimulation of the AA cascade, which generates intracellular ROS and thereby activates NF-κB. (*J. Clin. Invest.* 1997. 100:2054–2061.) Key words: cytosolic phospholipase A_2 • reactive oxygen species • mitogen-activated protein kinase • nuclear factor-κB • arachidonic acid

Introduction

Human cytomegalovirus (CMV)[1] is a ubiquitous virus that infects > 60% of the general population over age 35 yr (1), but is rarely considered to be the cause of clinical symptoms in healthy individuals (2). However, CMV has been implicated recently as a risk factor for restenosis after coronary angioplasty (3, 4) and for atherosclerosis (1). Because excessive accumulation of smooth muscle cells (SMCs) has been implicated in the development of both restenosis and atherosclerosis (5), we have examined the cellular response of SMCs to CMV infection.

We demonstrated recently by quantitative analysis of confocal microscopic images obtained using a fluorescent intracellular marker for reactive oxygen species (ROS) and by electrophoretic mobility shift assay that CMV infection of SMCs rapidly increases intracellular ROS and activates DNA binding of nuclear factor-kappa B (NF-κB) (6). The observation that NF-κB activation was inhibited by antioxidants suggested that the CMV-induced effect on NF-κB was mediated by ROS, known activators of this factor. Aspirin also inhibited ROS generation and NF-κB activation induced by CMV infection, effects found to be caused, at least in part, through inhibition of cyclooxygenase-2 (7). Because this enzyme plays a key role in AA metabolism, and because the AA cascade is known to be a source of ROS (8), these results suggested that CMV infection of SMCs increases intracellular ROS and activates NF-κB via stimulation of the AA cascade.

In an effort to identify more proximal components of the signal transduction pathway activated by CMV infection of SMCs, we focused on studies demonstrating that, in neutrophils, agonist-mediated release of AA is inhibited by pertussis toxin (PTx) (9), a potent inhibitor of certain guanine nucleotide–binding (G) proteins (e.g., Gi, Go, or transducin; see reference 10). Of note, AA is liberated from the *sn*-2 position of phospholipids in plasma membranes mainly by the action of high molecular weight cytosolic phospholipase A_2 ($cPLA_2$) (11), an enzyme also regulated by PTx-sensitive G proteins (9, 12, 13).

In their excellent review of the activation signals induced by CMV infection of cells, Albrecht et al. (14) observed that the activities of a number of membrane-associated enzymes (e.g., AA release and inositol triphosphate and diacylglycerol turnover) are very rapidly induced after exposure of fibroblasts to CMV. They also noted that when stimulated by exposure to serum and growth factors, these changes are generally linked to G protein–coupled receptors. Therefore, the authors implied that the signaling pathways stimulated by CMV infection involved activation of G proteins (14).

This investigation was undertaken to determine the proximal components of the signaling pathway by which CMV in-

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; $cPLA_2$, cytosolic phospholipase A_2; DCF, 2′,7′-dichlorofluorescein; G, guanine nucleotide–binding; HEL, human embryonic lung; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; NF-κB, nuclear factor κB; pi, postinfection; Ptx, pertussis toxin; ROS, reactive oxygen species; SMC, smooth muscle cell.

fection of SMCs leads ultimately to the release of AA, the generation of ROS, and the activation of NF-κB. Specifically, we examined the hypothesis that CMV infection of SMCs increases $cPLA_2$ activity through a PTx-sensitive G protein. This stimulates the AA cascade with the consequent generation of ROS, which then activates NF-κB, a cellular transcription factor that promotes the expression of many gene products. Because the phosphorylation of $cPLA_2$ by mitogen-activated protein (MAP) kinase is essential for an increase in $cPLA_2$ catalytic activity (15, 16), we also determined whether MAP kinase and its activator, MAP kinase kinase (MAPKK), are involved in this postulated G protein–mediated signaling pathway activated by CMV infection.

Methods

Cells, CMV, and PTx. Human aortic SMCs (passages 5–8) were purchased from Clonetics (San Diego, CA). These cells were determined to be > 95% pure based on positive staining for α-actin, negative staining for Factor VIII, and no uptake of acetylated LDL. Cells were grown in their optimal medium (SmGM2; Clonetics) containing 5% FBS at 37°C in an atmosphere of 95% room air and 5% CO_2.

Human CMV Towne strain was passaged in human embryonic lung fibroblasts (HEL 299; American Type Culture Collection, Rockville, MD) and titrated as described previously (17). Infection of aortic SMCs was performed with the supernatant of the CMV-infected HEL cells. Cells were infected at an moi of 5 in serum-free medium and adsorbed at 37°C. Under these conditions, we demonstrated previously that CMV infects SMCs, as evidenced by the appearance of CMV immediate early proteins (by immunohistochemistry) and by the development of cytopathic effects (6). For mock-infected cells in initial experiments, virus was removed from infectious media either by centrifugation at 100,000 *g* for 1 h or filtration through a 0.1-μm filter (Gelman Sciences, Inc., Ann Arbor, MI). Identical results were obtained in subsequent experiments when mock infection consisted of the identical media used for infection but without addition of CMV.

SMCs were pretreated with either 10 ng/ml PTx (List Biological Laboratories, Inc., Campbell, CA) or 10 ng/ml mutant PTx (lot 9K/129G, provided by Dr. Rino Rappuoli, Centro Ricerche, IRIS, Siena Italy) (18) for 1.5 h, or with 0.5–1 μM MAPKK inhibitor (PD 098059; Research Biochemicals, Inc., Natick, MA) for 30 min (19–21). After removal of the toxins or PD 098059, the cells were infected with CMV. The effectiveness of PTx treatment in causing ADP-ribosylation of G proteins present in SMCs, and the lack of effect of mutant PTx, were confirmed by the standard ADP-ribosylation assay.

Mutant PTx. PTx consists of an ADP-ribosyl transferase moiety, the A-protomer, and a binding moiety, the B-oligomer. The mutant toxin contains two amino acid substitutions in the ADP-ribosyltransferase subunit (Arg9-Lys and Glu129-Gly), which abolish the ADP-ribosyltransferase activity of PTx without any known effect on its binding capacity (18). Use of mutant PTx allowed us to control for any potential stimulatory effects caused by the binding of PTx to its cell surface receptor, and for any potential interactions between the B-oligomer and the virus, including the possibility that the toxin might compete with virus attachment to its receptors. We determined that mutant PTx competed (1,000-fold excess) with wild-type PTx for binding to SMC (thereby inhibiting PTx effects), using CMV-induced generation of ROS as the target effect (data not shown).

Assessment of intracellular redox state. Intracellular generation of ROS after CMV infection was measured as described previously (22). Briefly, cells were grown in 4-well chambered slides, washed with HBSS without phenol red, and then incubated for 5 min with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Inc., Eugene, OR), a nonpolar compound that diffuses readily into cells. It is deacetylated by cellular esterases to the membrane-impermeable, nonfluorescent derivative 2′,7′-dichlorodihydrofluorescein which, in the presence of intracellular H_2O_2 and peroxidases, is oxidized rapidly to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Fluorescence was monitored and recorded by laser scanning confocal microscopy (Leica TCS4D; Leica Lasertechnik GmbH, Heidelberg, Germany) as described previously (22).

Electrophoretic mobility shift assay. Cells were washed twice with PBS and harvested with a cell scraper. Nuclear extracts were prepared as described previously (23). Double-stranded oligonucleotides (Promega Corp., Madison, WI) containing a consensus NF-κB–binding sequence were end-labeled with polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA (40,000–80,000 cpm) was incubated with 3 μg nuclear extract in a final volume of 20 μl 10 mM Hepes (pH 7.9), 25 mM KCl, 0.2 mM EDTA, 1 mM DTT, 10% glycerol, 0.5% NP-40, and 100 ng of poly(dI–dC) copolymer for 30 min at room temperature. This mixture was then electrophoresed on 4% native acrylamide gel in 5 mM Tris/38 mM glycine running buffer.

Release of AA and its metabolites. Cells were plated into a 12-well cluster plate at 6×10^3 cells per well in growth medium. After a 48-h incubation, the cells were labeled with 0.5 μCi/ml [^{3}H]AA (221 Ci/mM; DuPont/NEN, Boston, MA) for 24 h at 37°C and washed three times with medium containing 0.1% fatty acid–free BSA (Sigma Chemical Co., St. Louis, MO). Radiolabeled cells were then treated with the toxins as indicated, washed with serum-free medium, and infected with the virus. The medium was then collected, and the radioactivity released by SMCs into the medium was determined by scintillation counting as described previously (24). The cells were solubilized with 1 N NaOH and used for protein determination and scintillation counting to determine the [^{3}H]AA content of the cells. The amount of [^{3}H]AA released into the medium was calculated as counts per minute per milligram of cell protein. Variation of total incorporated [^{3}H]AA per milligram of protein in each well was < 2%.

Western blot analysis of $cPLA_2$. Cells were scraped and lysed in 10 mM Hepes, pH 7.4, 250 mM sucrose, 5 mM EDTA, 20 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM aminoethylbenzenesulfonyl fluoride, and 100 μg/ml benzamidine by passing them 20 times through a 25-gauge needle. The cell lysate was centrifuged at 8,000 *g* for 30 min at 4°C, and the supernatant was then centrifuged at 100,000 *g* for 1 h at 4°C. After collection of the supernatant, the pellet (membrane) was washed twice and dispersed into the lysis buffer by sonication. The membrane fractions were used for Western blot analysis. 30 μg protein was dissolved in 2× SDS sample buffer and electrophoresed in 10% Tris/glycine–SDS gel. To increase the separation of native $cPLA_2$ and phosphorylated isoforms, electrophoresis was continued for 4 h at 150 V after tracking dye reached the bottom of the gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The blots were blocked in Tris-buffered saline containing 0.1% Tween 20 with 10% dry milk and then incubated with anti-$cPLA_2$ mAb (4-4B-3C, 1 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The signal was detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and quantified by densitometry.

Assay of PLA_2 activity. $cPLA_2$ activity was determined as previously described (24), except that 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine (54 mCi/mmol; Amersham Corp.) instead of 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine was used as a substrate. 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine was dried under N_2 and resuspended in dimethyl sulfoxide by vigorous mixing for 2 min. The substrate (2 μl containing 5 nmol) was incubated with 20 μl cell lysate containing 8 μg of the cytoplasm or 10 μg of the membrane, at 37°C for 30 min (total volume of 0.1 ml) in 0.1 M Tris-HCl, pH 8.5, containing 5 mM $CaCl_2$, 0.5 mg/ml fatty acid–free BSA, and 1 mM 2-mercaptoethanol. The reaction was stopped with 0.6 ml Dole's reagent (2-propanol/heptane/0.5 N H_2SO_4, 20:5:1 by vol). After addition of 10 μg AA, 0.4 ml heptane and 0.25 ml water were added. The mixtures were vortex-mixed, after which the upper phase was transferred to tubes containing 0.5 ml heptane and 40 mg 200-mesh Silica gel (Sigma Chemical Co.). After centrifugation, the supernatants were collected, and the radioactivity was quantified by scintillation counting.

Immunoprecipitation of MAP kinase and MAP kinase activity assay. To separate MAP kinase, cell lysates were incubated with 4 μg rabbit polyclonal anti–MAP kinase antibody (Erk1-CT; Upstate Biotechnology Inc., Lake Placid, NY) at 4°C overnight and then with washed protein A–agarose (Upstate Biotechnology Inc.). The immunoprecipitate was washed with PBS three times and then twice with assay buffer (20 mM Mops, pH 7.2, 25 mM glycerophosphate, 5 mM EGTA, 1 mM sodium ortho-vanadate, 1 mM DTT). MAP kinase activity in the immunoprecipitate was determined using a MAP kinase assay kit (Upstate Biotechnology Inc.) with myelin basic protein as a substrate and [γ-^{32}P]ATP (3,000 Ci/mmol; DuPont-NEN) according to the manufacturer's directions. The phosphorylated proteins were isolated by centrifugation of the reaction mixture through phosphocellulose membranes (SpinZyme; Pierce Chemical Co., Rockford, IL). The membrane containing the bound protein was washed twice with 75 mM phosphoric acid, and radioactivity on the membrane was counted.

Statistical analysis. All values are expressed as mean±SEM. Comparisons were made by Student's *t* test. Statistical significance was assumed if a null hypothesis could be rejected at the 0.05 level.

Results

We determined previously that CMV infection of coronary SMCs rapidly increases intracellular ROS generation, which leads to activation of NF-κB (6, 7). Therefore, we first determined whether these two effects of CMV infection are involved in a PTx-sensitive G protein–dependent signaling pathway.

Effect of PTx on CMV-induced ROS generation. The intensity of the fluorescence of DCF, which indicates the relative amount of intracellular ROS, was evaluated by quantitative measurements of relative fluorescence units (22). CMV infection, in comparison with mock infection, markedly increased fluorescence intensity by 15 min postinfection (pi), indicating an increase in intracellular ROS (Fig. 1, *A–C*). CMV-induced intracellular ROS generation seen at 15 min pi gradually decreased at 30 and 60 min pi (Fig. 1 *A*). PTx blocked the increase in intracellular ROS but mutant PTx did not (Fig. 1, *A* and *E*). These results demonstrate that CMV-induced ROS generation is mediated by PTx-sensitive G proteins.

Importantly, we determined that ROS production was due directly to CMV infection and not to a secondary event, i.e., production of secreted substances by CMV-infected cells that might then act in an autocrine fashion to stimulate ROS production. This was accomplished by preparing and testing mock-infection medium specifically depleted of viral particles by two distinct methods that exploited the relatively large diameter and mass of CMV compared with potential secreted substances. These mock controls consisted of (*a*) medium containing infectious virus produced by HEL fibroblasts (used to generate virus which was subsequently used to infect the

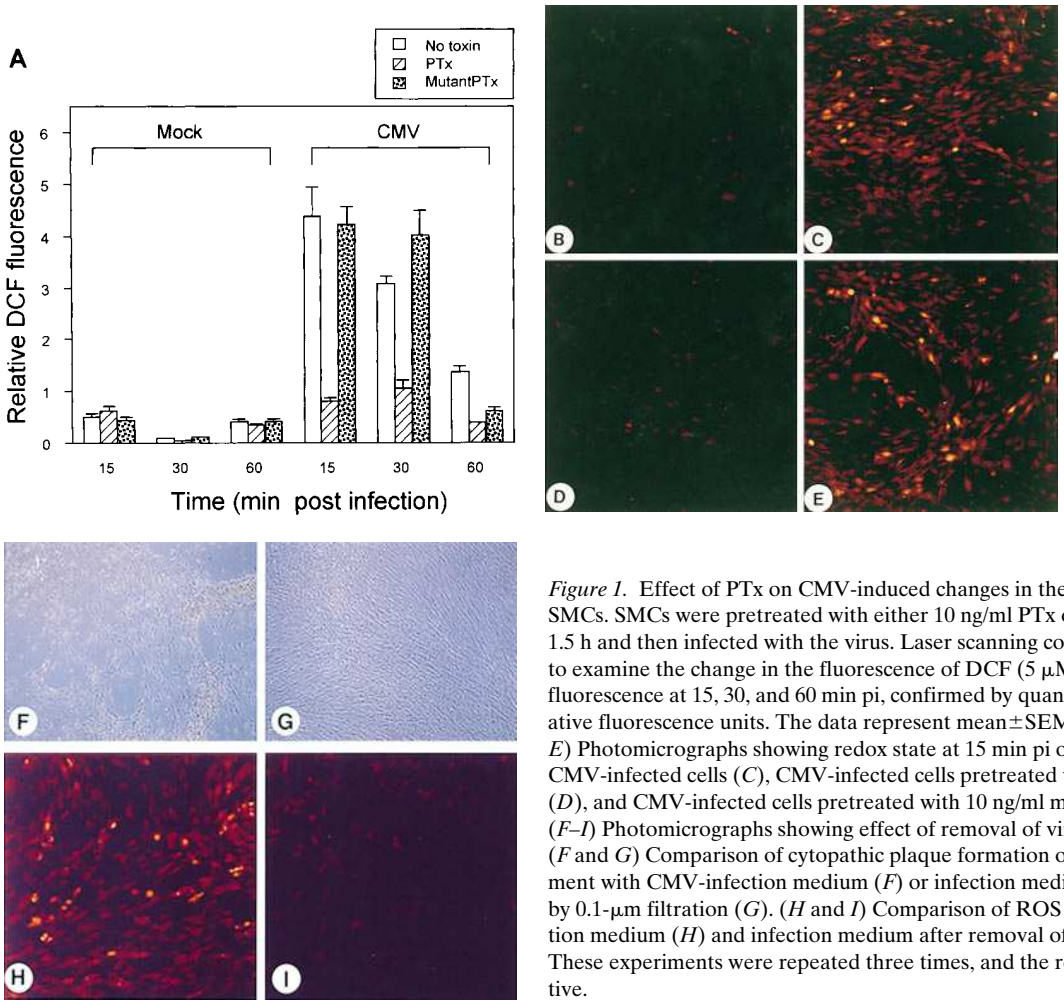

Figure 1. Effect of PTx on CMV-induced changes in the intracellular redox state of SMCs. SMCs were pretreated with either 10 ng/ml PTx or 10 ng/ml mutant PTx for 1.5 h and then infected with the virus. Laser scanning confocal microscopy was used to examine the change in the fluorescence of DCF (5 μM). (*A*) Relative intensity of fluorescence at 15, 30, and 60 min pi, confirmed by quantitative measurements of relative fluorescence units. The data represent mean±SEM of five different fields. (*B–E*) Photomicrographs showing redox state at 15 min pi of mock-infected cells (*B*), CMV-infected cells (*C*), CMV-infected cells pretreated with 10 ng/ml PTx for 1.5 h (*D*), and CMV-infected cells pretreated with 10 ng/ml mutant PTx for 1.5 h (*E*). (*F–I*) Photomicrographs showing effect of removal of virus from infectious medium. (*F* and *G*) Comparison of cytopathic plaque formation on fibroblasts 10 d after treatment with CMV-infection medium (*F*) or infection medium after removal of CMV by 0.1-μm filtration (*G*). (*H* and *I*) Comparison of ROS production by CMV-infection medium (*H*) and infection medium after removal of CMV by 0.1-μm filtration. These experiments were repeated three times, and the results shown are representative.

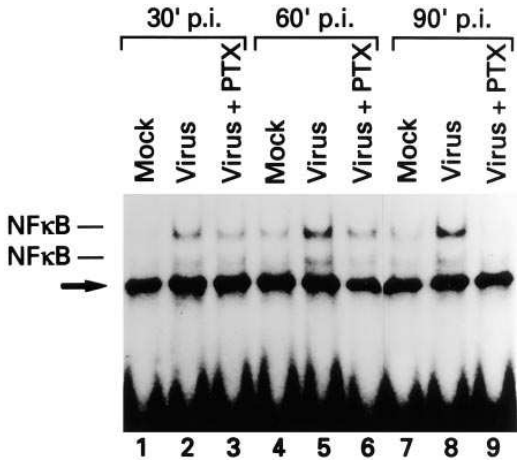

Figure 2. Effect of PTx on CMV-induced NF-κB activation in SMCs. SMCs were pretreated with 1 μg/ml PTx for 3 h and infected with CMV. Nuclear extracts of the cells were prepared at 30, 60, and 90 min pi, and 3 μg protein was used for the assay. Nuclear extracts of unstimulated or PMA-stimulated HeLa cells were used as negative and positive controls (data not shown). The upper two bands of the SMCs comigrated with the upper two bands of PMA-stimulated HeLa cells, indicating that these two bands represent specific NF-κB signals. The lowest band (*arrow*) indicates a constitutively expressed band. This experiment was repeated twice, and the results shown are representative.

SMCs) from which viral particles were removed by centrifugation at 100,000 *g* for 1 h, or (*b*) the same medium passed through a 0.1-μm filter, which successfully removes 100% of the infectious viral particles as indicated by lack of production of cytopathic effects when applied to HEL cells (Fig. 1, *F* and *G*). These two experiments control for the possibility that the infected HEL cells secrete soluble substances that might mediate the effects we observed when the virus (and the media in which it was harvested) was applied to the SMCs. Such mock-infection medium did not lead to the generation of ROS (data not shown).

In addition, to control for the possibility that the SMCs exposed to the virus secreted soluble substances that might in an autocrine fashion mediate the effects we observed, we performed the following control: infectious medium incubated for 15 min on SMCs was removed, and 0.1-μm filtered to remove viral particles and incubated for 15 min on fresh SMC cultures. Representative results of this experiment, demonstrating that viral particles mediate ROS production rather than secondarily secreted substances that would be unaffected by filtration, are shown in Fig. 1, *H* and *I*.

Effect of PTx on CMV-induced NF-κB activation. As determined by electrophoretic mobility shift assay, CMV infection increased the amount of NF-κB bound to a labeled oligonucleotide containing a consensus NF-κB–binding element (Fig. 2). This effect peaked at 60–90 min pi. PTx pretreatment inhibited CMV-associated NF-κB activation at 30, 60, and 90 min pi (Fig. 2).

In the subsequent experiments, we determined the upstream components of this CMV-triggered PTx-sensitive G protein pathway by which the virus causes ROS generation and activation of NF-κB in coronary SMCs.

Effect of PTx on CMV-induced release of [^{3}H]AA and its metabolites. To determine whether CMV-induced release of AA is mediated by PTx-sensitive G proteins, we next examined the effect of PTx on CMV-induced AA release. The release of [^{3}H]AA from SMCs increased significantly at 5, 15, 30, and 60 min pi (Fig. 3 *A*) in comparison with that found after mock infection. CMV-induced [^{3}H]AA release reached a maximum at 15 min pi (178% of that after mock infection, $P < 0.001$) and decreased gradually at 30 and 60 min pi (Fig. 3 *A*). PTx inhibited this CMV-induced [^{3}H]AA release at 5 and 15 min pi, but mutant PTx did not (Fig. 3 *B*).

Effect of CMV infection on phosphorylation and translocation of cPLA$_2$. As AA is released by the action of cPLA$_2$, we determined whether CMV infection stimulates cPLA$_2$ activation. Since activation of cPLA$_2$ requires both phosphorylation and translocation from cytoplasm to membrane (25), we took

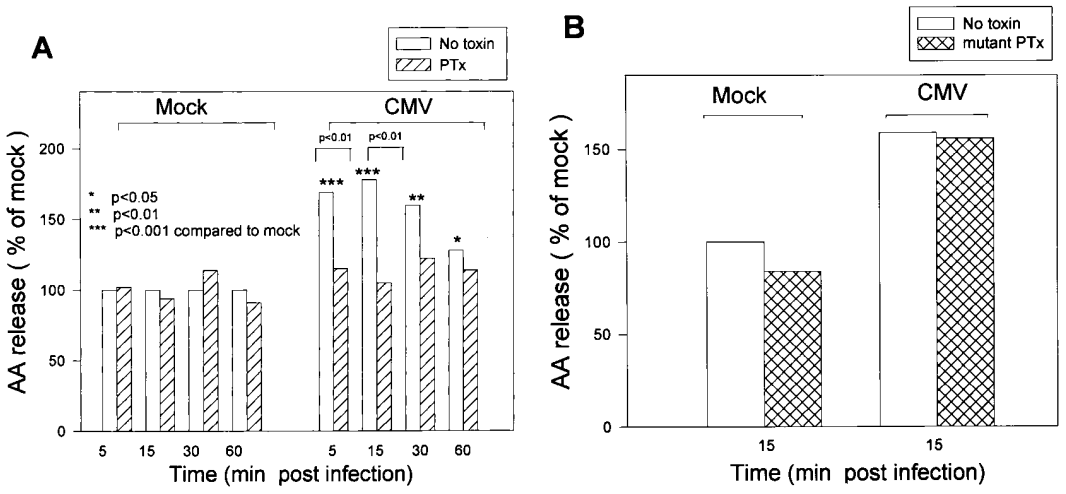

Figure 3. Effect of PTx on CMV-induced AA release from SMCs. Y axis shows percent change from values obtained with mock-infected cells in the absence of toxins. (*A*) [^{3}H]AA-prelabeled cells were pretreated with 10 ng/ml PTx for 1.5 h and then infected with 5 moi virus. The amount of [^{3}H]AA released into cultured media was quantified at 5, 15, 30, and 60 min pi by scintillation counting. This experiment, performed in triplicate, was repeated four times, and the results shown are representative. CMV infection increased AA release significantly, and PTx significantly decreased this effect ($P < 0.01$). (*B*) Same as *A*, except that cells were pretreated with mutant PTx. The medium was collected at 15 min pi. This experiment, performed in triplicate, was repeated twice, and the results shown are representative.

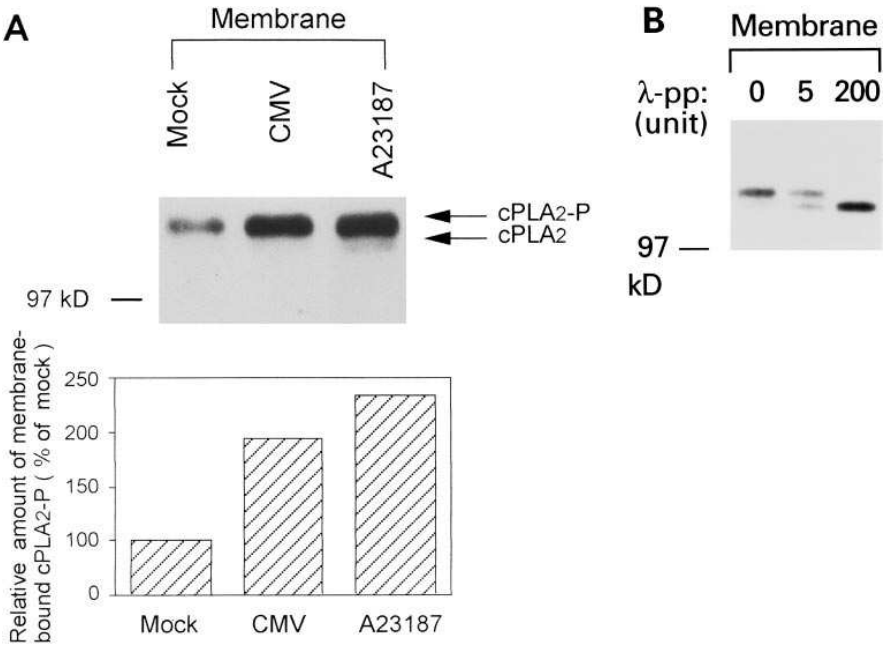

Figure 4. Effect of CMV infection on phosphorylation and translocation of $cPLA_2$ in SMCs. (*A*) SMCs were infected with 5 moi virus for 15 min, and the membrane fraction was separated from the cell lysate. Cells treated in the same way but not infected (*Mock*) were used as a control. 30 μg of each protein was subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-$cPLA_2$ mAb. *Arrows*, Positions of native and phosphorylated $cPLA_2$ (*cPLA2-P*). The amount of the membrane-bound phosphorylated $cPLA_2$ was quantified by densitometry and expressed as percentage of the mock-infected value. (*B*) Membrane fraction of CMV-infected cells was incubated with the indicated units of λ-protein phosphatase(*λ-PP*) for 30 min at 30%C. λ-Protein phosphatase treatment increased electrophoretic mobility. This experiment was repeated three times, and the results shown are representative.

advantage of the fact that the phosphorylated form of $cPLA_2$ has altered electrophoretic mobility to ascertain whether CMV infection increases the amount of phosphorylated membrane-associated $cPLA_2$. CMV infection increased the amount of phosphorylated $cPLA_2$ by 95% of the value in the mock-infected control (Fig. 4 *A*). Because $cPLA_2$ is known to be phosphorylated and to translocate to the cell membrane from the cytoplasm upon stimulation with agents that increase intracellular Ca^{2+} (25), calcium ionophore A23187 was used as a positive control. A23187 treatment increased phosphorylated $cPLA_2$ by 135% (Fig. 4 *A*). These results indicate that CMV infection of SMCs increases the amount of membrane-associated phosphorylated $cPLA_2$.

To confirm that the altered mobility was a result of phosphorylation, membranes from infected SMCs were treated with λ-protein phosphatase. The electrophoretic mobility of the phosphatase-treated $cPLA_2$ was increased, confirming that CMV infection increases membrane-associated phosphorylated $cPLA_2$ (Fig. 4 *B*).

Effect of PTx on CMV-induced $cPLA_2$ activation. We next assayed the catalytic activity of $cPLA_2$ in SMCs to confirm that the CMV-induced AA release is associated with $cPLA_2$ activation. Preliminary studies demonstrated that $cPLA_2$ activity was linear in relation to the amount of protein used in the assay, whether the protein was derived from total cell lysate or from cell membranes (data not shown). The amount of protein used to determine the effect of CMV and the toxins on $cPLA_2$ activation was within this range. $cPLA_2$ activities were determined in membrane (Fig. 5 *A*) and cytoplasmic fractions (Fig. 5 *B*). CMV infection increased significantly $cPLA_2$ activity in the

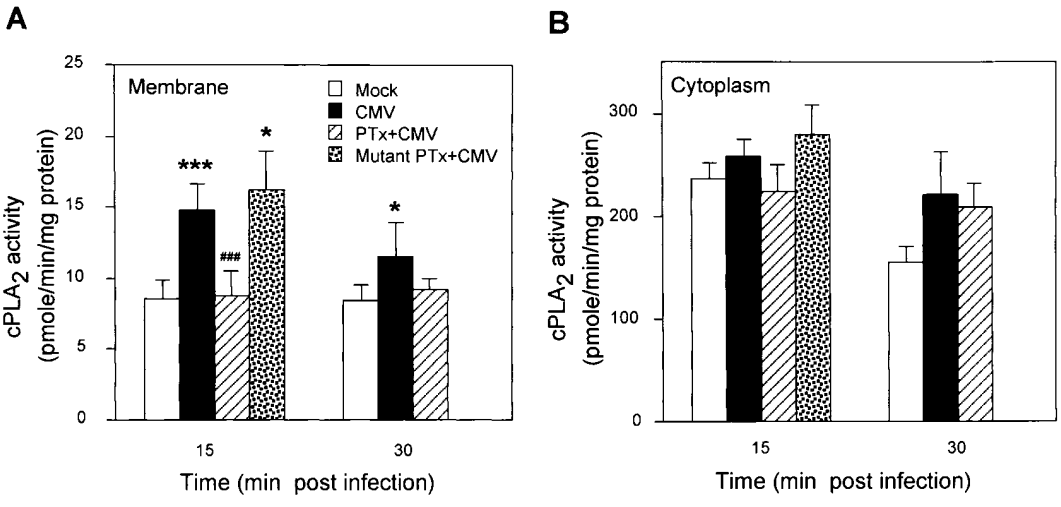

Figure 5. Effect of PTx on CMV-induced $cPLA_2$ activation. SMCs were pretreated with either 10 ng/ml PTx or 10 ng/ml mutant PTx for 1.5 h and then infected with 5 moi virus. Membrane (*A*) and cytoplasmic (*B*) fractions were prepared from the SMCs at 15 and 30 min pi. $cPLA_2$ activity in each fraction was measured by hydrolysis of 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine as described in Methods. 10 and 8 μg of the proteins, respectively, were used for the assay. The data represent mean±SEM of five separate experiments. $^{*}P < 0.05$. $^{***}P < 0.001$ vs. mock-infected cells. $^{\#\#\#}P < 0.001$ vs. CMV-infected cells without PTx preincubation. No significant changes were observed in the cytoplasmic fraction.

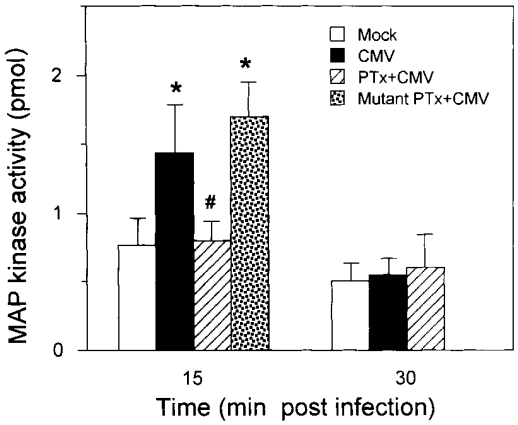

Figure 6. Effect of PTx on CMV-induced MAP kinase activation. SMCs were pretreated with either 10 ng/ml PTx or 10 ng/ml mutant PTx for 1.5 h and then infected with 5 moi virus. The SMCs were lysed at 15 and 30 min pi in sucrose lysis buffer, and MAP kinase was purified by immunoprecipitation using polyclonal anti–MAP kinase antibody. MAP kinase activities in the immunoprecipitates were assayed as described in Methods. The data represent mean±SEM of four separate experiments. $^{*}P < 0.05$ vs. mock-infected cells. $^{\#}P < 0.001$ vs. CMV-infected cells without PTx preincubation.

membranes, by 72 ($P < 0.001$) and 37% ($P < 0.05$) of the value in mock-infected cells, at 15 and 30 min pi, respectively. This CMV-induced increase in $cPLA_2$ activity was blocked completely by pretreatment with PTx but not with mutant PTx. The specific activity in the cytoplasm was ~ 10–20 times higher than in membranes. However, CMV infection did not increase significantly the activity of cytoplasmic $cPLA_2$.

Effect of PTx on CMV-induced MAP kinase activation. We next determined whether stimulation of the $cPLA_2$ pathway by CMV infection is associated with MAP kinase activation. CMV infection increased significantly MAP kinase activity, by 88% of the mock-infected control value at 15 min pi ($P < 0.05$); this increased activity returned to baseline levels by 30 min pi (Fig. 6). PTx inhibited the CMV-induced MAP kinase activation at 15 min pi, whereas mutant PTx did not (Fig. 6).

Effect of MAP kinase pathway inhibition on CMV-induced ROS generation. We next determined whether CMV-induced intracellular ROS generation is mediated by MAP kinase. The MAPKK inhibitor PD 098059 (19–21) was used for MAP kinase pathway inhibition. CMV infection increased markedly fluorescence intensity of DCF, indicating an increase in intracellular ROS generation in comparison with the mock-infected cells at 15 min pi (Fig. 7). PD 098059 inhibited this ROS generation in a concentration-dependent fashion (Fig. 7). Treatment of mock-infected cells with the same dose of the inhibitor did not alter significantly baseline fluorescence intensity (Fig. 7).

Discussion

We demonstrated recently that CMV infection of SMCs causes intracellular ROS generation, and that this appears to be due, at least in part, to activation of the AA cascade. The increase in ROS, in turn, appeared causally related to the CMV-induced activation of NF-κB (6, 7). This investigation demonstrates that a PTx-sensitive G protein–dependent signaling pathway mediates these cellular effects of CMV infection: the downstream events include phosphorylation and activation of MAP kinase through MAPKK, phosphorylation and activation of $cPLA_2$ (causing its translocation to cell membranes), and subsequent stimulation of the AA cascade, which generates intracellular ROS and thereby activates NF-κB.

To dissect this CMV infection–induced pathway, we first determined whether PTx-sensitive G proteins were, in fact, involved in the CMV-induced stimulation of the AA cascade and in the terminal products of the postulated pathway, ROS generation and NF-κB activation. We found that CMV infection increased significantly AA release, and that this effect was inhibited by PTx pretreatment (Fig. 3). In addition, both the ROS generation and NF-κB activation occurring after CMV infection of SMCs were blocked by PTx pretreatment (Figs. 1 and 2). Thus, CMV-induced stimulation of the AA cascade, generation of ROS, and activation of NF-κB are mediated by PTx-sensitive G proteins.

Given that AA is released through the catalytic activity of $cPLA_2$, we next determined whether CMV infection induces $cPLA_2$ activation and, if so, whether such an effect is mediated through a G protein signaling pathway. Three different electrophoretic shift assays on SDS-PAGE were used to identify phosphorylated $cPLA_2$, $cPLA_2$ activity, and AA release. We found that CMV infection increased the amount of membrane-associated phosphorylated $cPLA_2$, indicating either enhanced translocation of phosphorylated $cPLA_2$ to cell membranes or phosphorylation of membrane-associated $cPLA_2$. Increased intracellular Ca^{2+} content is needed for the translocation of $cPLA_2$ to the membrane. Since intracellular Ca^{2+} increases early after CMV infection, it is possible that this effect of the virus contributes to the CMV-induced increase in membrane-associated $cPLA_2$ (14). It should be noted that our experiments do not allow us to identify the cell membrane fraction to which these changes are localized, as $cPLA_2$ appears to

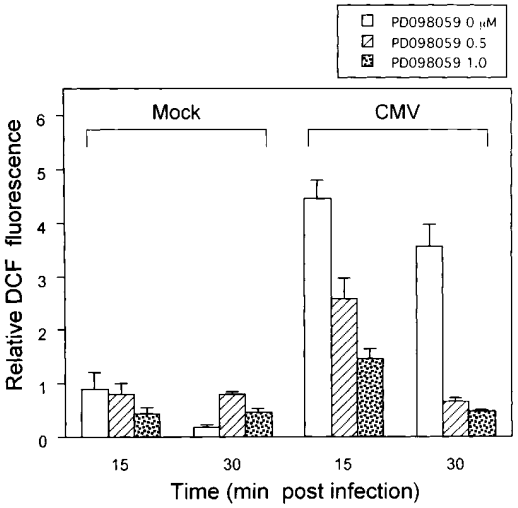

Figure 7. Effect of MAP kinase pathway inhibition on CMV-induced ROS generation. SMCs were incubated with MAPKK inhibitor for 30 min at 37°C, and infected with 5 moi virus for 15 and 30 min. Intracellular redox state was analyzed as described in Fig. 1. Graph shows relative fluorescence intensity measured by confocal image analysis, and the data represent the mean±SEM of five different fields. This experiment was repeated twice, and the results shown are representative.

translocate not only to the cell surface membrane but also to the nuclear envelope and endoplasmic reticulum (26).

CMV infection also increased membranous $cPLA_2$ catalytic activity (Figs. 4 and 5), an effect inhibited by PTx. Although $cPLA_2$ is known to be activated by products of the phospholipase C pathway, which is regulated by PTx-insensitive G proteins, a more recent study showed that $cPLA_2$ activation is also PTx sensitive, implying that members of the Gi/Go family are also involved in its regulation (9). Winitz et al. (13) demonstrated that functional Gi_2 protein is required specifically for G protein regulation of AA release in Chinese hamster ovary cells. Therefore, our results are compatible with the role of a PTx-sensitive pathway for $cPLA_2$ activation, and further indicate that $cPLA_2$ is a downstream target of the PTx-sensitive G protein signal transduction pathway activated by CMV infection.

The $cPLA_2$ family is divided into two major isozymes based on a difference in their sensitivity to intracellular Ca^{2+} ion content: Ca^{2+}-dependent $cPLA_2$ and a recently characterized Ca^{2+}-independent $cPLA_2$ (25). Although the two enzymes share many similarities, including high molecular weight, resistance to a reducing agent, and translocation to the membrane upon stimulation (27), the absolute difference between these two isozymes is that the Ca^{2+}-dependent $cPLA_2$ is phosphorylated exclusively at serine 505 residue by MAP kinase, as shown by electrophoretic mobility shifts on SDS-PAGE (15), whereas the Ca^{2+}-independent $cPLA_2$ is not. Thus, it is possible that the Ca^{2+}-independent $cPLA_2$ might have contributed to our results of the effects of CMV infection on $cPLA_2$ activity and AA release. However, our electrophoretic mobility shift experiments, demonstrating a CMV-induced increase in $cPLA_2$ phosphorylation, would be specific for Ca^{2+}-dependent $cPLA_2$.

Many AA-mobilizing agents, such as ATP, thrombin, and some growth factors, promote the phosphorylation of $cPLA_2$ and activate MAP kinase (13, 28, 29), suggesting that MAP kinase causes the agonist-induced phosphorylation of $cPLA_2$ and subsequent stimulation of the AA cascade. Several groups have demonstrated G protein–mediated MAP kinase activation (30, 31). Therefore, we examined the possibility that MAP kinase is a downstream target of the CMV-induced signaling pathway. We found this to be the case; MAP kinase activity was increased by CMV infection as early as $cPLA_2$ activation (Fig. 5 *A* and Fig. 6). Moreover, PTx pretreatment inhibited completely this increase in MAP kinase activity. Therefore, the next experiment we performed was to determine whether the PTx-sensitive and CMV-induced activation of MAP kinase was involved in generating the specific downstream products of the CMV-stimulated signaling pathway.

Diverse signaling pathways involving MAP kinase are used by many growth factors, hormones, and neurotransmitters. However, an obligate part of MAP kinase activation is the phosphorylation of tyrosine and threonine residues by MAPKK (32). Recently, a MAP kinase pathway inhibitor that specifically inhibits MAPKK was identified. We found that one of the terminal products of the CMV-stimulated signaling pathway, ROS generation, was reduced markedly by pretreatment with the MAPKK inhibitor in a dose-dependent manner (Fig. 7). This result confirms the role of MAP kinase in the signaling pathway that is stimulated by CMV infection of SMCs and leads to ROS generation, and also implicates MAPKK as an additional downstream target.

PTx consists of two moieties, an active protomer (A-protomer), which is an ADP-ribosyltransferase, and the B-oligomer. B-oligomer is responsible for binding of the toxin to its receptor. However, it may also have stimulatory effects in certain cell types (33). In addition, PTx appears to bind to cell surface glycoproteins (34). Moreover, the CMV envelope contains glycoproteins (35). Therefore, to exclude the possibility that our results could have been caused by PTx inhibiting CMV infection by competing with virus attachment to its receptors, or possibly through other interactions between the B-oligomer and the virus, we used a mutated PTx as an important control in this investigation. The mutated PTx lacks ADP-ribosyltransferase activity but has an intact B-oligomer, allowing it to bind normally to its receptor (17). We found that this mutant PTx indeed lacked the capacity to ADP-ribosylate PTx-sensitive G proteins, confirming the inactivation of the A-protomer. The mutant PTx competed with wild-type PTx (as assessed by CMV-induced ROS generation), confirming its intact binding domain (data not shown). In contrast to the inhibitory effects displayed by wild-type PTx, the mutant PTx had no impact on the CMV-induced effects.

In conclusion, this investigation demonstrates that a PTx-sensitive G protein–dependent signaling pathway mediates the cellular effects of CMV infection of human SMCs: the downstream targets include phosphorylation and activation of MAP kinase via MAPKK, phosphorylation and activation of $cPLA_2$ (with its translocation to cell membranes), and subsequent stimulation of the AA cascade, which generates intracellular ROS and thereby activates NF-κB. If CMV does contribute to restenosis and atherogenesis, these results might provide the basis for developing new therapeutic strategies, which would be targeted to the components of this signaling pathway and would inhibit the contribution of CMV to these disease processes.

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