Roles of Rac and p38 kinase in the activation of cytosolic phospholipase A₂ in response to PMA

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The roles of Rac and p38 kinase in the activation of cPLA₂ (cytosolic PLA₂) in Rat-2 fibroblasts were investigated. In the present study, we found that PMA activates cPLA₂ by a Rac-p38 kinase-dependent pathway. Consistent with this, Rac, if activated, was shown to stimulate cPLA₂ in a p38 kinase-dependent manner. In another experiment to understand the signalling mechanism by which the Rac-p38 kinase cascade mediates cPLA₂ activation in response to PMA, we observed that PMA-induced cPLA₂ translocation to the perinuclear region is completely inhibited by the expression of Rac1^{N17} or treatment with SB203580 (inhibitor of

p38 kinase), suggesting that Rac-p38 kinase cascade acts in this instance by mediating the translocation of cPLA₂. The mediatory role of p38 kinase in cPLA₂ activation was further demonstrated after a treatment with anisomycin, a very effective activator of p38 kinase. Consistent with the mediatory role of p38 kinase in stimulating cPLA₂, anisomycin induced the translocation and activation of cPLA₂ in a p38 kinase-dependent manner.

Key words: anisomycin, arachidonic acid, cPLA₂, p38 kinase, PMA, Rac.

INTRODUCTION

Phospholipase A₂ catalyses the hydrolysis of the central (*sn*-2) ester bond of glycerophospholipids, producing a non-esterified fatty acid and lysophospholipid [1]. These products have a variety of cellular functions: they contribute to cell signalling, phospholipid remodelling, membrane perturbation and inflammation [1,2]. Mammalian cells possess structurally diverse forms of phospholipase A₂, notably cPLA₂ (cytosolic PLA₂), sPLA₂ (secretory sPLA₂) and iPLA₂ (Ca²⁺-independent) forms [2]. Among them, cPLA₂ is an 85 kDa cytosolic enzyme that requires micromolar free-Ca²⁺ and is believed to be a major producer of AA (arachidonic acid) under physiological conditions. AA, a C₂₀ fatty acid released by cPLA₂, is further metabolized into eicosanoids such as prostaglandins and leukotrienes by cyclo-oxygenase and lipoxygenase respectively [2,3]. These eicosanoids are potent lipid mediators involved in various homeostatic functions and inflammation. Although the physiological characteristics of cPLA₂ make it an important target for treating eicosanoid-mediated inflammation, the upstream signalling pathway to cPLA₂ is still not clear.

cPLA₂ is mainly regulated by Ca²⁺ influx and phosphorylation. It is translocated to the perinuclear membrane region by its C2 domain in a Ca²⁺-dependent manner [4,5] and it is phosphorylated by MAPK (mitogen-activated protein kinases) such as ERK (extracellular signal-regulated kinase), c-Jun N-terminal kinase or p38 kinase [6–10]. However, the detailed mechanisms of its regulation remain to be elucidated. Previously, it was demonstrated that cPLA₂ is regulated by Rac1, a member of the Rho family of G-proteins, which acts as a molecular switch by cycling between active GTP-bound and inactive GDP-bound forms. Switching is regulated by guanine nucleotide exchange factors, GTPase-activating factors and GDP-dissociation-inhibitory factors [11]. Rac1 binds to effector molecules in its GTP bound form, and

influences cellular processes such as cell motility, invasion, degranulation, cell–cell interaction, cell proliferation, cytoskeletal rearrangements and differentiation [12–14]. Consistent with the idea of cPLA₂ as a downstream mediator of Rac signalling, cPLA₂ has been shown to be necessary for Rac in mediating actin remodelling, *c-fos* serum response element activation, transformation, or c-Jun N-terminal kinase stimulation [13–16], and we have obtained evidence of a role for Rac in cPLA₂ activation and the subsequent release of AA [15–18].

In an approach to understand the signalling mechanism that accounts for Rac-mediated cPLA₂ activation, in the present study, we have tested whether PMA stimulates cPLA₂ by a Rac-dependent pathway and which signalling mechanism would be involved. PMA is a well-known activator of classical and novel isotypes of PKC (protein kinase C) [19]. We and others have reported that it stimulates AA release in macrophages and fibroblasts probably without the involvement of Ca²⁺ influx [20–22]. In the present study, we report that activation of cPLA₂ by Rac is mainly in a p38 kinase-dependent manner. Furthermore, we observed that PMA-induced cPLA₂ translocation to the perinuclear membrane region is completely inhibited by the expression of Rac1^{N17} or treatment with SB203580 (a p38 kinase inhibitor), revealing a novel regulatory mechanism of cPLA₂ through the Rac-p38 kinase cascade. Similarly, anisomycin, a very effective activator of p38 kinase, induced cPLA₂ activation and translocation to the perinuclear region, further supporting an involved role of p38 kinase in the translocation process of cPLA₂.

EXPERIMENTAL

Materials

PMA and EGTA were from Sigma–Aldrich (St. Louis, MO, U.S.A.); GF109203X (PKC inhibitor), SB203580, PD98059

Abbreviations used: AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; CHOP, C/EBP homologous protein-10; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GST, glutathione S-transferase; LTB₄, leukotriene B₄; MAPK, mitogen-activated protein kinase; PAK, p21-activated protein kinase; PBD, p21-binding domain; PI3K, phosphoinositide 3-kinase; PI4K, phosphoinositide 4-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SAPC, 1-stearoyl-2-¹⁴C-arachidonyl phosphatidyl choline.

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(MAPK/ERK kinase inhibitor) and anisomycin were from Calbiochem (Darmstadt, Germany); wortmannin [23], FBS (fetal bovine serum), DMEM (Dulbecco's modified Eagle's medium), and non-essential amino acids were from GIBCO™ Invitrogen (Carlsbad, CA, U.S.A.); and SAPC (1-stearoyl-2-¹⁴C-arachidonyl phosphatidyl choline) was from Amersham Biosciences (Piscataway, NJ, U.S.A.). Silica gel 60 F254 plates were obtained from Merck (Darmstadt, Germany). All other chemicals were from standard sources and were of molecular biology grade or higher. Mouse monoclonal anti-cPLA₂ or anti- α -tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal anti-phospho-cPLA₂, anti-(phospho-p38 kinase) and anti-(p38 kinase) antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.). The myc-tagged expression vector pEXV, pEXV-Rac1^{V12} that encodes Rac1^{V12} (a constitutively active form of Rac1) and pEXV-Rac1^{N17} that encodes Rac1^{N17} (a dominant-negative form of Rac1) were gifts from Dr A. Hall (University College, London, U.K.). cDNAs encoding Rac1^{V12} and Rac1^{N17} were cloned between the EcoRI and XhoI sites of HA-tagged pcDNA3 plasmids and were used for the transfection of Rat-2 fibroblasts. pGFP-cPLA₂ was a gift from Dr T. Shimizu (University of Tokyo, Tokyo, Japan) [24].

Cell culture, transfection and luciferase assay

Rat-2 fibroblasts were obtained from the A.T.C.C. (CRL 1764). The cells were grown in DMEM, supplemented with 0.1 mM non-essential amino acids, 10% (v/v) FBS, penicillin (50 units/ml) and streptomycin (50 μ g/ml), at 37 °C in a humidified mixture of air and CO₂ (95:5). The stable Rat-2^{Rac1^{N17}} clones expressing Rac1^{N17}, a dominant-negative Rac1 mutant, were described previously [17,25]. Transient transfection was performed by plating approx. 3×10^5 cells in 60 mm dishes for 24 h and adding calcium phosphate/DNA precipitates prepared with 20 μ g of DNA/ml of transfection medium. The quantity of DNA used in each transfection was kept constant (20 μ g/ml transfection medium) by adding sonicated calf thymus DNA (Sigma). To control the variation in cell number and transfection efficiency, all clones were co-transfected with 0.3 μ g of pCMV- β GAL, a eukaryotic expression vector containing the *Escherichia coli* β -galactosidase (*lacZ*) structural gene under the control of the cytomegalovirus promoter. After incubating for 6 h with the calcium phosphate/DNA precipitates, the cells were rinsed twice in PBS before incubating them in fresh DMEM containing 0.5% FBS for an additional 24 h. Each dish of cells was then rinsed twice in PBS and lysed in 0.1 ml of lysis solution (0.2 M Tris (pH 7.6, and 0.1%, v/v, Triton X-100), after which the lysed cells were scraped off and centrifuged for 1 min. The supernatants were assayed for protein and β -galactosidase activity and subjected to Western blotting or subcellular fractionation.

To measure p38 kinase activity with the PathDetect trans-reporting system (Stratagene, La Jolla, CA, U.S.A.; cat. no. 219005), CHOP (C/EBP homologous protein-10, also known as GADD153) fused to a trans-activator plasmid (pFA2-CHOP) was co-transfected with the pFR-Luc reporter plasmid according to the manufacturer's instructions. To examine the role of Rac1 in p38 kinase activation, pcDNA-HA-Rac1^{V12} was co-transfected with pFR-Luc or pFA2-CHOP using calcium phosphate methods. After 36 h, each dish of cells was rinsed twice in PBS and lysed in 0.1 ml of a lysis solution (0.2 M Tris, pH 7.6, and 0.1% Triton X-100). Next, the supernatants were assayed for luciferase activity as well as protein concentration and β -galactosidase activity.

Luciferase activity was assayed in 10 μ l samples of the extract using the Luciferase Assay System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions; luciferase

luminescence was counted in a luminometer (Turner Designs, Sunnyvale, CA, U.S.A.; TD-20/20) and normalized to co-transfected β -galactosidase activity. Transfection experiments were performed in triplicate with two independently isolated sets of cells and results are expressed as means $\pm$ S.D. relative to the control.

Assay of cPLA₂ activity

The Rat-2 cells Rat-2^{Rac1^{N17}} cells were starved for 24 h in DMEM containing 0.5% FBS, then rinsed twice in PBS and lysed in a lysis solution (20 mM Tris/Cl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM dithiothreitol and protease inhibitors). The cells were lysed by passing with a 26-gauge needle and fractionated by ultracentrifugation at 100 000 g for 1 h. The supernatants were assayed for cPLA₂ activity using sonicated liposomes prepared as described previously [26,27]. Briefly, a stock solution of the [¹⁴C]SAPC radioactive substrate was prepared as follows: the [¹⁴C]SAPC substrate (10 nmol) was dried under nitrogen and resuspended in 0.5 ml of distilled water by sonication (3 $\times$ 10 s) in a bath-type sonicator (Ultrasonik 300, J. M. Ney, Broomfield, CT, U.S.A.). The reaction was started by adding each cell lysate (containing $\sim$ 10 μ g of protein/50 μ l) to make 200 μ l of total reaction mixture that included 50 μ l of [¹⁴C]SAPC (final concentration 5 μ M), 1 mM CaCl₂ and 20 mM Tris/Cl at pH 8.5. The assay mixtures were incubated at 37 °C for 30 min and then quenched by adding 500 μ l of Dole's reagent (32%, v/v propan-2-ol, 67%, v/v, n-heptane and 1%, v/v, 0.5 M H₂SO₄) and vortex-mixing. Non-esterified fatty acid was extracted as follows: in the reaction mixture, 300 μ l of n-heptane and 200 μ l of distilled water were added, the samples were centrifuged and the upper phase ($\sim$ 400 μ l) was transferred to a prepared silicic gel column [28]. The released [¹⁴C]AA was extracted by adding equal volumes of diethyl ether to silica gel and the eluate was counted for radioactivity in a liquid-scintillation counter. In all experiments, samples were tested in duplicate and adjusted for non-specific release by subtracting the value of the control without cell lysate.

Rac activity assay

The PAK (p21-activated protein kinase)-PBD (p21-binding domain) binding assay was performed essentially as described in [29]. In brief, the PBD of human PAK1, comprising amino acids 68–166, was subcloned into the bacterial expression vector pGEX-2TK (Amersham Biosciences) and expressed in *E. coli* as the GST-PAK-PBD fusion protein (where GST stands for glutathione S-transferase), according to the manufacturer's instructions. A 50% (v/v) slurry (15 μ l) of GST-PAK-PBD glutathione-Sepharose 4B was added to lysates of Rat-2 fibroblasts and continuously rotated at 4 °C for 60 min. The bound proteins were collected by centrifugation and the pellets were washed three times with cell lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 25 mM NaF, 10%, v/v, glycerol, 0.25%, w/v, sodium deoxycholate, 10 mM MgCl₂, 1 mM EDTA and 1% Triton X-100) and finally suspended in SDS sample buffer. Proteins were size-fractionated by SDS/PAGE, and Rho family GTPases were identified by Western blotting with Rac1 and Cdc42 antibodies.

Subcellular fractionation of lysates

Rat-2 cells were serum-starved for 18 h in serum-free DMEM and incubated with appropriate agonists for the indicated times. The medium was removed and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation and resuspended in 0.2 ml of buffer A (137 mM NaCl, 8.1 mM Na₂HPO₄,

2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 10 µg/ml leupeptin, pH 7.5). The resuspended cells were then lysed on ice by 20 passes through a 21.1-gauge needle. Lysates were centrifuged at 100 000 *g* for 1 h to prepare cytosolic and particulate membrane fractions and the particulate membrane fractions were washed and suspended in 50 µl of buffer A. The protein concentration was determined routinely with the Bradford reagent and BSA as standard.

Western blotting

Protein samples were heated at 95 °C for 5 min and subjected to SDS/PAGE (8–10 % polyacrylamide), followed by transfer to PVDF membranes at 100 V with a Novex wet transfer unit (Novex, San Diego, CA, U.S.A.). The membranes were blocked overnight in Tris-buffered saline with 0.01 % (v/v) Tween 20 and 5 % (w/v) non-fat dried milk, after which they were incubated for 2 h with primary antibody in Tris-buffered saline, and then for 1 h with horseradish peroxidase-conjugated secondary antibody. The blots were developed using an enhanced chemiluminescence kit (ECL[®], Amersham Biosciences).

Confocal microscopy

To localize cPLA₂, cells were plated on coverslips and grown for 24 h in DMEM containing 10 % FBS. After transfection with calcium phosphate/DNA precipitates (10 µg of DNA/ml transfection medium), the cells were starved in DMEM containing 0.5 % FBS for 36 h, and exposed to 20 nM PMA for the indicated times. The reaction was stopped by washing twice with ice-cold PBS and fixing with 4 % (w/v) paraformaldehyde. Thereafter, they were mounted on a glass slip and sealed. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal system (Bio-Rad Laboratories, Hercules, CA, U.S.A.); multiple image sections of thickness 0.2 µm were recorded over the depth of the cells and analysed.

Data analysis and statistics

Data are expressed as means ± S.D. or as percentage of control ± S.D. Statistical comparisons between groups were made with Student's *t* test. Values of *P* < 0.01 were considered significant.

RESULTS

Rac1^{V12} activates cPLA₂

Previously, we have suggested a potential role of Rac as an upstream mediator of cPLA₂ stimulation [15]. Consistent with this idea, we observed that overexpression of Rac1^{V12} induces a highly elevated cPLA₂ activity as assayed by the release of ¹⁴C-AA. For this assay, subconfluent Rat-2 fibroblasts were transiently transfected with pcDNA-HA-Rac1^{V12} or pcDNA-HA-Rac1^{N17} and incubated for 24 h in a serum-free DMEM. As shown in Figure 1, cPLA₂ activity was stimulated by Rac1^{V12} overexpression (Figure 1A) and there was only a small increase of cPLA₂ activity in Rac1^{N17}-overexpressing cells when compared with pcDNA3-transfected cells (Figure 1B), suggesting that activation of Rac1 is sufficient for cPLA₂ stimulation. Next, we investigated the signalling mechanism by which Rac mediates cPLA₂ activation. Subconfluent cells were transiently transfected with the plasmid (5 µg) pcDNA-HA-Rac1^{V12} or pcDNA3 and incubated for an additional 24 h in serum-free DMEM. Various inhibitors (SB203580, PD98059 or EGTA) or DMSO (vehicle) were then added 6 h before harvest. As shown in Figure 1(C), SB203580 (10 µM) significantly attenuated the activation of cPLA₂ by

Figure 1 Constitutive activation of Rac stimulates cPLA₂

(A) Rat-2 cells were transiently transfected with pcDNA3 or pcDNA-HA-Rac1^{V12} by the calcium phosphate precipitation method; after 24 h, the cells were harvested and assayed for cPLA₂ activity as described in the Experimental section. Results are expressed as percentage of control and are the means ± S.D. for three independent experiments. (B) Cells were transiently transfected with pcDNA3, pcDNA-HA-Rac1^{V12} or pcDNA-HA-Rac1^{N17} (5 µg/ml transfection media); after 24 h, the cells were harvested and assayed for cPLA₂ activity. Results are expressed as percentage of control and are the means ± S.D. for three independent experiments. (C) Subconfluent Rat-2 fibroblasts were transiently transfected with pcDNA-HA-Rac1^{V12} or pcDNA3 (5 µg/ml transfection media) by the calcium phosphate method, starved in DMEM containing 0.5 % FBS for 24 h and then harvested. The cells were treated with PD98059 (10 µM), SB203580 (10 µM), EGTA (5 mM) or DMSO for 6 h before harvesting, and lysates were assayed for cPLA₂. Results are expressed as means ± S.D. of control for three independent experiments. Statistical significance was assessed using unpaired *t*-tests (*P* < 0.01).

Rac1^{V12} overexpression, whereas an inhibitor of ERKs (PD98059; 10 µM) or a calcium chelator (EGTA; 5 mM) had little effect.

p38 kinase as a downstream mediator of Rac1 in the signalling to cPLA₂ activation

The above results suggest that Rac stimulates cPLA₂ by a p38 kinase-dependent pathway, pointing to p38 kinase as a downstream mediator of Rac1 in signalling to cPLA₂ stimulation. To determine further the hierarchical relationship of Rac1 and p38 kinase, we performed Western-blot analysis and phosphorylation-dependent reporter gene assays using the PathDetect transporter system (Stratagene) as described in the Experimental section. To investigate whether activation of Rac1 itself can induce the phosphorylation of p38 kinase, p38 kinase phosphorylation was examined in Rat-2 cells transfected with pcDNA-HA-Rac1^{V12} by Western blotting. p38 kinase was activated by overexpression of Rac1^{V12} in a dose-dependent manner (Figure 2A) and p38 kinase

Figure 2 p38 kinase as a downstream mediator of Rac in the signalling to cPLA₂

(A) Levels of phospho-p38 kinase and total p38 kinase were compared in subconfluent Rat-2 fibroblasts transfected with pcDNA3 or pcDNA-HA-Rac1^{V12} and starved in DMEM containing 0.5% FBS for 24 h. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health, Bethesda, MD, U.S.A.) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$). (B) Subconfluent Rat-2 cells were co-transfected with pcDNA3 or pcDNA-HA-Rac1^{V12} along with pFA2-CHOP or pFR-Luc by the calcium phosphate precipitation method, after which the transfectants were starved in DMEM containing 0.5% FBS for 36 h and lysed. The lysates were assayed for p38 kinase activity by the CHOP-luciferase trans-reporter system (see the Experimental section for details). Results are expressed as means $\pm$ S.D. of control from three independent experiments. Statistical significance of differences was assessed using unpaired Student's *t* tests ($P < 0.01$).

luciferase reporter. Gene assays also showed a dose-dependent activation of p38 kinase by overexpressing Rac1^{V12} (Figure 2B).

Essential role of Rac in the PMA-induced signalling to cPLA₂ activation

Next, we examined whether Rac plays a similar mediatory role in the cPLA₂ activation in response to PMA in Rat-2 fibroblasts. The cells were exposed to 20 nM PMA and lysates were assayed for cPLA₂ activity as described in the Experimental section. As expected, PMA stimulated cPLA₂ activity (Figure 3A), and activation was inhibited in Rat-2 cells overexpressing the dominant-negative mutant of Rac1, namely Rat-2^{Rac1N17} (Figure 3B), indicating that Rac1 plays an essential role in the PMA-induced cPLA₂ activation.

To study further the role of Rac1 in PMA-induced signalling, we tested if PMA activates Rac or not. For this assay, we performed a Rac-PAK binding assay [29]; Rat-2 cells (1×10^6 cells) were plated on to 100 mm dishes and incubated in DMEM containing 10% FBS for 24 h. Subconfluent cells were starved in DMEM containing 0.5% FBS for 24 h, and exposed to 20 nM PMA for the indicated times, after which the cells were lysed. Supernatants of lysates were assayed for active Rac1 protein as described in the Experimental section. Rac1 was indeed activated by PMA (20 nM) treatment as shown in Figure 3(C), reaching a maximum at 10 min. At the same time, it appeared to translocate from the cytosolic to particulate membrane regions (Figure 3D), reflecting an activation of Rac1.

Figure 3 PMA activates cPLA₂ through Rac in Rat-2 fibroblasts

(A) cPLA₂ activity was assayed in PMA-treated Rat-2 fibroblasts as described in the Experimental section. Results are expressed as percentage of control and are the means $\pm$ S.D. for three independent experiments. (B) cPLA₂ activity in PMA (20 nM, 10 min)-treated Rat-2 and Rat-2^{Rac1N17} fibroblasts was compared. Results are expressed as percentage of control and are the means $\pm$ S.D. for three independent experiments. Statistical significance of differences was assessed using unpaired *t* tests ($P < 0.01$). (C) Rat-2 cells (2×10^6) were plated in 100-mm dishes, incubated in DMEM containing 10% FBS for 24 h, starved (0.5% FBS) for 24 h, and then exposed to 20 nM PMA for the indicated times. Cell lysates were incubated with GST-PAK-PBD coupled with glutathione-agarose beads. Bound activated GTP-bound Rac was resolved by SDS/PAGE (15% polyacrylamide) and transferred on to PVDF. Activated Rac protein was detected using an anti-Rac1 antibody and, as a control, total Rac protein was also determined by immunoblotting with Rac1 antibody. The results shown are representative of at least three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of differences was assessed using unpaired Student's *t* tests ($P < 0.05$). (D) Rat-2 cells (2×10^6) were exposed to 20 nM PMA for 10 min and cytosolic and membrane fractions were prepared as described in the Experimental section. The results shown are representative of at least three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of differences was assessed using unpaired Student's *t* tests ($P < 0.05$).

PMA activates cPLA₂ in a Rac-p38 kinase-dependent pathway

Next, we examined the signalling mechanism by which PMA mediates cPLA₂ activation. For this assay, serum-starved Rat-2 fibroblasts were exposed to various inhibitors 30 min before treatment with PMA (20 nM, 30 min), and then cPLA₂ was assayed. In agreement with the above results, SB203580 inhibited the

Figure 4 PMA activates cPLA₂ in a Rac-p38 kinase-cascade-dependent manner

(A) Rat-2 fibroblasts were preincubated for 30 min with PD98059 (10 µM), SB203580 (10 µM), EGTA (5 mM), GF109203X (5 µM), wortmannin (100 nM) or DMSO and then exposed to 20 nM PMA for 30 min. Thereafter, the cells were harvested and assayed for cPLA₂ activity. Results are expressed as means $\pm$ S.D. of control for three independent experiments. Statistical significance was assessed using unpaired Student's *t* tests ($P < 0.01$). (B) Levels of phospho-p38 kinase and total p38 kinase were examined in subconfluent Rat-2 fibroblasts exposed to 50 nM PMA (0, 5, 15, 30 and 45 min). The cells were lysed and the lysates were probed for phospho-p38 kinase and total p38 kinase. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. (C) Levels of phospho-p38 kinase and total p38 kinase were compared in subconfluent Rat-2 and Rat-2^{Rac1N17} fibroblasts exposed to 20 nM PMA (10 min). The cells were lysed and the lysates were probed for phospho-p38 kinase and total p38 kinase. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of differences was assessed using unpaired Student's *t* tests ($P < 0.05$). (D) Subconfluent Rat-2 cells were transfected with pFA2-CHOP or pFR-Luc by the calcium phosphate precipitation method, after which the transfectants were starved in DMEM containing 0.5 % FBS for 36 h and lysed. The cells were exposed to 50 nM PMA in the presence or absence of SB203580 (10 µM) for 6 h before cell harvest and treated with inhibitor for 30 min before PMA treatment. The lysates were assayed for p38 kinase activity by the CHOP-luciferase trans-reporter system (see the Experimental section for details). Results are expressed as means $\pm$ S.D. of control for three independent experiments. Statistical significance of differences was assessed using unpaired Student's *t* tests ($P < 0.01$).

activation of cPLA₂ in the PMA-stimulated Rat-2 cells, whereas PD98059 and EGTA had little effect (Figure 4A). GF109203X (5 µM), a general inhibitor of PKC isotypes, also inhibited the activation of cPLA₂ by PMA, thus supporting a role for PKC in PMA-induced cPLA₂ activation; this is consistent with the fact that PMA is a potent PKC activator [19]. Wortmannin (100 nM), a PI3K (phosphoinositide 3-kinase)-inhibitor, had no effect. To demonstrate the role of p38 kinase in the PMA signalling to cPLA₂ activation, we first examined the time course of p38 kinase phosphorylation in response to PMA treatment. As shown in Figure 4(B), p38 kinase was phosphorylated by PMA (50 nM) treatment in a time-dependent manner, reaching a maximum at 45 min. In addition, PMA treatment led to the phosphorylation of p38 kinase in wild-type Rat-2 cells but not in Rat-2^{Rac1N17} cells (Figure 4C), indicating that PMA activates p38 kinase through Rac. We have also examined whether PMA activates the p38

Figure 5 Anisomycin induces cPLA₂ activation of Rat-2 fibroblasts

(A) Levels of phospho-p38 kinase and total p38 kinase were compared in subconfluent Rat-2 and Rat-2^{Rac1N17} fibroblasts exposed to 100 ng/ml anisomycin for the indicated times (0, 5 and 15 min). The cells were lysed and the lysates were probed for phospho-p38 kinase and total p38 kinase. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$). (B) The inhibitory effects of SB203580 (10 µM), PD98059 (10 µM) or DMSO on the anisomycin-induced p38 kinase phosphorylation were examined in Rat-2 cells. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$). (C) Rat-2 fibroblasts were preincubated for 30 min with SB203580 (10 µM) or DMSO and then exposed to 100 ng/ml anisomycin for 60 min. Thereafter, the cells were harvested and assayed for cPLA₂ activity. Results are expressed as means $\pm$ S.D. of control for three independent experiments. Statistical significance was assessed using unpaired *t*-tests ($P < 0.01$).

kinase/CHOP-luciferase reporter gene activity. As shown in Figure 4(D), a significant stimulation of p38 kinase activity (~2.3-fold increase compared with the control) was observed in response to PMA (50 nM), and this activation was significantly attenuated in Rat-2^{Rac1N17} cells. Together, p38 kinase is suspected to act as a downstream mediator of cPLA₂ stimulation in response to PMA or activated Rac1.

To assess further the involvement of p38 kinase in the activation of cPLA₂, Rat-2 cells were exposed to anisomycin, a very effective p38 kinase activator [30]. As expected, anisomycin induced the activation of p38 kinase in Rat-2 fibroblasts, leading to the activation of cPLA₂ activity (Figures 5A and 5C). Interestingly, activation of p38 kinase in response to anisomycin was also observed in Rat-2^{Rac1N17} cells (Figure 5A), suggesting that anisomycin activates p38 kinase in a Rac-independent manner.

p38 kinase-linked cascade mediates the translocation of cPLA₂

A previous study has reported that, in primary murine astrocytes, PMA stimulated the phosphorylation of ERKs and cPLA₂ as well

Figure 6 Rac-p38 kinase mediates the translocation of cPLA₂

(A) Rat-2 and Rat-2^{Rac1N17} fibroblasts were starved in DMEM containing 0.5% FBS for 24 h and then exposed to 20 nM PMA or DMSO for 10 min. Cell lysates were probed for phospho-Ser⁵⁰⁵ of cPLA₂ and total cPLA₂. Additionally, the inhibitory effects of PD98059 (10 μ M) or SB203580 (10 μ M) on the PMA-induced cPLA₂ phosphorylation on Ser⁵⁰⁵ were examined in Rat-2 cells. The results shown are representative of at least three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. (B) Rat-2 fibroblasts were transiently transfected with pcDNA3 or pcDNA-HA-Rac1^{V12} (5 μ g) by the calcium phosphate precipitation method. The cells were then starved in DMEM containing 0.5% FBS for 24 h, incubated for 6 h in the presence of SB203580 (10 μ M) or DMSO and lysed. Then, cytosolic and membrane fractions were prepared and assayed for cPLA₂ translocation. Protein and β -galactosidase activities were analysed for the normalization of transfection efficiency and protein amount. The level of α -tubulin was used as a control to confirm equal amounts of proteins/lane. The results shown are representative of three independent experiments. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$). (C) Rat-2 cells were starved in DMEM containing 0.5% (v/v) FBS for 24 h, exposed to 20 nM PMA for 10 min and then lysed, after which cytosolic and membrane fractions were prepared for cPLA₂ translocation analysis as described in Experimental section. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$). (D) Rat-2 and Rat-2^{Rac1N17} fibroblasts were transfected with pEGFP-cPLA₂ by the calcium phosphate precipitation method. Cells were exposed to 20 nM PMA for 10 min and then fixed with 4% paraformaldehyde. GFP-cPLA₂ was visualized by confocal microscopy as described in Experimental section. (E) Rat-2 fibroblasts were transfected with pEGFP-cPLA₂ by the calcium phosphate precipitation method. The cells were then starved in DMEM containing 0.5% FBS for 36 h, preincubated for 30 min in the presence of SB203580 (10 μ M) or DMSO and then exposed to 20 nM PMA for 10 min. The cells were fixed with 4% paraformaldehyde and GFP-cPLA₂ was visualized as described in the Experimental section. (F) Rat-2 cells were starved in DMEM containing 0.5% FBS for 24 h, exposed to 50 ng/ml anisomycin for 15 min in the presence or absence of SB203580 (10 μ M) and then lysed, after which cytosolic and membrane fractions were prepared for cPLA₂ translocation analysis as described in the Experimental section. Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health) and expressed in terms of fold increase of control for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's *t* tests ($P < 0.05$).

as evoked AA release and that, however, complete inhibition of phospho-ERK by U0126, an inhibitor of MAPK/ERK kinase, did not completely inhibit PMA-stimulated cPLA₂ and AA release, suggesting that phosphorylation of cPLA₂ due to phospho-ERK is not sufficient to evoke AA release [31]. Therefore, similar to that report, we undertook to test whether Rac-p38 kinase-mediated activation of cPLA₂ is not enhanced by phosphorylation of cPLA₂ through ERKs in the PMA signalling to cPLA₂ activation.

We therefore examined the phosphorylation of cPLA₂ on Ser⁵⁰⁵ in subconfluent Rat-2 and Rat-2^{Rac1N17} fibroblasts using an antibody to Ser⁵⁰⁵-phosphorylated cPLA₂ [32–34]. As shown in Figure 6(A), although PMA caused a significant phosphorylation of cPLA₂ on Ser⁵⁰⁵, this phosphorylation on Ser⁵⁰⁵ by PMA was not inhibited by Rac1^{N17}, suggesting that the Rac-linked pathway is independent of the phosphorylation mediated by ERKs. Similarly, phosphorylation on Ser⁵⁰⁵ was not blocked by pretreatment with

Scheme 1 A hypothetical model for the role of the Rac-p38 kinase cascade in the activation of cPLA₂

SB203580, although PD98059 completely decreased the cPLA₂ phosphorylation by PMA (Figure 6A). Consistent with these results, transient expression of Rac1^{V12} did not show any enhanced level of Ser⁵⁰⁵ phosphorylation (results not shown). Together, these and earlier results (Figures 1C and 4A) suggest that PMA-Rac-p38 kinase-mediated cPLA₂ activation does not involve ERK-mediated phosphorylation of cPLA₂ on Ser⁵⁰⁵, at least in Rat-2 cells.

In a further approach to determine the signalling mechanism by which the Rac-p38 kinase cascade mediates cPLA₂ activation in response to PMA, we next examined whether cPLA₂ translocation is affected by this cascade or not. By subcellular fractionation of lysates, Rac-induced cPLA₂ translocation was significantly inhibited by SB203580 (Figure 6B) and, similarly, PMA was shown to induce the translocation of cPLA₂ from the cytosol to the membrane fraction in a Rac-dependent manner, and this was further confirmed by confocal microscopy of GFP-cPLA₂ in Rat-2 fibroblasts (Figures 6C and 6D). Clearly, translocation of cPLA₂ was inhibited in Rat-2^{Rac1N17} cells, suggesting a mediatory role for the Rac-p38 kinase cascade in the translocation of cPLA₂. By pretreatment with SB203580 (Figure 6E), PMA-induced cPLA₂ translocation was completely blocked, suggesting the involvement of p38 kinase in the translocation of cPLA₂ in response to PMA.

In addition, anisomycin caused cPLA₂ to be translocated to the membrane fraction from the cytosolic fraction (Figure 6F). Therefore we propose a hypothetical model, and, in this model, the Rac-p38 kinase cascade mediates the translocation of cPLA₂ and this pathway does not involve ERK-dependent phosphorylation of cPLA₂ on Ser⁵⁰⁵ in Rat-2 fibroblasts (Scheme 1).

DISCUSSION

The present study showed that Rac1 stimulates cPLA₂ by a signalling pathway in a p38 kinase-dependent manner. The activation of cPLA₂ after PMA stimulation or Rac1^{V12} overexpression was completely inhibited by SB203580, but not by PD98059 (Figures 1C and 4A), suggesting that Rac1 stimulates cPLA₂ by an ERK-independent pathway. An essential role of Rac1 was demonstrated by comparing the effects of Rac1^{V12} overexpression in Rat-2 fibroblasts, and the effect of PMA on cPLA₂ activation

in Rat-2 and Rat-2^{Rac1N17} cells (Figures 1 and 3). These findings support our previous results showing that Rac liberates AA by a cPLA₂-dependent pathway [15–18]. Activation of Rac1 by PMA was further confirmed by the fact that it is rapidly translocated from the cytosol to the membrane fraction when cells are exposed to PMA (Figure 3D).

Although PMA caused the phosphorylation of cPLA₂ on Ser⁵⁰⁵ [32–34], the phosphorylation on Ser⁵⁰⁵ by PMA was not inhibited by Rac1^{N17} (Figure 6A), suggesting that the Rac-linked pathway is independent of ERK-mediated phosphorylation of cPLA₂. Similarly, phosphorylation on Ser⁵⁰⁵ was not blocked by pretreatment with SB203580, although PD98059 completely decreased cPLA₂ phosphorylation by PMA (Figure 6A). While p38 kinase is involved in the phosphorylation of cPLA₂ on Ser⁵⁰⁵ in thrombin-stimulated platelets [7], cPLA₂ is also phosphorylated by p38 kinase on four residues, namely Ser⁴³⁷, Ser⁴⁵⁴, Ser⁵⁰⁵ and Ser⁷²⁷ [10,31–34], and thus it remains to be determined whether p38 kinase causes phosphorylation on any of the other serine residues under our experimental conditions. For instance, p38 kinase has been reported to lead to phosphorylation of cPLA₂ on Ser⁷²⁷ through MAPK-interacting kinase 1 in Chinese-hamster ovary and HEK-293 cells (human embryonic kidney 293 cells) [32]. Interestingly, a significant fraction of cPLA₂ is constitutively phosphorylated on Ser⁵⁰⁵ in Sf9 cells, yet AA is not released unless the cells are treated with okadaic acid or agonists that increase intracellular Ca²⁺ [32]. Previously, we reported that Rac1-ERK signalling to cPLA₂ is implicated in LTB₄ (leukotriene B₄)-induced ROS (reactive oxygen species) generation for chemotaxis [35]. Thus, depending on the agonists, ERKs or p38 kinase may be involved in cPLA₂ regulation and in fact, for an LTB₄-induced cPLA₂ stimulation, cPLA₂ was assumed to act in a positive feedback loop (Rac-ERK-cPLA₂-AA-NADPH oxidase cascade) to modulate NADPH oxidase to mediate ROS generation. Therefore an ERK-cPLA₂-linked cascade does not constitute the main signalling route to ROS generation in LTB₄ signalling. In contrast, for PMA, the Rac-p38 kinase-cascade is the main signalling mediator of the PMA-induced signalling leading to cPLA₂ stimulation, regulating the formation of eicosanoids. Thus we suspect that p38 kinase acts as a general downstream mediator of Rac in cPLA₂ stimulation in response to a variety of agonists acting through Rac. In any event, from these reports and our present results, we conclude that the role of Ser⁵⁰⁵ phosphorylation in the regulation of cPLA₂ is cell-type- and agonist-dependent and that it is not essential for the Rac-p38 kinase-stimulated cPLA₂ activation in Rat-2 fibroblasts.

We were then interested in the translocation of cPLA₂, the other major mechanism of cPLA₂ activation, and found that PMA caused the translocation of cPLA₂, especially to the perinuclear membrane region, through the p38 kinase-linked cascade. This was further supported by the effect of anisomycin on cPLA₂ translocation in Rat-2 fibroblasts. Recently, Evans et al. [36,37] reported that the [Ca²⁺]_i level controls the targeting of cPLA₂ to intracellular membranes such as the Golgi, endoplasmic reticulum and perinuclear membrane. According to Evans et al., cPLA₂ translocation to the perinuclear membrane region requires high [Ca²⁺]_i. However, it has been shown that PMA provides cPLA₂-activating signals without inducing Ca²⁺ influx [20–22]. This has led to the suggestion that, under certain conditions, cPLA₂ activity may be modulated independent of Ca²⁺ mobilization in some cell types [19]; it remains to be elucidated whether this is the case for Rac-p38 kinase-dependent cPLA₂ translocation in Rat-2 fibroblasts. Although removal of extracellular free-Ca²⁺ with EGTA did not inhibit PMA- or Rac1-induced activation of cPLA₂ (Figures 1C and 4A), the effect of Ca²⁺ in the translocation of cPLA₂ by the Rac-p38 kinase cascade is still not clear.

The detailed mechanism by which the Rac-p38 kinase cascade mediates cPLA₂ stimulation in response to PMA remains to be characterized, especially regarding the downstream targets of p38 kinase.

Another potential mechanism by which Rac mediates cPLA₂ activation has been previously reported. For example, Dennis and co-workers [38,39] reported that cPLA₂ could be activated by direct binding to phosphatidylinositol 4,5-bisphosphate, a product of PI4K (phosphoinositide 4-kinase), which is activated by Rac1 [38,39]. In addition, polyphosphoinositides decreased the Ca²⁺ requirement of cPLA₂ such that, under certain conditions, its activity was truly Ca²⁺-independent [24]. However, we have shown that PMA/Rac1-mediated cPLA₂ activation in Rat-2 fibroblasts is independent of PI4K, since translocation of cPLA₂ was not blocked by 2,3-dihydroxy-benzaldehyde, a PI4K inhibitor (results not shown) [40,41]. The involvement of PI3K, another molecule closely related to PI4K, in the PMA-mediated cPLA₂ activation, was also excluded by the fact that wortmannin did not attenuate cPLA₂ activation in response to PMA (Figure 4A). The C2 domain of cPLA₂ is known to be involved in the preferential translocation of cPLA₂ to the nuclear region [5,6,36]. However, we found that PMA induced the translocation of full-length cPLA₂ but not the C2 domain alone (results not shown). Our results thus suggest that a region other than C2 is required, depending on the agonists, for the translocation of cPLA₂.

Taken together, our findings indicate that Rac1 induces cPLA₂ activation in Rat-2 fibroblasts through a novel mechanism involving a p38 kinase. Although the downstream targets of p38 kinase still need to be identified, our present results suggest that the Rac-p38 kinase cascade acts by mediating the translocation of cPLA₂. Further characterization of the mechanism of cPLA₂ activation by the Rac-p38 kinase cascade would be a pivotal step towards a better understanding of the regulatory mechanism leading to cPLA₂ activation.

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