Involvement of cytosolic phospholipase A_2, and the subsequent release of arachidonic acid, in signalling by Rac for the generation of intracellular reactive oxygen species in Rat-2 fibroblasts

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Although there have been a number of recent studies on the role of Rac in the generation of reactive oxygen species (ROS), details of the signalling pathway remain unclear. In the present study we analysed the extent to which the activation of cytosolic phospholipase A_2 and the resultant release of arachidonic acid (AA) are involved in the Rac-mediated generation of ROS. Transfection of Rat-2 cells with RacV12, a constitutively active form of Rac1, induced elevated levels of ROS, as reflected by increased H_2O_2-sensitive fluorescence of 2′,7′-dichlorofluorescein. These effects could be blocked by inhibiting phospholipase A_2 or 5-lipoxygenase but not by inhibiting cyclo-oxygenase. The application of exogenous AA increased levels of ROS but the effect was dependent on the further metabolism of AA to leukotrienes $C_4/D_4/E_4$ by 5-lipoxygenase. Indeed, the exogenous application of a mixture of leukotrienes $C_4/D_4/E_4$ elicited transient elevations in the levels of ROS that were blocked by catalase. These findings indicate that phospholipase A_2 and subsequent AA metabolism by 5-lipoxygenase act as downstream mediators in a Rac signalling pathway leading to the generation of ROS.

Key words: hydrogen peroxide, leukotrienes, 5-lipoxygenase, signal transduction.

INTRODUCTION

Rac, a member of the Rho family of small GTPases, mediates such cellular functions as the reorganization of the actin cytoskeleton, cell growth and transformation, cell motility and migration, and response to stress [1–6]. In addition, Rac was recently shown to serve as a regulator of the intracellular redox state of non-phagocytic cells [7–10]. Therefore, after ligand-evoked activation, Rac might function to regulate the levels of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and H_2O_2. For example, Rac1 seems to mediate increases in intracellular ROS in NIH 3T3 cells exposed to tumour necrosis factor α or interleukin 1β [9]. However, the downstream pathway leading from Rac1 activity to ROS generation remains poorly characterized, especially in fibroblasts.

It has been shown that, when activated, Rac in turn activates cytosolic phospholipase A_2 ($cPLA_2$), and there is a resultant release of arachidonic acid (AA), a principal product of $cPLA_2$ activity [11,12]. This makes it likely that $cPLA_2$ is a major downstream mediator of Rac signalling. For instance, Rac-induced release of AA has been shown to be a component of the biochemical pathways by which Rac stimulates both Ca^{2+} influx and Rho-dependent cytoskeletal organization in fibroblasts [11]. In addition, we observed that in response to tumour necrosis factor α or exogenously applied ceramide (a proposed lipid second messenger generated by sphingomyelin hydrolysis in response to various stresses and proinflammatory cytokines [13,14]), a Rac–$cPLA_2$–AA cascade mediates c-*fos* serum response element (SRE) activation and nuclear signalling [15,16].

To improve our understanding of the molecular mechanism responsible, in the present study we assessed the respective roles of $cPLA_2$, AA and 5-lipoxygenase (5-LO) in the Rac-mediated generation of ROS. Here we show that $cPLA_2$ and subsequent AA metabolism by 5-LO are involved in a Rac signalling pathway leading to the generation of ROS.

MATERIALS AND METHODS

Chemicals and plasmids

2′,7′-Dichlorofluorescein diacetate (DCFDA) was obtained from Molecular Probes (Eugene, OR, U.S.A.); AA, MK-886, indomethacin, AA-861 and arachidonyltrifluoromethyl ketone ($AACOCF_3$) were from Biomol (Plymouth Meeting, PA, U.S.A.); leukotrienes and *N*-acetylcysteine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); anti-$cPLA_2$ antibody was from Santa Cruz (Santa Cruz, CA, U.S.A.); and Dulbecco's modified Eagle's medium (DMEM), Phenol Red-free DMEM, non-essential amino acids and fetal bovine serum (FBS) were from Gibco-BRL (Gaithersburg, MD, U.S.A.). All other chemicals were from standard sources and were of molecular biology grade or higher. pEXV, a Myc-tagged expression vector, and pEXV-RacV12, an expression vector encoding RacV12 (a constitutively activated form of Rac1), were gifts from Dr Alan Hall (University College London, London, U.K.).

Cell culture, transfections and luciferase assay

Rat-2 fibroblasts were obtained from the American Type Culture Collection (A. T. C. C., Manassas, VA, U.S.A.) (CRL 1764) and maintained in DMEM supplemented with 0.1 mM non-essential amino acids, 10% (v/v) FBS and penicillin (50 i.u./ml)/streptomycin (50 μg/ml) (Gibco-BRL) at 37 °C under a humidified air/CO_2 (19:1) atmosphere. Rat2-RacV12 cells were prepared by transfecting Rat-2 cells with pEXV-RacV12 and a

Abbreviations used: AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; $cPLA_2$, cytosolic phospholipase A_2; DCF, 2′,7′-dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; 5-LO, 5-lipoxygenase; ROS, reactive oxygen species; SRE, serum response element.

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neomycin resistance vector (pSV-Neo), after which clones were selected for 2 weeks in the presence of G418 antibiotic (0.4 mg/ml), as described previously [17]. Expression of RacV12 protein by the transfectants was confirmed by Western blot analysis with an anti-Myc epitope antibody as a probe. As a control, Rat-2 cells were transfected with the empty pEXV vector along with the neomycin resistance vector.

Transient transfection was performed by plating approx. 5×10^5 cells in 100 mm dishes for 24 h and then adding calcium phosphate/DNA precipitates prepared with 20 μg of DNA per dish. To control for variations in cell number and transfection efficiency, all clones were co-transfected with 1 μg of pCMV-βGAL, a eukaryotic expression vector containing the *Escherichia coli* β-galactosidase (*lacZ*) structural gene under the transcriptional control of the CMV promoter. In each transfection, the quantity of DNA used was held constant at 20 μg by the addition of sonicated calf thymus DNA (Sigma). After incubation for 6 h with the calcium phosphate/DNA precipitates, the cells were rinsed twice with PBS before incubation in fresh DMEM supplemented with 0.5 % (v/v) FBS. Each dish of cells was then rinsed twice with PBS and lysed in 0.2 ml of lysis solution [0.2 M Tris/HCl (pH 7.6)/0.1 % (v/v) Triton X-100], after which the lysed cells were scraped and centrifuged for 1 min. Supernatants were assayed for protein concentration and β-galactosidase activity. Luciferase activity was assayed with 10 μl of extract in accordance with the manufacturer's protocol (Promega Luciferase Assay System; Promega, Madison, WI, U.S.A.) and counted for 10 s in a Beckman liquid-scintillation spectrometer, using the tritium channel with the coincidence circuit disconnected. Transfection experiments were performed in triplicate with two independently isolated sets of cells; the results were averaged [16].

cPLA$_2$ translocation by RacV12 transfection

Rat-2 cells transiently transfected with pEXV-RacV12 were serum-starved for 24 h in DMEM containing 0.5 % (v/v) FBS. The medium was then 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 Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4/2.5 mM EDTA/1 mM dithiothreitol/0.1 mM PMSF/10 μg/ml leupeptin (pH 7.5)]. The resuspended cells were then mechanically lysed on ice by trituration with a 21.1-gauge needle. To prepare cytosolic and total particulate fractions, the lysates were first centrifuged at 100000 ***g*** for 1 h. The supernatants (cytosolic fraction) were then precipitated with 5 vol. of acetone, incubated for 5 min on ice and centrifuged at 14000 rev./min for 20 min at 4 °C. The resultant pellet was resuspended in buffer A containing 1 % (v/v) Triton X-100. The particulate fractions, which contained the membrane fraction, were washed twice and resuspended in 50 μl of buffer A containing 1 % (v/v) Triton X-100. The protein in each fraction was quantified with a Bradford procedure with 1 μl of extract, 799 μl of water and 200 μl of Bio-Rad Dye Reagent. Aliquots of protein sample (20 μl containing approx. 40 μg of protein) were then loaded on polyacrylamide gels for subsequent immunoblot assay for cPLA$_2$.

Leukotriene $C_4/D_4/E_4$ assays

Cells were seeded at 3×10^5 per 60 mm dish in DMEM containing 10 % (v/v) FBS for 24 h. Cells were then rinsed twice with cold PBS and mixed with 4 vol. of ethanol and left at 4 °C for 30 min. The resulting precipitate was removed by centrifugation at 15000 ***g*** (10000 rev./min) for 30 min at 4 °C. The ethanolic supernatant containing leukotrienes was collected. The ethanol was then removed by evaporation under vacuum and the samples were stored under argon at −50 °C before assay for the leukotriene $C_4/D_4/E_4$ levels as described in the Leukotriene $C_4/D_4/E_4$ enzyme immunoassay system (RPN 224; Amersham Pharmacia Biotech).

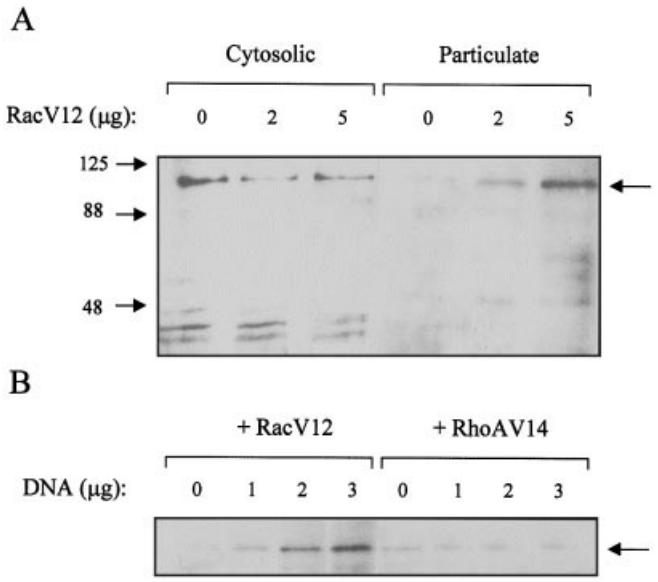

Figure 1 cPLA$_2$ translocation evoked by transfection with RacV12

(**A**) Western blot illustrating the shift in the anti-cPLA$_2$-reactive band (arrowed at the right) from the cytosolic fraction to the particulate fraction in Rat-2 cells transiently transfected with 0, 2 or 5 μg of the RacV12 expression vector, pEXV-RacV12. The quantity of transfected vector was kept constant by adding decreasing amounts of pEXV. Cells were then serum-starved for 24 h in DMEM containing 0.5 % (v/v) FBS, after which the cytosolic and particulate fractions were prepared as described in the Materials and methods section. The positions of molecular mass markers are indicated (in kDa) at the left. (**B**) Western blot performed as described for (**A**) and showing the effect of transiently transfecting Rat-2 cells with 0, 1, 2 or 3 μg of pEXV-RacV12 or pEXV-RhoV14 (a RhoV14 expression vector encoding a constitutively activated form of RhoA) on cPLA$_2$ translocation to the particulate fraction. Note that, transfection with RacV12 but not RhoAV14 evoked cPLA$_2$ translocation (arrowed at the right). Results are representative of three independent experiments.

Measurement of intracellular H_2O_2

Intracellular H_2O_2 was measured as a function of 2′,7′-dichlorofluorescein (DCF) fluorescence by using the procedures of Ohba et al. [18]. In brief, cells were grown on coverslips for 2 days and then serum-starved in DMEM containing 0.5 % (v/v) FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM without Phenol Red for at least 30 min before exposure to agonists (AA or leukotrienes) for the indicated durations. When the effects of inhibitors were being assessed, cells were pretreated with the respective inhibitor for 30 min. To measure intracellular H_2O_2, cells were then incubated for 10 min with the H_2O_2-sensitive fluorophore DCFDA (5 μg/ml), which, when taken up, fluorescently labels intracellular H_2O_2 with DCF. The cells were then immediately observed under a laser-scanning confocal microscope (Carl Zeiss LSM 410). DCF-labelled H_2O_2 was excited by a 488 nm argon laser, and the evoked emission was filtered with a 515 nm long-pass filter. DCF fluorescence was measured in 30 randomly selected cells. Values are means ± S.D. for DCF fluorescence intensity in three independent experiments.

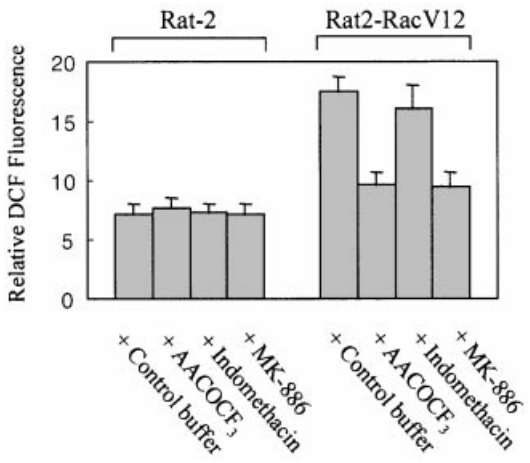

Figure 2 Role of $cPLA_2$ in RacV12-mediated generation of ROS

Serum-starved Rat-2 and Rat2-RacV12 stable cells were pretreated for 30 min with control buffer, $AACOCF_3$ (10 μM), indomethacin (10 μM) or MK886 (50 nM) before the measurement of levels of fluorescence. The relative intensities of H_2O_2-sensitive DCF fluorescence (arbitrary units), which served as an index of the levels of ROS, were measured in DCFDA-loaded cells. Results are means $\pm$ S.D. ($n = 30$ cells).

RESULTS

Rac-induced translocation of $cPLA_2$

We have previously shown that transiently transfecting cells with the constitutively active RacV12 induced a dose-dependent release of AA that was completely abolished by pretreatment by mepacrine, a potent inhibitor of PLA_2 [12]. In the present study the activation of $cPLA_2$ by Rac was further characterized in Rat-2 fibroblasts by examining the effect of RacV12 expression on $cPLA_2$ translocation [19,20] (Figure 1). Transient transfection of Rat-2 fibroblasts with 0, 2 or 5 μg of pEXV-RacV12 elicited rapid and dose-dependent translocation of $cPLA_2$ (molecular mass approx. 100 kDa) from the cytosolic fraction to the particulate fraction (Figure 1A). As an example, transfection with 5 μg of pEXV-RacV12 elicited an approx. 4.8-fold increase in the size of the particulate $cPLA_2$ fraction and a concomitant decline in the cytosolic fraction, which is indicative of the activation of $cPLA_2$ by RacV12. In contrast, there was no detectable translocation in response to transfection of pEXV-RhoV14 (0, 1, 2 and 3 μg), an expression vector encoding a constitutively activated form of RhoA (Figure 1B). These results are therefore in good agreement with the proposed role of $cPLA_2$ as one of the signalling mediators downstream of Rac.

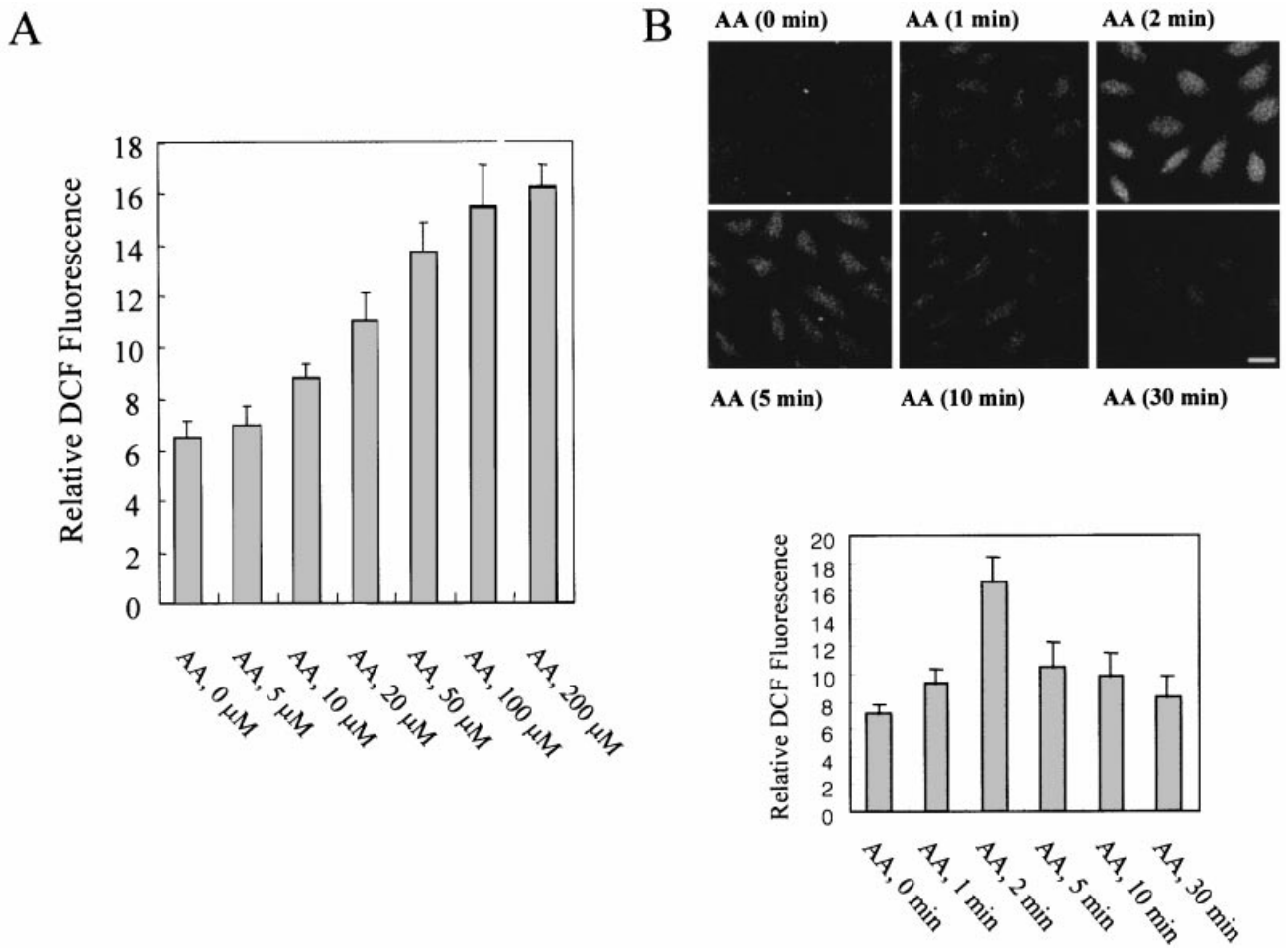

Figure 3 Activation of ROS by exogenous AA in a dose- and time-dependent manner

Rat-2 cells were serum-starved for 2 days and then stimulated with either AA at the indicated doses (0, 5, 10, 20, 50, 100 and 200 μM) for 2 min (**A**) or AA (100 μM) for the indicated durations (0, 1, 2, 5, 10 and 30 min) (**B**). DFC fluorescence intensities reflecting the relative levels of ROS (arbitrary units) were imaged with a confocal laser scanning fluorescence microscope and quantified. Results are means $\pm$ S.D. ($n = 30$ cells). Scale bar, 30 μm.

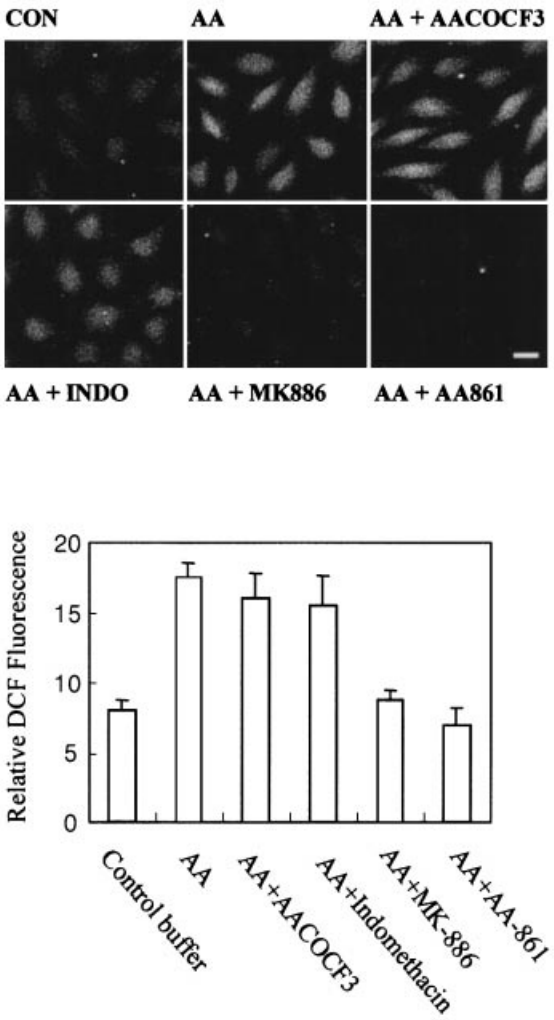

Figure 4 Effect of MK-886 and AA-861 on the AA-induced generation of ROS

Serum-starved Rat-2 cells were exposed to 100 μM AA for 2 min in the presence or absence of $AACOCF_3$ (10 μM), indomethacin (10 μM), AA-861 (1 μM) and/or MK-886 (50 nM), as indicated. Inhibitors were added 30 min before the addition of AA. DFC fluorescence was imaged and quantified as in Figure 3. Scale bar, 30 μm. Results are means $\pm$ S.D. ($n = 30$ cells).

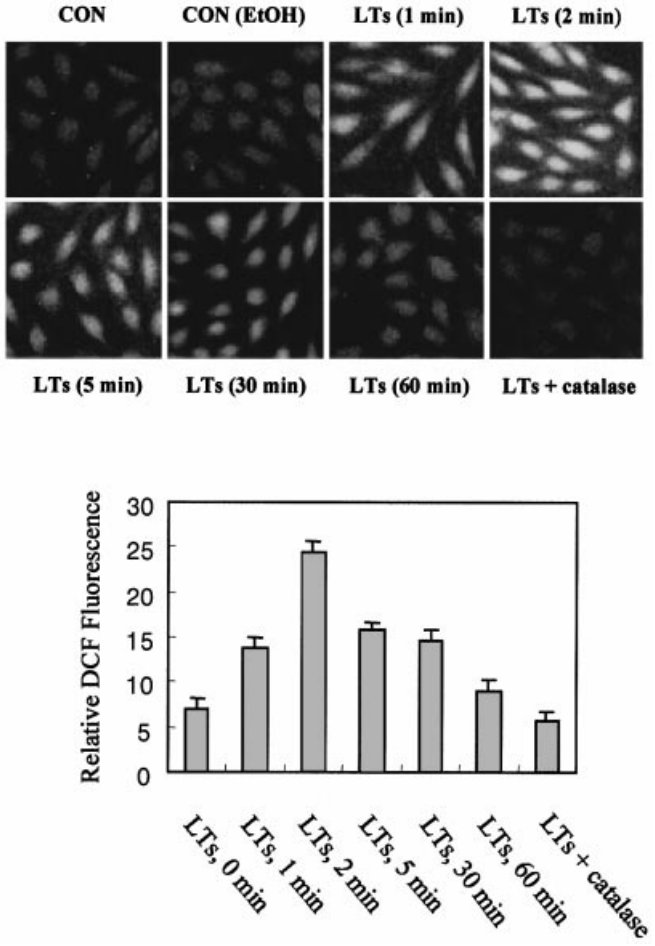

Figure 5 Application of exogenous leukotrienes induces the generation of ROS

Serum-starved Rat-2 cells were exposed to mixture of leukotrienes $C_4/D_4/E_4$ (LTs; 200 nM mixture) for the indicated durations (0, 1, 2, 5, 30 and 60 min). In some cases, cells were exposed to the mixture of leukotrienes for 2 min in the presence of catalase (10 units). DCF fluorescence was imaged and quantified as in Figure 3. Results are means $\pm$ S.D. ($n = 30$ cells).

Effect of $cPLA_2$ inhibition on the generation of ROS in RacV12 cells

One potential mechanism by which Rac might regulate the generation of ROS is via the $cPLA_2$–AA cascade. Therefore, by analysing images of H_2O_2-sensitive DCF fluorescence, we assessed the extent to which pretreatment with specific inhibitors of $cPLA_2$ or AA metabolism attenuate Rac-mediated ROS generation. In comparison with Rat-2 controls, Rat2-RacV12 cells, stably expressing RacV12, showed significantly increased basal DCF fluorescence (Figure 2). Pretreatment with 10 μM $AACOCF_3$, a specific $cPLA_2$ inhibitor, almost completely blocked ROS generation in Rat2-RacV12 cells (more than 90% inhibition). Similar effects were exerted by MK-886, a specific 5-LO inhibitor, suggesting that $cPLA_2$ activity and the subsequent metabolism of AA by 5-LO are involved in the Rac signalling leading to ROS generation. In contrast, levels of ROS were unaffected by 10 μM indomethacin, a non-specific cyclo-oxygenase inhibitor. Consistent with these results was the observation that Rat2-RacV12 cells generated a significantly enhanced level of leukotrienes, products of 5-LO, in comparison with control Rat-2 cells. For example, the level of leukotrienes $C_4/D_4/E_4$ generated in RacV12 cells was approx. 2-fold higher than that in control cells (Table 1).

Table 1 Enhanced generation of leukotrienes $C_4/D_4/E_4$ by RacV12

Rat-2, Rat2-RacN17 (stable cells expressing RacN17, a dominant-negative mutant of Rac1 [17]) and Rat2-RacV12 cells were grown for 24 h in DMEM containing 10% (v/v) FBS and then harvested for the analysis of intracellular levels of leukotrienes $C_4/D_4/E_4$ mixture as described in the Materials and methods section. Results are averages of three independent experiments.

Cell type	Concentration of leukotrienes $C_4/D_4/E_4$ (pg/ml)
Rat-2	16 ± 4
Rat2-RacN17	12 ± 5
Rat2-RacV12	32 ± 3

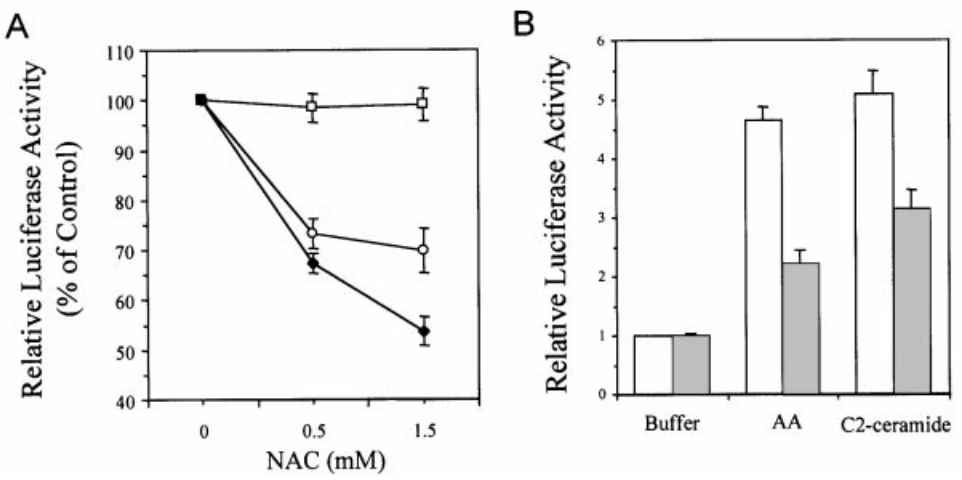

Figure 6 Essential role of ROS in the activation of SRE by C2-ceramide and AA

(**A**) Rat-2 cells were transiently transfected with 3 μg of pSRE-Luc reporter plasmid and then serum-starved for 36 h. Thereafter, cells were incubated for 30 min with the indicated concentrations of *N*-acetylcysteine (NAC) (0.5 and 1.5 mM) prior to incubation for 1 h with buffer (control) (□), C2-ceramide (5 μM) (○) or AA (100 μM) (◆). Cells were then harvested and relative luciferase activity was assessed. Results are expressed as percentages of control. The results shown are representative of at least three independent transfections. (**B**) Rat-2 cells were transiently co-transfected with 3 μg of pRc (vector) (open bars) or pRc-superoxide dismutase (SOD) (filled bars) along with 3 μg of pSRE-Luc reporter plasmid. DNA sample size was held at 20 μg by the addition of calf thymus carrier DNA. Transfectants were serum-starved for 36 h then incubated with buffer (control), AA (100 μM) or C2-ceramide (5 μM) for 1 h after which they were harvested and relative luciferase activity was assessed. Results are expressed as percentages of control. The results shown are representative of at least three independent transfections.

Application of exogenous AA generates ROS via 5-LO-dependent pathway

The foregoing results encouraged us to examine in more detail the role of $cPLA_2$ in the Rac-mediated generation of ROS. To do this, we first tested whether exogenous AA could stimulate ROS generation. After serum starvation for 48 h DCDFA-loaded Rat-2 cells were exposed to AA at the indicated doses (0, 5, 10, 20, 50, 100 and 200 μM) and for the indicated durations (0, 1, 2, 5, 10 and 30 min). Consistent with the proposed role of $cPLA_2$ as a downstream mediator of Rac in ROS generation, the application of exogenous AA significantly increased DCF fluorescence intensities in dose- and time-dependent ways, reflecting increases in the levels of ROS (Figure 3). For example, maximum ROS generation (approx. 2.4-fold increase over control) was observed within 2 min of exposure; levels declined thereafter.

The aforementioned effect of MK-886 in Rat2-RacV12 cells suggests that 5-LO activity is required for Rac-mediated ROS generation. To investigate the role of 5-LO further, we tested the effect of MK-886 and AA-861, two unrelated inhibitors of 5-LO, on ROS generation elicited by AA. As shown in Figure 4, each of the inhibitors almost entirely abolished the AA-induced generation of ROS (more than 90% inhibition). In contrast, neither $AACOCF_3$ nor indomethacin affected AA-induced ROS generation.

Exogenous leukotrienes $C_4/D_4/E_4$ stimulate ROS generation

To demonstrate further the involvement of 5-LO in ROS generation, we then assessed the effect of leukotrienes $C_4/D_4/E_4$, products of AA metabolism by 5-LO, on levels of ROS. As shown in Figure 5, the exogenous application of a mixture of leukotrienes $C_4/D_4/E_4$ (200 nM) to DCFDA-loaded Rat-2 cells induced an approx. 3.5-fold increase in the levels of ROS within 2 min. Leukotriene-evoked ROS generation was not inhibited by pretreatment with either MK-886 or AA-861 (results not shown), although the addition of catalase (10 units), an oxygen radical scavenger, completely abolished the response. Thus 5-LO, but not cyclo-oxygenase, seems to have a critical role in mediating ROS generation via the Rac–AA cascade.

DISCUSSION

In the present study, the crucial role played by the $cPLA_2$–AA-linked cascade in the signalling cascade leading from Rac activation to the generation of ROS was demonstrated by using three approaches: first, pretreatment with $AACOCF_3$ was shown to block RacV12-induced ROS generation (Figure 2); secondly, exogenous AA was shown to increase levels of ROS significantly within 2 min of application (Figure 3); and thirdly, the metabolism of AA by 5-LO, yielding leukotrienes $C_4/D_4/E_4$, was also shown to be essential for the generation of ROS (Figure 4). The role of 5-LO in mediating ROS generation was further demonstrated by a significant increase in the ROS level after the direct addition of leukotrienes $C_4/D_4/E_4$ to the cells (Figure 5). Our proposed mode of Rac activation of ROS via the '$cPLA_2$–AA' signalling cascade seems very similar to that of c-*fos* SRE activation [12]. In c-*fos* SRE activation by Rac, essential roles of $cPLA_2$ activation and subsequent AA production have already been demonstrated [12]. Additionally, in a previous study we reported that 5-LO has a critical role as a downstream mediator of 'Rac–$cPLA_2$–AA' signalling to stimulate c-*fos* SRE [12]. Thus, the $cPLA_2$–AA signalling cascade is apparently common to the Rac-mediated regulation of both ROS and SRE. To study whether there is any signalling link between ROS generation and c-*fos* SRE activation, we have tested whether ROS generation is necessary for SRE activation by AA or C2-ceramide, which has previously been shown to activate SRE via the Rac–$cPLA_2$-dependent pathway [16]. As shown in Figure 6(A), pretreatment with 0.5 mM *N*-acetylcysteine, a free-radical scavenger, significantly inhibited the SRE stimulation by C2-ceramide or exogenous AA. In addition, co-transfection with an expression

plasmid of superoxide dismutase markedly diminished the activation of SRE by these agonists (Figure 6B). Together, these results suggest that the ROS generated via the Rac–$cPLA_2$–AA-linked cascade is crucial for the nuclear signalling cascade leading to c-*fos* SRE activation, in response to various agonists acting through Rac. Recently, Shin et al. [21] reported that AA-induced stress-activated protein kinase ('SAPK') activation and ROS generation were partly prevented by RacN17, thus indicating that AA might act upstream of Rac for the generation of ROS. Their results are quite in contrast with previous reports of ours and others [11,12,15,16]; the reason for this discrepancy is not clear. Interestingly, we could not reproduce the results as published [21] and we therefore suspect that the experimental conditions might have been different, or there could have been a positive feedback loop regulation by AA acting on Rac, as claimed by the same authors [21]. However, we predict that the role of feedback regulation would be minimal even if there were any. In any event, our present findings make us confident that $cPLA_2$ is essential for generating ROS in response to Rac activation. In support of our observation, there is an increasing amount of previous evidence supporting a signalling link between ROS and $cPLA_2$-mediated AA metabolism [22–25]. In summary, the results presented are indicative of the crucial role of $cPLA_2$ in the regulation of the Rac-mediated generation of ROS. Although we do not know the details of the downstream mediators of $cPLA_2$ in a Rac signalling pathway leading to the generation of ROS, our findings indicate that AA and subsequent AA metabolism by 5-LO are involved. Further studies should provide additional information about the specific steps linking 5-LO-catalysed leukotriene synthesis with ROS generation.

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