Role of cytosolic phospholipase A_2 in arachidonic acid release of rat-liver macrophages: regulation by Ca^{2+} and phosphorylation

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In this study we have verified the existence of a cytosolic phospholipase A_2 ($cPLA_2$) in rat-liver macrophages. Stimulation of these cells with phorbol 12-myristate 13-acetate (PMA), zymosan and lipopolysaccharide (LPS), but not with the Ca^{2+}-ionophore A23187, leads to phosphorylation of $cPLA_2$ and activation of mitogen-activated protein (MAP) kinase, supporting the hypothesis that MAP kinase is involved in $cPLA_2$ phosphorylation. We show furthermore, that the tyrosine kinase inhibitor genistein prevents the LPS- but not the PMA- or zymosan-induced phosphorylation of $cPLA_2$ and activation of MAP kinase, indicating that tyrosine kinases participate in LPS- but not in PMA- and zymosan-induced $cPLA_2$ phosphorylation and MAP kinase activation. Phosphorylation of $cPLA_2$ does not strongly correlate with stimulation of the arachidonic acid (AA) cascade: (1) A23187, a potent stimulator of AA release, fails to induce $cPLA_2$ phosphorylation; (2) withdrawal of extracellular Ca^{2+}, which inhibits PMA-stimulated AA release (Dieter, Schulze-Specking and Decker (1988) Eur. J. Biochem. **177**, 61–67), has no effect on PMA-induced phosphorylation of $cPLA_2$; (3) LPS induces $cPLA_2$ phosphorylation within minutes, whereas increased AA release upon treatment with LPS is detectable for the first time after 4 h; and (4) genistein, which prevents LPS-induced $cPLA_2$ phosphorylation, does not inhibit AA release in response to LPS. From these data we suggest that a rise in intracellular Ca^{2+}, but not phosphorylation of $cPLA_2$, is essential for activation of the AA cascade in rat-liver macrophages.

INTRODUCTION

Rat-liver macrophages, activated with a variety of agents, elicit a number of responses, including production and release of prostanoids which are potent lipid mediators of inflammation [1–4]. Activation of the arachidonic acid (AA) cascade can be obtained in the short-term (within minutes) after treatment with phorbol 12-myristate 13-acetate (PMA), zymosan or Ca^{2+} ionophore [1]. The synthesis of prostanoids has an obligate requirement for Ca^{2+}, since omission of extracellular Ca^{2+} from rat-liver macrophages has been shown to decrease agonist-elicited prostaglandin E_2 production [5]. It should be noted that an increase in intracellular free Ca^{2+} only occurs after treatment with Ca^{2+} ionophore and zymosan but not in response to PMA [6]. We have previously demonstrated that the PMA- and zymosan-induced release of prostanoids depends, besides the presence of extracellular Ca^{2+}, on the activation of protein kinase C (PKC), whereas the generation of prostanoids in response to the Ca^{2+} ionophore is independent of PKC [7–9]. In the long-term (within hours), the AA cascade in rat-liver macrophages can be activated with lipopolysaccharide (LPS), which leads to an increase in prostanoid levels for the first time after 4–6 h [10]. Ca^{2+} seems not to be involved in the LPS signal-transduction pathway [11].

The rate of prostanoid synthesis in rat-liver macrophages is thought to be determined by the availability of free AA, controlled by its liberation from and its re-esterification into phospholipids [12]. The question of the enzyme(s) involved in liberation of AA is still controversial. In rat-liver macrophages, we have provided evidence that there exist at least two different pathways that may both contribute to stimulus-induced AA release and the subsequent production of prostanoids. One pathway, which seems to provide the major part of PMA- and 50% of zymosan-elicited AA release, involves the activation of a phospholipase C (PLC) leading to the formation of diacylglycerol (DAG), which becomes deacylated by a DAG lipase to liberate free AA [6]. The second, and best known, pathway for AA liberation is elicited by the activation of a phospholipase A_2 (PLA_2) hydrolysing the ester-bond in the *sn*-2 position of phospholipids leading thereby to free AA and lysophospholipid (for reviews, see [13–15]). An intracellular cytosolic PLA_2 ($cPLA_2$) with a molecular mass of 85 kDa [16,17] has been characterized in several different cell types, such as the human monocytic cell lines U937 [18,19] and THP-1 [20], rat-kidney [21] and mouse macrophages [22,23]. This enzyme is strictly dependent on the presence of Ca^{2+} in the range 10^{-7}–10^{-6} M [18–23], has a preference for AA in the *sn*-2 position of phospholipids [16,18,24] and an alkaline pH optimum [18,21,22], cannot be inhibited by reducing agents like dithiothreitol (DTT) [18] and shows a Ca^{2+}-dependent translocation to cellular membranes [16,20,25]. A Ca^{2+}-dependent PLA_2 activity in rat-liver macrophages that undergoes Ca^{2+}-dependent translocation to cellular membranes has been identified previously by Krause et al. [26,27].

Several investigators have shown recently that activation of the AA cascade is associated with increased phosphorylation of $cPLA_2$ accompanied by stimulation of $cPLA_2$ activity [28–32]; however, others have shown that phosphorylation of $cPLA_2$ does

Abbreviations used: AA, arachidonic acid; DAG, diacylglycerol; DTT, dithiothreitol; MAP, mitogen-activated protein; ERK, extracellularly regulated kinase; LPS, lipopolysaccharide; NCS, newborn-calf serum; PC, phosphatidylcholine; PLC, phospholipase C; PKC, protein kinase C; $cPLA_2$, cytosolic phospholipase A_2; PMA, phorbol 12-myristate 13-acetate.

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not always correlate with increased AA production [33–35]. There is accumulating evidence that mitogen-activated protein (MAP) kinase is involved in phosphorylation of $cPLA_2$. $cPLA_2$ possesses a MAP kinase consensus sequence and MAP kinase is able to phosphorylate $cPLA_2$ *in vitro*, thereby increasing its activity [36,37]. Moreover, evidence has been provided by Qiu and Leslie [38] that $cPLA_2$ is a substrate for MAP kinase *in vivo* in mouse macrophages.

In this report we demonstrate that rat-liver macrophages express an 85 kDa $cPLA_2$ that is phosphorylated upon stimulation with PMA, zymosan and LPS. The aim of this study was to elucidate the signal-transduction pathways leading to $cPLA_2$ phosphorylation and to discuss the role of this phosphorylation in activation of AA release in these cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and newborn calf serum (NCS) were obtained from Biochrom; NCS was heat inactivated at 56 °C for 30 min. Zymosan, fatty-acid-free BSA, potato acid phosphatase, benzamidin, leupeptin, soybean trypsin inhibitor and pepstatin A were purchased from Sigma. A23187 was obtained from Calbiochem. Genistein and microcystein LR were from Biomol. PMA was purchased from Pharmacia. [5,6,8,9,11,12,14,15-^{3}H]AA (180 Ci/mmol) and L-3-phosphatidylcholine-1-stearoyl-2-[1-^{14}C]arachidonyl (PC) were obtained from Amersham. Polyclonal antibodies raised against full-length U937 $cPLA_2$ and against a peptide corresponding to amino acids 42–58 of U937 $cPLA_2$ and purified U937 $cPLA_2$ were a gift from Dr. J. D. Clark (Genetics Institute, Massachusetts, MA, U.S.A.) LPS from *Salmonella minnesota* R595 was provided by Dr. C. Galanos (Freiburg, FRG).

Cell culture

Rat-liver macrophages from male Wistar rats (250–350 g) obtained from Interfauna Süddeutsche Versuchstierfarm (Tuttlingen, Germany) were removed under Nembutal anaesthesia by a centrifugal elutriation procedure [39]. The cells were maintained in primary culture with RPMI medium containing 30% NCS. All experiments were performed with rat-liver macrophages kept in primary culture for either 48 or 72 h as indicated.

Determination of [^{3}H]AA release

Rat-liver macrophages were incubated in RPMI 1640 containing 30% NCS with 1 μCi of [^{3}H]AA/ml for 24 h. Then the media were removed, the cells washed thoroughly and incubated without or with the various stimuli in Hanks' solution (122 mM NaCl/5.4 mM KCl/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/0.4 mM $MgSO_4$/0.38 mM KH_2PO_4/0.34 mM Na_2HPO_4/10 mM glucose/20 mM Hepes, pH 7.5) containing 2% (w/v) BSA (Behring-Werke, Germany) [12] for different times. The media were removed, centrifuged and the radioactivity determined in the supernatants. We have previously shown that under these conditions radioactivity consists predominantly of AA (80%) and to a minor extent AA metabolites (20%) [12].

Preparation of cell-free extracts

Rat-liver macrophages were washed thoroughly and incubated in Hanks' solution for the indicated times with or without the various stimuli. Then the cells were washed with cold PBS (137 mM NaCl/2.7 mM KCl/6.5 mM Na_2HPO_4/1.4 mM KH_2PO_4, pH 7.5) and frozen in liquid nitrogen. After thawing, cells were scraped off the culture dish with homogenization buffer (1 mM DTT/0.1 μM microcystin LR/100 μM Na_3VO_4/1 μg/ml benzamidin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 μg/ml soybean trypsin inhibitor/100 mM Tris/HCl, pH 9) using a rubber policeman. Cells were homogenized with 20 strokes in a Dounce homogenizer.

Translocation of $cPLA_2$

Translocation of $cPLA_2$ was performed as described previously [26]. The cells were scraped off the dishes with homogenization buffer (1 mM EGTA/1 mM DTT/100 μM Na_3VO_4/0.1 μM microcystein LR/1 μg/ml benzamidin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 μg/ml soybean trypsin inhibitor/100 mM Tris/HCl, pH 7.5). The lysates were matched for protein content and diluted to 1 mg/ml protein and sonicated (3 s, 100 W, 35 kHz) in an ice-cold water bath. After centrifugation at 350000 *g* for 30 min (TLA-100.2 fixed-angle rotor, Beckman TL-100 Tabletop Ultracentrifuge) the supernatant was removed. The remaining pellet was resuspended in homogenization buffer by sonication (3 s, 100 W, 35 kHz).

Determination of PLA_2 activity

PLA_2 activity was determined according to the method described previously [26]. [^{14}C]PC was dried under N_2 and resuspended by sonication in substrate solution (1.25 μM $CaCl_2$/12.5 mM $MgCl_2$/3 mM DTT/0.1 μM microcystin LR/100 μM Na_3VO_4/0.125% (w/v) fatty-acid-free BSA/125 mM Tris/HCl, pH 9) to a final concentration of 2.2 μM [^{14}C]PC. The reaction was started by adding 80 μl of substrate solution to 20 μl of cell extract. After 5 min incubation at 37 °C the reaction was stopped by the addition of 750 μl of Dole's reagent [propan-2-ol/1 M HCl; 700:60 (v/v) [40]]. After addition of 400 μl of H_2O, AA was extracted by the heptane/silica method with 700 μl of n-heptane [40]. Contaminating phospholipids were adsorbed on silica using a modified version of [41]. A 500 μl volume of the upper phase was added to silica-60-H (100 μg; Merck, Darmstadt, Germany) with 200 μl of n-heptane. After centrifugation (8000 *g*, 10 min) the radioactivity of the supernatant was measured in a scintillation counter.

SDS/PAGE and Immunoblotting

Samples (20 μg) were separated on 10% gels (1.5 mm thick) for 3.5 h at 60 mA [42] and transferred to nitrocellulose membranes (Schleicher & Schuell). The blot was probed with polyclonal antibodies raised against the full-length $cPLA_2$ from U937 cells (diluted 1:1000) or polyclonal antibodies directed against a peptide corresponding to amino acids 42–58 from $cPLA_2$ of U937 cells (diluted 1:200). Detection was performed using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham) or using the Western Blue-stabilized substrate for alkaline phosphatase (Serva).

Phosphatase treatment

Translocation of $cPLA_2$ in the presence of 1 mM free Ca^{2+} was performed as described above. It has been shown that at free-Ca^{2+} concentrations of > 1 μM, $cPLA_2$ is attached to cellular membranes [26]. The homogenate was sedimented at 350000 *g* for 30 min to remove cytoplasmic proteases. The remaining pellet was resuspended by sonication in 40 mM Pipes, pH 6, containing 1 mM EGTA/1 mM DTT/1 μg/ml benzamidin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 μg/ml soybean trypsin inhibitor. The resuspended pellets were incubated for 120 min at

30 °C with 0.1 unit of potato acid phosphatase type VII per μg of cellular protein. Then the lysates were subjected to SDS/PAGE [42], or lysates were assayed for PLA_2 activity.

In situ MAP-kinase assay

Cells were scraped off the plates in ice-cold Frackelton's buffer (10 mM Tris/HCl/50 mM NaCl/1% (v/v) Triton X-100/ 30 mM Na_3PO_4/50 mM NaF/100 μM Na_3VO_4/2 μM $ZnCl_2$/ 1 mM phenylmethanesulphonyl fluoride, vortexed for 30 s and centrifuged at 15800 *g* for 30 min. The supernatants were matched for protein content and diluted to 1 mg/ml protein in 62.5 mM Tris/HCl, pH 6.8, containing 2.3% (w/v) SDS, 5 mM EDTA, 10% (v/v) glycerol and 100 mM DTT and heated at 85 °C for 5 min before SDS/PAGE. The gels were polymerized with 0.2 mg/ml myelin basic protein and, after electrophoresis, denatured in 6 M guanidine hydrochloride, renatured and assayed for kinase activity as described by Chao et al. [43].

Quantification of protein

Protein was determined according to the method of Bradford using BSA as a standard [44].

RESULTS

Immunochemical detection of $cPLA_2$ in rat-liver macrophages

We have previously demonstrated that rat-liver macrophages contain an intracellular PLA_2 activity that becomes maximally activated by between 10^{-7} and 10^{-6} M free Ca^{2+}, which shows a Ca^{2+}-dependent translocation to cellular membranes [26,27]. In order to determine whether rat-liver macrophages express a $cPLA_2$ we performed Western-blot analysis with polyclonal antibodies raised against the full-length $cPLA_2$ of U937 cells [16] or with polyclonal antibodies raised against a peptide corresponding to amino acids 42–58 of U937 $cPLA_2$. As shown in Figure 1(a), both antibodies recognized a protein with an apparent molecular mass of 100 kDa that co-migrates with the purified U937 $cPLA_2$ and with $cPLA_2$ in lysates of U937 cells. $cPLA_2$ was associated with membranes in the presence of Ca^{2+}, whereas after Ca^{2+} removal it could only be detected in the cytosolic fraction (Figure 1b). Krause et al. [26] have shown that PLA_2 activity exhibits a similar Ca^{2+}-dependent distribution: PLA_2 activity could be detected in the cytosol at free-Ca^{2+} concentrations below 1 μM and associated with cellular membranes at 1 μM or higher free-Ca^{2+} concentrations. Thus, the PLA_2 activity in rat-liver macrophages previously described [26,27] seems to be evoked by a $cPLA_2$ immunologically related to the $cPLA_2$ of human U937 cells.

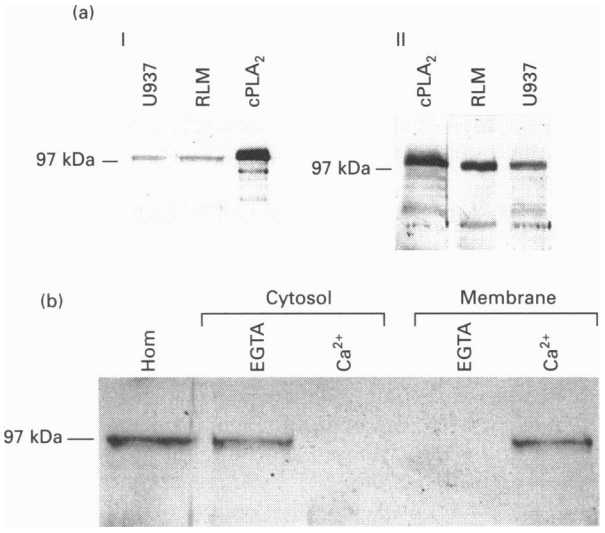

Figure 1 Immunochemical identification and Ca^{2+}-dependent translocation of $cPLA_2$ in rat-liver macrophages

(**a**) The lysates (20 μg) of rat-liver macrophages (RLM) and U937 cells and purified $cPLA_2$ (20 ng) from U937 cells were subjected to SDS/PAGE (10% gel) and transferred to nitrocellulose membrane. The blots were probed with rabbit polyclonal antibodies raised against full-length $cPLA_2$ of U937 cells (panel I) and rabbit polyclonal antibodies raised against a peptide corresponding to amino acids 42–58 of U937 $cPLA_2$ (panel II). (**b**) Rat-liver macrophages (72 h in primary culture) were homogenized and fractionated either in the presence (1 mM Ca^{2+}) or absence of Ca^{2+} (1 mM EGTA), as described in the Materials and methods section. Homogenate (Hom), cytosol and membrane fraction (20 μg of each) were subjected to SDS/PAGE and immunoblotted with rabbit polyclonal antibodies raised against the full-length $cPLA_2$ from U937 cells. Blots were developed using the Western Blue*-stabilized substrate for alkaline phosphatase.

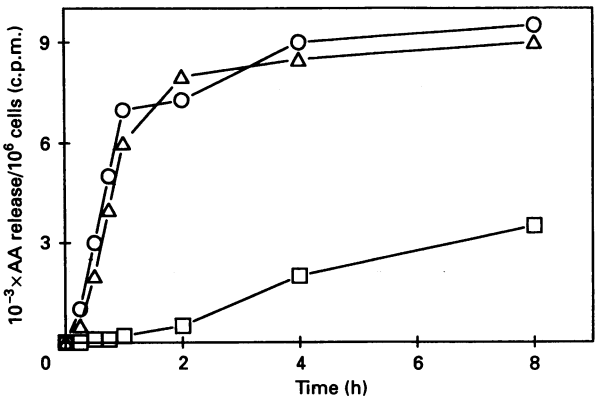

Figure 2 Time-dependence of AA release in response to PMA, zymosan and LPS

Rat-liver macrophages (48 h in primary culture) were incubated with 1 μCi/ml [^{3}H]AA. After 24 h the cells were incubated in Hanks' solution containing 2% BSA without or with PMA (○, 1 μM), zymosan (△, 0.5 mg/ml) or LPS (□, 0.5 μg/ml) for different times. The release of [^{3}H]AA was determined as described in the Materials and methods section. Values (in c.p.m.) represent differences between stimulated and unstimulated cells.

Phosphorylation of $cPLA_2$

Rat-liver macrophages respond upon PMA- and zymosan-treatment within minutes with increased AA release (Figure 2). PMA, zymosan and the Ca^{2+} ionophore A23187 lead to comparable levels of AA release after 60 min (Table 1). In contrast to these agents, LPS induces an increase in AA release that becomes detectable for the first time after 4 h (Figure 2). Several groups have reported that phosphorylation of $cPLA_2$ may be involved in activation of $cPLA_2$ and stimulation of AA release [28–32]. Phosphorylation of $cPLA_2$ can be measured by a shift to higher apparent molecular mass in SDS/PAGE [28,29,32–37]. In order to investigate whether phosphorylation might also be involved in activation of $cPLA_2$ in rat-liver macrophages we determined the electrophoretic mobility of $cPLA_2$ from unstimulated and stimulated cells (with PMA, zymosan, A23187 or LPS for 10 min). Figure 3(a) shows that in unstimulated cells, $cPLA_2$ is present in a rapidly migrating form, whereas PMA, zymosan and LPS, but not A23187, cause a complete shift to a more slowly migrating

Table 1 PLA_2 activity and AA release in response to PMA, zymosan, A23187 and LPS

PLA_2 activity: rat liver macrophages (72 h in primary culture) were incubated in Hanks' solution without (control) or with PMA (1 μM), zymosan (0.5 mg/ml), A23187 (1 μM) or LPS (0.5 μg/ml) for 10 min. PLA_2 activity in the lysates (2.5 μg) was determined as described in the Materials and methods section. A value of 100% corresponds to a PLA_2 activity of 1.5 ± 0.4 nmol AA/min per mg of protein. AA release: rat liver macrophages (48 h in primary culture) were incubated with 1 μCi/ml [^{3}H]AA. After 24 h the cells were incubated in Hanks' solution containing 2% BSA without (control) or with PMA (1 μM), zymosan (0.5 mg/ml), A23187 (1 μM) or LPS (0.5 μg/ml) for 60 min. The release of [^{3}H]AA was determined as described in the Materials and methods section. Results are means $\pm$ S.D. of three independent experiments. *P* values were calculated using Student's *t*-test calculated versus no stimulus; * $P < 0.006$, ** not significant.

Stimulus	PLA_2 activity (% of control)	$10^{-3} \times$ [^{3}H]AA release/10^6 cells (c.p.m.)
Control	100	8.7 ± 1.1
PMA	145 ± 9*	20.2 ± 3
Zymosan	142 ± 7*	27.3 ± 3.9
A23187	104 ± 13**	21.4 ± 3.2
LPS	135 ± 2*	8.5 ± 3.6

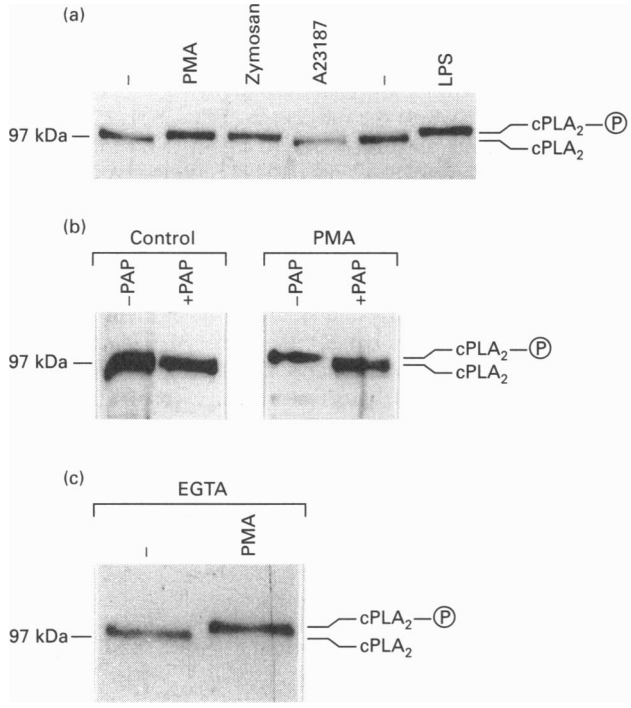

Figure 3 Mobility-shift of $cPLA_2$ in SDS/PAGE

(**a**) Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution without (–) or with PMA (1 μM), zymosan (0.5 mg/ml), A23187 (1 μM) or LPS (0.5 μg/ml) for 10 min. Then the lysates (20 μg) were analysed by SDS/PAGE (10% gel). (**b**) Treatment with potato acid phosphatase. Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution without (control) or with PMA (1 μM) for 10 min. Then membrane fractions were obtained as described in the Materials and methods section. Membranes (60 μg) were incubated for 120 min at 30 °C without (—PAP) or with 6 units of potato acid phosphatase (+PAP) and analysed by SDS/PAGE (10% gel; 20 μg). (**c**) Ca^{2+}-dependence of $cPLA_2$ phosphorylation. Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution containing no Ca^{2+} but 1 mM EGTA without (—) or with PMA (1 μM) for 10 min. Then the cells were homogenized and the lysates (20 μg) were analysed by SDS/PAGE (10% gel). Immunoblotting was performed with rabbit polyclonal antibodies raised against full-length $cPLA_2$ from U937 cells.

form of $cPLA_2$ in SDS/PAGE. The rapidly migrating form of the enzyme is no longer detectable by Western-blot analysis after stimulation with PMA, zymosan and LPS. In order to verify that the altered mobility of $cPLA_2$ is due to phosphorylation of the enzyme we incubated cell lysates of control and PMA-treated cells with potato acid phosphatase. Figure 3(b) demonstrates that phosphatase treatment converts the slower migrating (PMA-treated cells) completely into the faster migrating form (control cells), while it causes no change in electrophoretic mobility of $cPLA_2$ from control cells. Phosphorylation of $cPLA_2$ has also been verified by incorporation of ^{32}P into the protein (results not shown). We have previously shown that PMA-induced AA release in rat liver macrophages requires extracellular Ca^{2+} [5]. Therefore, we examined the electrophoretic mobility of $cPLA_2$ in cells incubated without Ca^{2+}. Figure 3(c) shows that PMA-induced phosphorylation of $cPLA_2$ occurs also in the absence of extracellular Ca^{2+}, indicating that extracellular Ca^{2+} is not necessary for $cPLA_2$ phosphorylation.

$cPLA_2$ phosphorylation and activity

It has been reported by other investigators that phosphorylation of $cPLA_2$ causes an increase in enzyme activity [28–32]. In rat-liver macrophages, phosphorylation of $cPLA_2$ (after PMA, zymosan and LPS; see Figure 3a) induces a small and significant increase in enzyme activity (about 140% of unphosphorylated $cPLA_2$; Table 1). A23187, which did not induce an altered mobility of $cPLA_2$ in SDS/PAGE (Figure 3a) causes no significant change of $cPLA_2$ activity (Table 1). Similar results were obtained using another PLA_2 assay according to Lin et al. [28]. In order to investigate whether the enhanced enzymic activity is caused by phosphorylation of $cPLA_2$ we measured PLA_2 activity in cell lysates which had been treated beforehand with phosphatase for 120 min to dephosphorylate $cPLA_2$ (Figure 3b). While phosphatase treatment has no effect on PLA_2 activity in lysates from unstimulated cells (from 0.55 ± 0.04 to 0.53 ± 0.06 nmol AA/min per mg of protein), PLA_2 activity in lysates from PMA-stimulated cells is decreased from 0.74 ± 0.06 to 0.54 ± 0.02 nmol AA/min per mg of protein.

Effect of PMA, zymosan, A23187 and LPS on MAP kinase activation

Recent studies have indicated that $cPLA_2$ serves as a substrate for MAP kinase, leading to $cPLA_2$ phosphorylation [36–38]. We therefore examined whether agents that induce $cPLA_2$ phosphorylation (see Figure 3a) are able to activate the MAP kinase isoenzymes, extracellularly regulated kinase (ERK)-1 and ERK-2, in rat-liver macrophages. Figure 4 demonstrates that treatment with PMA, zymosan and LPS, but not with A23187, induces an activation of the MAP kinase isoenzymes ERK-1 and ERK-2, suggesting that phosphorylation of $cPLA_2$ may be mediated by MAP kinase.

Effects of the tyrosine kinase inhibitor genistein on agonist-induced $cPLA_2$ phosphorylation, MAP kinase activation and AA release

It has been shown that tyrosine kinases play a significant role in the signal-transduction pathway of LPS [45,46]. Hence we used the tyrosine kinase inhibitor genistein to investigate whether tyrosine kinases participate in the signal-transduction pathway of agonists that induce $cPLA_2$ phosphorylation, MAP kinase activation and AA release. In rat-liver macrophages, genistein suppresses LPS-induced $cPLA_2$ phosphorylation but does not alter phosphorylation of $cPLA_2$ after treatment with PMA and

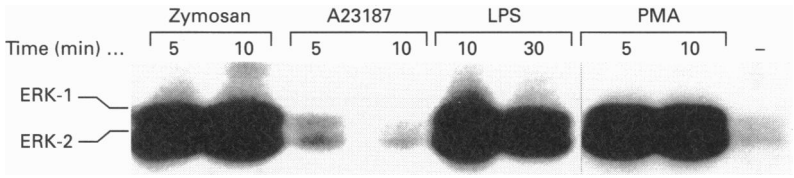

Figure 4 'In gel' kinase assay of MAP kinase activity

Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution without (—) or with PMA (1 μM), zymosan (0.5 mg/ml), A23187 (1 μM) or LPS (0.5 μg/ml) for the indicated times. Cells were lysed and subjected to SDS/PAGE with 0.2 mg/ml myelin basic protein polymerized in the separating gel, and 'in gel' kinase assay of MAP kinase was performed as described in the Materials and methods section. The positions of the MAP kinase isoenzymes ERK-1 and ERK-2 are indicated.

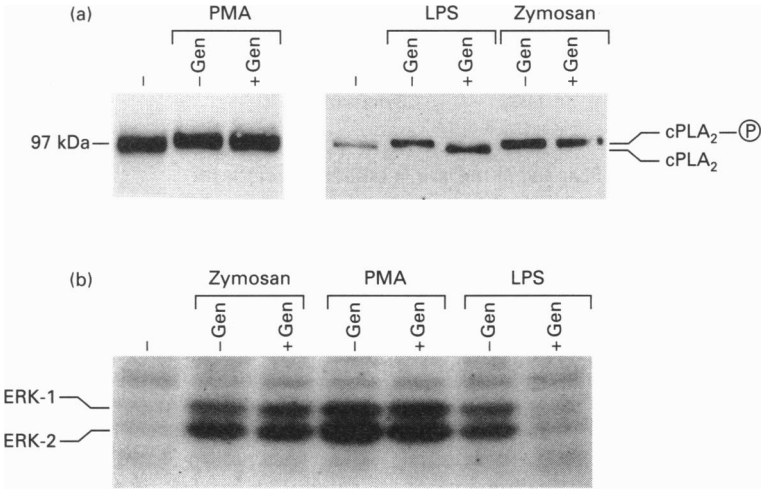

Figure 5 Effect of the tyrosine kinase inhibitor genistein on phosphorylation of $cPLA_2$, activation of MAP kinase and release of AA

(**a**) Phosphorylation of $cPLA_2$. Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution for 1 h in the absence (—Gen) or presence (+Gen) of the tyrosine kinase inhibitor genistein (50 μM). Thereafter, no agent (–), PMA (1 μM), zymosan (0.5 mg/ml) or LPS (0.5 μg/ml) was added for 10 min. Then the lysates (20 μg) were analysed by SDS/PAGE (10% gel). Immunoblotting was performed with rabbit polyclonal antibodies raised against full-length $cPLA_2$ from U937 cells. (**b**) Activation of MAP kinase. Rat-liver macrophages (72 h in primary culture) were incubated in Hanks' solution for 1 h in the absence (—Gen) or presence (+Gen) of the tyrosine kinase inhibitor genistein (50 μM). Thereafter, no agent (—), PMA (1 μM), zymosan (0.5 mg/ml) or LPS (0.5 μg/ml) was added for 10 min. Cells were lysed and subjected to SDS/PAGE with 0.2 mg/ml myelin basic protein polymerized in the separating gel, and 'in gel' kinase assay of MAP kinase was performed as described in the Materials and methods section. The positions of the MAP kinase isoenzymes ERK-1 and ERK-2 are indicated.

Table 2 Effect of the tyrosine kinase inhibitor genistein on AA release

Rat-liver macrophages (48 h in primary culture) were incubated with 1 μCi/ml [^{3}H]AA. After 24 h the cells were incubated in Hanks solution containing 2% BSA for 1 h in the absence or presence of the tyrosine kinase inhibitor genistein (50 μM). Thereafter, PMA (1 μM), zymosan (0.5 mg/ml) or A23187 (1 μM) was added for 1 h and LPS (0.5 μg/ml) was added for 5 h. The release of [^{3}H]AA was determined as described in the Materials and methods section. Results are means ± S.D. of three independent experiments. Values (in c.p.m.) represent differences between stimulated and unstimulated cells.

	10^{-3} × [^{3}H]AA release/10^6 cells (c.p.m.)	
Stimulus	—Genistein	+Genistein
PMA	9.3 ± 0.4	8.4 ± 0.4
Zymosan	11.8 ± 3.2	11.0 ± 2.9
A23187	9.4 ± 1.1	8.3 ± 0.9
LPS	4.8 ± 1.3	13.6 ± 0.9

zymosan (Figure 5a). Accordingly, genistein inhibits activation of MAP kinase in response to LPS but has no effect on the PMA- and zymosan-elicited stimulation of MAP kinase (Figure 5b). These data indicate a role for tyrosine kinases in LPS-mediated MAP kinase activation and $cPLA_2$ phosphorylation. LPS-induced release of AA does not seem to require tyrosine kinase activation, since genistein does not inhibit but rather stimulates LPS-elicited AA release (Table 2). The reason for the stimulatory effect of genistein on LPS-induced AA release is not yet known. However, the effect seems to be specific for LPS, since genistein does not affect AA release from unstimulated cells (results not shown), or AA release upon addition of zymosan, PMA or A23187 (Table 2).

DISCUSSION

In the present study we have demonstrated that rat-liver macrophages express a $cPLA_2$ with an apparent molecular mass of 100 kDa on SDS/PAGE (Figure 1a). $cPLA_2$ undergoes a Ca^{2+}-dependent translocation to cellular membranes (Figure 1b) and therefore probably resembles PLA_2 activity, which has been described recently in rat-liver macrophages to be stimulated and to translocate to membranes at free Ca^{2+}-concentrations of $> 10^{-7}$ M [26].

It has been reported previously that $cPLA_2$ becomes phosphorylated in different cell types upon stimulation with

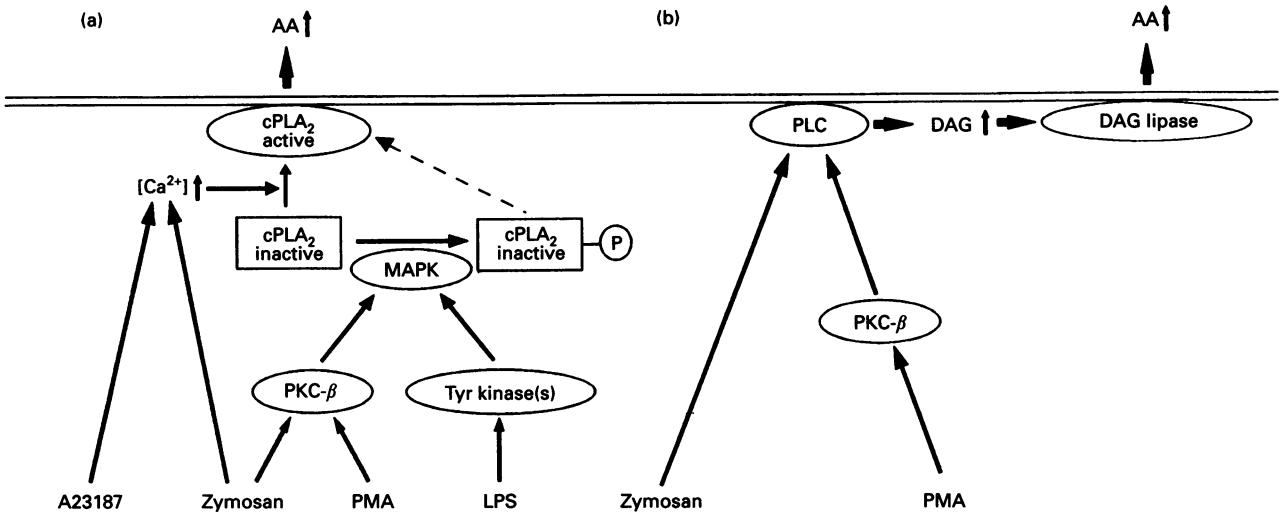

Figure 6 A proposed model for the regulation of cPLA$_2$ and AA release in rat-liver macrophages

MAPK, MAP kinase; Tyr kinase, tyrosine kinase.

PMA [28,30,31], zymosan [30,31] and LPS [35]. In rat-liver macrophages, we also observe cPLA$_2$ phosphorylation in response to these agents, as shown by a shift of cPLA$_2$ to a higher molecular mass on SDS/PAGE (Figure 3a), which is reversed by phosphatase treatment (Figure 3b). The shift of cPLA$_2$ becomes detectable 2 min after addition of PMA (at this time-point cPLA$_2$ appears as a double band) and is complete after 5 min (results not shown). At this and later time-points, the unphosphorylated form of cPLA$_2$ can no longer be observed by Western-blot analysis, suggesting that cPLA$_2$ is completely phosphorylated by PMA, zymosan and LPS. No altered electrophoretic mobility of cPLA$_2$ could be detected after treatment with the Ca^{2+} ionophore A23187 (Figure 3a). This is in contrast with results obtained in Chinese hamster ovary cells overexpressing cPLA$_2$ [28], and mouse peritoneal macrophages [31] where the Ca^{2+} ionophore was shown to stimulate cPLA$_2$ phosphorylation. Furthermore, we observed a small increase in enzymic activity of the phosphorylated form of cPLA$_2$ (1.4-fold; Table 1), which is low compared with the 2–3-fold stimulation of cPLA$_2$ activity found in other cells [28–32]. Treatment of rat-liver macrophages with the tyrosine kinase inhibitor genistein indicates that the LPS- but not the zymosan- or PMA-induced phosphorylation of cPLA$_2$ is mediated by tyrosine kinases (Figure 5a). The hypothesis that MAP kinase activation is involved in phosphorylation of cPLA$_2$ is further supported by the fact that both are induced by zymosan, PMA and LPS, but not by A23187 (Figure 4). Moreover, genistein, which inhibits the LPS-induced phosphorylation of cPLA$_2$ (Figure 5a), also prevents the LPS-induced activation of MAP-kinase, whereas MAP-kinase activation and cPLA$_2$ phosphorylation in response to PMA and zymosan are not affected by the inhibitor (Figure 5b). These results, together with recent observations that tyrosine kinase inhibitors are able to prevent LPS-mediated cytotoxicity and MAP kinase activation [46,47], indicate that tyrosine kinases have an important role in the signal-transduction pathway of LPS.

Several mechanisms have been postulated to be involved in agonist-induced activation of cPLA$_2$ and concomitant synthesis and release of eicosanoids, including increased expression [29,33,34,48], coupling to G-proteins [49,50], phosphorylation [28–32] or rise of intracellular free Ca^{2+} [25,28,33–35]. Data presented in this paper, together with earlier findings, suggest that in rat-liver macrophages an increase in cellular Ca^{2+}, but not phosphorylation of cPLA$_2$, is essential for activation of the AA cascade: (1) cPLA$_2$ is strongly dependent on free-Ca^{2+} concentrations of $> 10^{-7}$ M for enzymic activity and becomes attached to cellular membranes at those Ca^{2+} concentrations (Figure 1b; [18,20,25,26]); (2) removal of extracellular Ca^{2+} inhibits almost totally the release of AA and its metabolites [5] but does not affect phosphorylation of cPLA$_2$ (Figure 3c); (3) the Ca^{2+} ionophore A23187 elicits a release of AA to a similar extent as zymosan or PMA (Table 1; [1]) but does not induce phosphorylation of cPLA$_2$ (Figure 3a); (4) LPS induces cPLA$_2$ phosphorylation (Figure 3a) and an increase in cPLA$_2$ activity within 10 min (Table 1), but a release of AA can be detected for the first time after 4 h (Figure 2); (5) genistein suppresses LPS-induced phosphorylation of cPLA$_2$ but stimulates AA release in response to LPS (Table 2). These conclusions are consistent with observations in human neutrophils [35] and the human fibroblast cell line WI-38 [33], where it has been shown that phosphorylation of cPLA$_2$ in response to LPS and interleukin 1α alone was not able to stimulate a release of AA or eicosanoids. Based on these data, Lin et al. have proposed that phosphorylated cPLA$_2$ awaits a second stimulus for its full activation [28,33].

If the hypothesis is true that in rat liver macrophages cPLA$_2$ becomes activated mainly by a rise in intracellular free Ca^{2+}, such an increase of free Ca^{2+} has to precede AA release. This has been demonstrated for the Ca^{2+} ionophore A23187 and zymosan, but not for PMA [6]. Therefore, the question remains of how PMA is able to induce AA release in liver macrophages. We have previously demonstrated that in these cells a second pathway exists leading to AA release, which involves the activation of a PLC, leading to generation of DAG, and a DAG lipase that liberates AA from DAG [6]. We presented evidence that AA release upon treatment with PMA mainly results from the PLC/DAG lipase pathway and not from the cPLA$_2$ pathway [6], whereas A23187 induces AA release via the cPLA$_2$ pathway and zymosan leads to the activation of both pathways [6]. The

importance of the PLC/DAG lipase pathway for AA release has been demonstrated recently by Konrad et al. [51] for glucose- and carbachol-stimulated AA release from isolated islets.

The signal-transduction pathway of LPS, leading to AA release in rat-liver macrophages, is not yet fully understood. LPS leads to phosphorylation of $cPLA_2$ and an increase in PLA_2 activity, but it induces neither a rise in intracellular free Ca^{2+} nor generation of DAG [11]. However, recent data indicate that LPS induces enhanced expression of $cPLA_2$ [48], which may explain the delayed AA release after LPS (Figure 2).

Based on these data, we conclude the following hypothesis, shown in Figure 6. In rat-liver macrophages at least two different pathways exist leading to AA release. The first pathway (Figure 6a) leads to the activation of a $cPLA_2$ by a rise in intracellular free Ca^{2+}, which induces translocation of the enzyme to cellular membranes. This pathway is used by the Ca^{2+} ionophore A23187 and zymosan, but not by PMA and LPS. Phosphorylation of $cPLA_2$ is induced upon treatment with PMA, zymosan and LPS. These agents also lead to activation of MAP kinase, which might directly phosphorylate $cPLA_2$ [36–38]. Tyrosine kinases seem to mediate LPS-induced $cPLA_2$ phosphorylation, while PMA and zymosan have been shown to activate PKC-β [9]. The role of $cPLA_2$ phosphorylation for activation of AA release in rat liver macrophages is not yet understood. PMA and zymosan induce AA release by activation of a second pathway (Figure 6b) that involves the activation of a PLC generating free DAG [6], which serves as substrate for DAG lipase. The PMA- but not zymosan-induced generation of DAG has been shown to be mediated by activation of PKC-β [6].

All these data indicate that, with respect to liberation of AA and formation of eicosanoids in mammalian cells, one has to consider not only the question how $cPLA_2$ becomes activated but also the fact that different agonists may use other pathways, like for example the PLC/DAG lipase pathway that can also lead to liberation of AA and consequent release of eicosanoids.

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