Granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes phosphorylation and an increase in the activity of cytosolic phospholipase A_2 in human neutrophils

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Incubation of human neutrophils with 500 pM granulocyte–macrophage colony-stimulating factor (GM-CSF) results in a rapid and time-dependent increase in the phosphorylation of cytosolic phospholipase A_2 (cPLA$_2$), which was reflected in a slower electrophoretic mobility of the enzyme. The GM-CSF-induced phosphorylation of cPLA$_2$ was accompanied by a parallel and time-dependent increase in the enzyme activity. Preincubation of neutrophils with the tyrosine kinase inhibitor genistein caused inhibition of the GM-CSF-stimulated phosphorylation and activity of cPLA$_2$. Immunoprecipitation of the enzyme following incubation of neutrophils with [^{32}P]P$_i$ shows that cPLA$_2$ is phosphorylated by GM-CSF. Potato acid phosphatase caused dephosphorylation of the enzyme, indicating that cPLA$_2$ is indeed phosphorylated by GM-CSF. The subcellular distribution of cPLA$_2$ in GM-CSF-stimulated neutrophils revealed that the enzyme resides almost completely in the cytosolic fraction. Addition of Ca^{2+} to the lysis buffer before homogenization results in the translocation of the phosphorylated and the dephosphorylated forms of the enzyme to the membranes. Translocation of cPLA$_2$ was also achieved after incubation with 0.1 μM *N*-formylmethionyl-leucyl-phenylalanine (fMLP) after GM-CSF stimulation and when neutrophils were challenged with the Ca^{2+} ionophore A23187. EDTA and EGTA were unable to solubilize the translocated enzyme from the neutrophil membranes, indicating that cPLA$_2$ is attached to the membranes by strong bonds and not merely due to ionic forces exerted by Ca^{2+}. The inability of GM-CSF to promote arachidonic acid mobilization is probably due to the fact that GM-CSF does not cause an increase in intracellular Ca^{2+}, which is necessary for the translocation of the enzyme to the membranes where its substrate(s) reside.

INTRODUCTION

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is one of several growth factors responsible for proliferation and differentiation of myeloid progenitor cells [1–5]. In addition, GM-CSF acts on the mature neutrophils by potentiating their cellular functions and responsiveness to various stimuli. These functions include superoxide generation [6], phagocytosis [7], arachidonic acid (AA) release [8], platelet-activating factor (PAF) production [9] and degranulation [10].

Despite the dramatic effect of GM-CSF on many cellular responses of neutrophils, very little is known about the mechanism underlying this priming phenomenon. It was found that GM-CSF promotes the up-regulation of the receptor for *N*-formylmethionyl-leucyl-phenylalanine (fMLP) [11], but it does not activate the phosphoinositide cycle, nor does it increase the intracellular Ca^{2+} concentration [12–14]. The effect of GM-CSF on neutrophils is mediated through a single class high-affinity receptor consisting of α and β subunits. Although GM-CSF receptors do not contain a tyrosine kinase domain, GM-CSF has been shown to stimulate the rapid tyrosine phosphorylation of six unique and distinct proteins in neutrophils [14–17], including the p42 and p44 isoforms of the mitogen-activated protein kinase (MAP kinase) [18]. Inhibition of GM-CSF-induced tyrosine phosphorylation blocks many of the biological effects of GM-CSF in neutrophils, including GM-CSF-induced alkalinization and fMLP-induced Ca^{2+} mobilization, demonstrating the significance of tyrosine phosphorylation in the signalling pathway of GM-CSF [14]. There is accumulating evidence showing that G-protein are affected by GM-CSF-mediated responses in neutrophils. GM-CSF priming of certain neutrophil processes, such as induction of c-fos mRNA, enhancement of fMLP-induced superoxide generation and PAF-induced increase in intracellular Ca^{2+}, are inhibited by pertussis toxin [19,20]. GM-CSF can also augment the effect of receptor-independent G-protein activators, such as guanosine 5′-[γ-thio]trisphosphate, on the respiratory burst [21], implying that GM-CSF affects neutrophil response to a subsequent agonist independently of the expression of cell-surface receptors. Recently, it has been demonstrated that GM-CSF induces the up-regulation of the amount of $G_i2\alpha$ subunit of the G_i2 protein associated with the plasma membranes, which may account for the priming effect seen when cells are stimulated with G-protein-dependent chemoattractant [22]. However, pertussis toxin sensitive G-proteins do not appear to be involved in either the direct or the priming effect on AA release in neutrophils [23]. Recent studies utilizing pertussis toxin have demonstrated inhibition of AA release and subsequent leukotriene synthesis in neutrophils treated with the Ca^{2+} ionophore A23187 [24]. Although GM-CSF by itself does not provoke AA release, many of the responses of neutrophils to various agonists which are primed

Abbreviations used: MAP kinase, mitogen-activated protein kinase; GM-CSF, granulocyte–macrophage colony-stimulating factor; HBSS, Hanks balanced salt solution; ECL, enhanced chemiluminescence; cPLA$_2$, cytosolic phospholipase A_2; AA, arachidonic acid; PAF, platelet-activating factor; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PAP, potato acid phosphatase.

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and enhanced by GM-CSF are mediated by the release of AA, which is metabolized to produce prostaglandins, thromboxanes and leukotrienes [25,26]. AA can be cleaved from phospholipids by various mechanisms. The recently discovered cytosolic phospholipase A_2 ($cPLA_2$), which is also called the hormonally regulated PLA_2, is regarded as an important enzyme that has been implicated in many agonist-induced AA release actions. It also plays an essential role in the release of PAF [9]. This enzyme is specific for the release of AA from the *sn*-2 position of phospholipids, has a low K_m for Ca^{2+}, is phosphorylated and translocated to the cell membrane following appropriate stimuli [27,28] and shares no amino acid sequence similarity with the secretory phospholipase A_2 ($sPLA_2$) [29]. Unlike $sPLA_2$, $cPLA_2$ is insensitive to thiol groups and is not inhibited by β-mercaptoethanol and dithiothreitol [30,31]. Although GM-CSF by itself does not promote AA release and eicosanoid production, the present study demonstrates that GM-CSF induces phosphorylation and increased activity of $cPLA_2$, but does not cause translocation of the enzyme to the neutrophil membranes. The lack of translocation is probably due to the inability of GM-CSF to increase intracellular Ca^{2+} concentrations.

MATERIALS AND METHODS

Materials

GM-CSF was purchased from R & D Systems, Minneapolis, MN, U.S.A. Polyvinylidine difluoride protein transfer membrane was from Millipore Corp., Bedford, MA, U.S.A. Electrophoresis reagents and molecular-mass markers were from Bio-Rad Laboratories, Melville, NY, U.S.A. Enhanced chemiluminescence (ECL) reagent for Western blotting was purchased from Amersham, Arlington Heights, IL, U.S.A. 1-Stearoyl-2-[1-^{14}C] arachidonylphosphatidylcholine was the product of New England Nuclear. Genistein was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Hanks Balanced Salt Solution (HBSS) devoid of Ca^{2+} and Mg^{2+} was obtained from JRH Biosciences, Lenexa, KS, U.S.A. Anti-$cPLA_2$ polyclonal and monoclonal antibodies were generously given by Dr. J. L. Knopf, Genetics Institute, Cambridge, MA, U.S.A. [27,32]. LK6DF TLC plates were the product of Whatman. Budget-solve scintillation-counting cocktail was the product of Research Product International Corp., Mount Prospect, IL, U.S.A. All other reagents were products of Sigma Chemical Co., St. Louis, MO, U.S.A.

Isolation and incubation of neutrophils

Neutrophils were isolated from citrated peripheral blood of healthy volunteers by the method of English and Anderson [33]. Contaminating erythrocytes were lysed by hypotonic shock. Neutrophils were suspended at a concentration of 10^7 cells/ml in modified Hanks Balanced Salt Solution containing 10 mM Hepes (pH 7.35) and devoid of Ca^{2+} and Mg^{2+} (HBSS). Samples (2 ml) of cells were preincubated for 5–10 min at 37 °C, and then incubated with GM-CSF and other effectors for various time intervals. The reaction was stopped by adding 30 ml of ice-cold HBSS, and cells were centrifuged at 250 ***g*** for 5 min at 4 °C and resuspended in 0.4 ml of lysis buffer, consisting of 250 mM sucrose, 50 mM Hepes, 1 mM EGTA, 1 mM EDTA, 20 μM leupeptin, 20 μM pepstatin, 1 mM PMSF, 20 μg/ml aprotinin, 1 mM sodium orthovanadate and 5 mM dithiothreitol. The cells were transferred to microfuge tubes and sonicated twice for 5–7 s bursts with a Kontes Micro-Ultrasonic cell disrupter at setting 4. The cell homogenate was centrifuged at 150000 ***g*** for 20 min at 4 °C in a Beckman TL-100 Ultracentrifuge. The supernatant obtained was assayed for $cPLA_2$ activity.

Assay of PLA_2 activity

PLA_2 activity was measured as described by Bonventre et al. [34]. Briefly, 1-stearoyl-2-[1-^{14}C]arachidonylphosphatidylcholine was used as substrate. The substrate was dried under a stream of N_2 and resuspended in DMSO with vigorous vortex-mixing. A 2 μl portion of the substrate (final concentration 15 μM) and 5 μl of 16 mM $CaCl_2$ (final concentration 2 mM) were added to Eppendorf microcentrifuge tubes. The reaction was initiated by adding 33 μl (30–40 μg of protein) of the cytosolic fraction of neutrophil homogenate. The reaction was carried out for 10 min at 37 °C. The reaction was stopped by adding 40 μl of ice-cold quench solution, comprising 40 μg/ml AA in ethanol containing 2 % (v/v) acetic acid. A 50 μl portion of the solution was spotted on LK5DF silica-gel TLC plates and developed with the organic phase of ethyl acetate/iso-octane/acetic acid/water (55:75:8:100, by vol.). The areas corresponding to AA were revealed by brief exposure to iodine vapour, scraped off into scintillation vials containing 3.0 ml of Budget-solve counting cocktail, and the radioactivity was counted in an LKB (model 1211) liquid-scintillation counter.

Western blotting

Immunoblotting was carried out as described previously [15]. Cells were incubated with GM-CSF and other effectors for various time intervals as indicated in the Figure legends. The reaction was stopped by adding 30 ml of ice-cold HBSS. Cells were pelleted by centrifugation, then resuspended in sucrose buffer at a concentration of 10^8 cells/ml. Neutrophils were disrupted by sonication as described above, then centrifuged at 150000 ***g*** for 20 min. A 50 μl portion of the protein sample (80–100 μg of protein) was mixed with Laemmli stopping solution, which contains 9 % (w/v) SDS, 6 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol and a trace amount of Bromophenol Blue in 0.2 M Tris/HCl (pH 6.7). The mixture was boiled and electrophoresed through an SDS/8 %-polyacrylamide gel. The proteins were then transferred to Immobilon-P transfer membrane and probed with polyclonal or monoclonal anti-$cPLA_2$ antibody. Bound antibodies were detected by ECL using horseradish-peroxidase-conjugated anti-rabbit immunoglobulin and exposed briefly to Kodak X-Omat films at room temperature.

Immunoprecipitation

Immunoprecipitation was carried out as described previously [35] with slight modifications. Briefly, 5×10^7 neutrophils were washed and resuspended in 1 ml of 20 mM Hepes/120 mM NaCl/5 mM KCl/1 mM $MgCl_2$/5 mM glucose, pH 7.4 (buffer A). Then 0.5 mCi of [^{32}P]P_i was added and cells were incubated for 1 h at 37 °C. Cells were washed with 3×10 ml of buffer A, resuspended in 1 ml of HBSS with 1 mg/ml BSA and incubated without or with 500 pM GM-CSF for 15 min at 37 °C. Cells were precipitated by brief centrifugation, and 100 μl of boiling 1 % SDS in 10 mM Hepes, pH 7.4, was added to the cells, and the mixture was then boiled for 15 min. Next, 400 μl of ice-cold water was added, and 500 μl of 200 mM Tris/HCl/300 mM NaCl/2 % Triton X-100/1 % Nonidet P-40/2 mM EDTA/2 mM EGTA, pH 7.4 ($2 \times$ lysis buffer), was added to the samples. After centrifugation at 15000 ***g*** for 10 min, supernatants were transferred to tubes containing anti-rabbit IgG–agarose beads to

which anti-$cPLA_2$ antibody had been conjugated and incubated overnight at 4 °C. The beads were washed twice with 1 × lysis buffer containing 1 mM PMSF, 1 mM di-isopropyl fluorophosphate, 20 μM leupeptin, 20 μM pepstatin and 20 μg/ml aprotinin. The beads were boiled with 50 μl of Laemmli sample buffer (3% SDS, 10% 2-mercaptoethanol, 10% glycerol, 5 mM Na_2HPO_4, 0.05% Bromophenol Blue) and subjected to SDS/PAGE (8% gel). Gels were dried and autoradiographed by using X-Omat Kodak scientific imaging films.

RESULTS

The activity of $cPLA_2$ was measured in the cytosolic fraction (150 000 ***g*** supernatant) of neutrophil homogenate with 1-stearoyl-2-[1-^{14}C]arachidonylphosphatidylcholine as substrate, since this enzyme shows high selectivity for arachidonyl residues at the *sn*-2 position of phospholipids. The concentration of 2 mM $CaCl_2$ in the assay mixture was 0.4 mM higher than the combined concentration of EDTA (0.8 mM) and EGTA (0.8 mM) present in the assay mixture. Slightly higher activity of $cPLA_2$ was observed with higher concentrations of $CaCl_2$; however, we found that this concentration of $CaCl_2$ (2 mM) in relation to the concentration of chelators in the assay mixture ensures better and more consistent results, especially when using agonists that stimulate $cPLA_2$ activity. Under the assay conditions employed, the activity of $cPLA_2$ was linear for 40 min or up until 4% of the substrate was degraded by the enzyme (Figure 1).

Changes in the electrophoretic mobility and catalytic activity of $cPLA_2$ following GM-CSF treatment

We examined whether GM-CSF, which is known to prime neutrophils for subsequent stimulation by other agonists, is capable of phosphorylating and activating $cPLA_2$. Neutrophils were incubated in the absence and presence of 500 pM GM-CSF at 37 °C for various time intervals, and the cytosolic fraction obtained from these neutrophils was subjected to SDS/PAGE immunoblotting. As shown in Figure 2, the extent of $cPLA_2$ phosphorylation was markedly increased with GM-CSF stimulation, as indicated by its slower electrophoretic mobility. The phosphorylation of $cPLA_2$ was apparent 2 min after incubation, and was progressive with the time for which neutrophils were exposed to GM-CSF. In order to examine whether the phosphorylation of $cPLA_2$ is reflected in an increased activity of the enzyme, we measured $cPLA_2$ activity in the cytosol of unstimulated and GM-CSF-stimulated neutrophils. As shown in Figure 3, incubation of neutrophils with GM-CSF caused a progressive and time-dependent increase in $cPLA_2$ activity. An increase of 150% in $cPLA_2$ was obtained after 15 min of incubation, and a slight decrease in the enzyme activity was noticed at 20 min of incubation. These results suggest a correlation between the increased activity of $cPLA_2$ and its conversion into the slower-migrating $cPLA_2$ (i.e. phosphorylated form). Incubation of the cytosolic fraction of unstimulated and GM-CSF-stimulated neutrophils with anti-$cPLA_2$ antibody

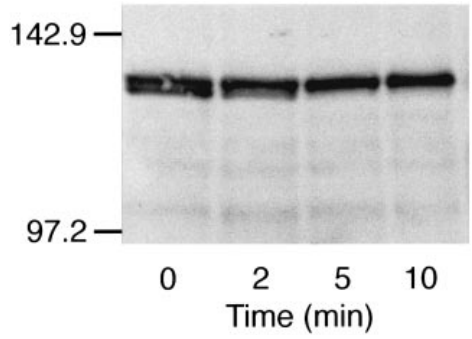

Figure 2 Phosphorylation of $cPLA_2$ in response to stimulation of neutrophils with GM-CSF

Neutrophils (2×10^7 cells) in 2 ml of HBSS were preincubated at 37 °C for 10 min. Cells were incubated without (0) or with 500 pM GM-CSF for 2, 5 or 10 min. The incubation was stopped by the addition of 30 ml of ice-cold HBSS. Neutrophils were suspended in 0.4 ml of sucrose buffer, sonicated (2×5 s bursts) and centrifuged at 150 000 ***g*** for 20 min. A sample (80–100 μg of protein) from the supernatant was loaded on SDS/PAGE, followed by immunoblotting with anti-$cPLA_2$ antibody as explained in the Materials and methods section. These results are representative of five different experiments. Here and in Figures 4 and 5, 142.9 and 97.2 (kDa) indicate positions of molecular-mass standards.

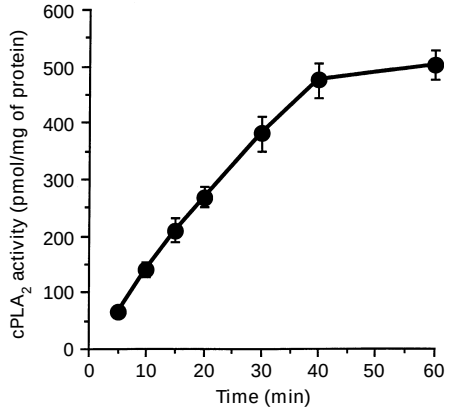

Figure 1 Time course of $cPLA_2$ activity after incubation for various time intervals

Neutrophils (5×10^7 cells) were suspended in 1.2 ml of lysis buffer. Cells were disrupted by sonication, then centrifuged at 150 000 ***g*** for 20 min. The supernatant obtained was used to measure $cPLA_2$ activity after incubation for various time intervals as explained in the Materials and methods section. The results are means ± S.E.M. of three different experiments.

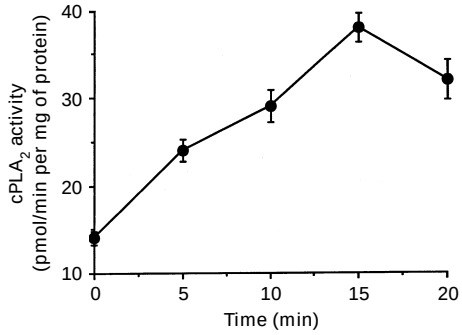

Figure 3 Stimulation of $cPLA_2$ activity by GM-CSF

Neutrophils (2×10^7 cells/ml) were incubated at 37 °C for 10 min in a shaking water bath. GM-CSF (500 pM) was added, and the incubation was continued for various time intervals. The reaction was stopped by adding 30 ml of ice-cold HBSS. Cells were centrifuged at 250 ***g*** for 5 min at 4 °C, resuspended in 0.4 ml of lysis buffer and sonicated. The supernatant obtained after centrifugation at 150 000 ***g*** for 20 min was used to measure $cPLA_2$ activity. The results are means ± S.E.M. of three different experiments.

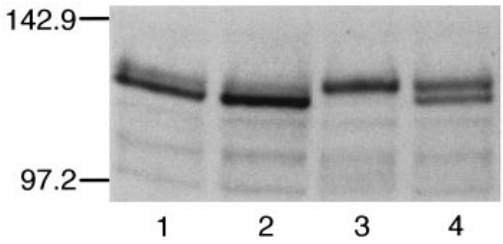

Figure 4 Dephosphorylation of $cPLA_2$ by PAP

The cytosolic fraction obtained from unstimulated (1) and GM-CSF (500 pM)-stimulated neutrophils (3) was incubated with PAP at a concentration of 50 μg/mg of protein for 30 min at 37 °C and loaded on SDS/PAGE, followed by immunoblotting with anti-$cPLA_2$ antibody. Other lanes: 2, unstimulated neutrophils after PAP treatment; 4, GM-CSF-stimulated neutrophils after PAP treatment. The results represent one of two experiments.

(10 μg/mg of protein) for 15 min completely abolished the $cPLA_2$ activity (results not shown). To verify whether the slower migration of $cPLA_2$ observed in neutrophils after GM-CSF stimulation is indeed the result of phosphorylation of the enzyme, the cytosol obtained from neutrophils stimulated with GM-CSF was treated with potato acid phosphatase (PAP). As shown in Figure 4, PAP treatment of the cytosolic fraction obtained from GM-CSF-stimulated neutrophils caused a conversion of the $cPLA_2$ from the slower-migrating (phosphorylated) form into the faster-migrating (dephosphorylated) form. These results show indeed that the GM-CSF-induced slower migration of $cPLA_2$ is due to phosphorylation of the enzyme. Similar results were also obtained by Lin et al. [29], using $cPLA_2$ from Chinese hamster ovary cells, and by Kramer et al. [36] in platelets. The presence of a doublet of $cPLA_2$ is indicative of the presence of two forms of the enzyme. The inability of PAP to induce complete dephosphorylation of $cPLA_2$, is because PAP is a non-specific phosphatase, or because $cPLA_2$ is a poor substrate for PAP.

Effect of genistein on GM-CSF-induced phosphorylation and activity of $cPLA_2$

It is well known that GM-CSF promotes tyrosine phosphorylation of several proteins in neutrophils, including the p42 and p44 isoforms of MAP kinase [15,17,18]. In order to verify whether MAP kinase is implicated in the GM-CSF-induced phosphorylation and increased activity of $cPLA_2$, neutrophils were preincubated with 1–100 μg of genistein/ml for 30 min before GM-CSF stimulation. It was found that genistein caused a concentration-dependent inhibition of GM-CSF-induced phosphorylation of $cPLA_2$, which was apparent even at a concentration of 1 μg/ml. The maximum effect of genistein was obtained at a concentration of 50 μg/ml (Figure 5). Pre-treatment of neutrophils with 50 μg/ml genistein also inhibited the GM-CSF-induced increase in the catalytic activity of $cPLA_2$ (Table 1).

Immunoprecipitation of $cPLA_2$

In order to substantiate further the notion that GM-CSF induces phosphorylation of $cPLA_2$, neutrophils were stimulated with 500 pM GM-CSF after preincubation of the cells with $[^{32}P]P_i$, and the $cPLA_2$ was subjected to immunoprecipitation using monoclonal anti-$cPLA_2$ conjugated to anti-rabbit IgG–agarose beads. The results presented in Figure 6 show that GM-CSF caused an increase in the phosphorylation of $cPLA_2$ as compared with the enzyme from resting cells.

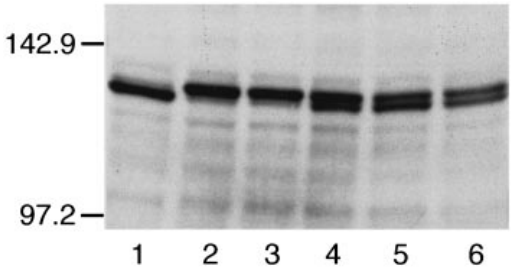

Figure 5 Effect of genistein on GM-CSF-stimulated phosphorylation of $cPLA_2$

Neutrophils (2 ml; 1×10^7 cells/ml) were preincubated without (lanes 1 and 2) or with 1, 25, 50 or 100 μg/ml genistein (lanes 3–6 respectively) for 30 min at 37 °C in a shaking water bath. GM-CSF was added to a final concentration of 500 pM (lanes 2–6) and incubation was continued for 10 min. Incubation was stopped by adding 30 ml of ice-cold HBSS, and neutrophils were pelleted by centrifugation (250 ***g*** for 5 min at 4 °C). Then 0.4 ml of sucrose buffer was added, and cells were transferred to microfuge tubes and sonicated. The supernatant obtained after centrifugation at 150 000 ***g*** for 20 min was loaded (80–100 μg of protein) on SDS/PAGE and immunoblotted with anti-$cPLA_2$ antibody as explained in the Materials and methods section. The results represent one of four experiments.

Table 1 Effect of genistein on GM-CSF stimulation of $cPLA_2$ activity

Neutrophils (2×10^7 cells) were suspended in 2 ml of HBSS and incubated at 37 °C for 30 min in a shaking water bath in the presence and absence of 50 μg/ml genistein. GM-CSF (500 pM) was added and incubation was continued for 10 min. The reaction was stopped by adding 30 ml of ice-cold HBSS, and neutrophils were precipitated by centrifugation at 250 ***g*** for 5 min at 4 °C, then resuspended in 0.4 ml of lysis buffer. Cells were disrupted by sonication, and the homogenate was centrifuged at 150 000 ***g*** for 20 min. The resulting supernatant was used for measuring $cPLA_2$ activity. The results are means ± S.E.M. of three experiments.

Treatment	$cPLA_2$ activity (pmol/min per mg of protein)
Control	14.5 ± 1.2
Genistein	15.6 ± 1.9
GM-CSF	31.7 ± 3.3
Genistein + GM-CSF	19.2 ± 2.1

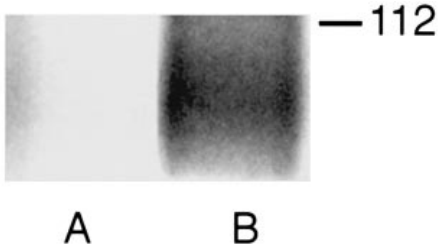

Figure 6 Immunoprecipitation of $cPLA_2$

Neutrophils (5×10^7) were washed and incubated with 0.5 mCi of $[^{32}P]P_i$. Cells were washed three times with buffer A, resuspended in HBSS and divided into two tubes. One sample was stimulated with 500 pM GM-CSF and the other was used as control. After incubation with GM-CSF for 15 min at 37 °C, cells were centrifuged, and 100 μl of boiling 1% SDS was added to the pellet and the mixture was immediately boiled for 15 min. Supernatant obtained after centrifugation at 15 000 ***g*** for 10 min was transferred to tubes containing anti-rabbit IgG–agarose bends to which monoclonal anti-$cPLA_2$ antibody was conjugated. After overnight incubation, the agarose beads were washed and boiled with 50 μl of Laemmli buffer for 5 min. The supernatant obtained after brief centrifugation was loaded for SDS/PAGE (8% gels). Gels were dried and subjected to autoradiography. The results represent one of two identical experiments. (A) Control; (B) GM-CSF-stimulated. In Figures 6–9, 112 (kDa) indicates the position of a molecular-mass standard.

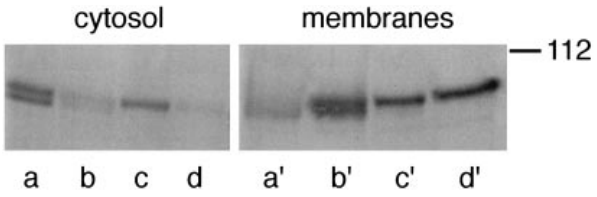

Figure 7 Effect of A23187 on the phosphorylation and distribution of $cPLA_2$ between the cytosol and the membranes

Neutrophils (3×10^7) were incubated in HBSS without (a, b) or with (c, d) 1 μM A23187 and 1 mM Ca^{2+}. After incubation for 5 min at 37 °C, cells were centrifuged, washed with 2 × 20 ml of HBSS and resuspended in 0.4 ml of lysis buffer (which contains 1 mM EGTA and 1 mM EDTA) without (a, c) or with 2.5 mM Ca^{2+} (b, d) and sonicated. The homogenate was centrifuged at 15000 ***g*** for 10 min to precipitate unbroken cells and nuclei, and the resulting supernatants were centrifuged at 150000 ***g*** for 30 min. The corresponding pellets were resuspended in the same buffers by brief sonication. Samples (80 μg of protein) from the supernatants and membranes were treated with Laemmli sample buffer, loaded for SDS/PAGE (8% gels) and probed with polyclonal anti-$cPLA_2$ antibody. The results represent one of four identical experiments. Lanes: a, b, $cPLA_2$ from the cytosol of unstimulated cells in the absence and presence of Ca^{2+}, respectively; a′, b′, $cPLA_2$ from membranes obtained from a and b respectively; c, d, $cPLA_2$ from A23187-treated cells in the absence and presence of Ca^{2+}, respectively. c′, d′, $cPLA_2$ from membranes obtained from c and d, respectively.

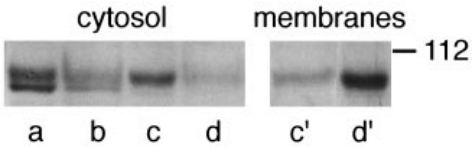

Figure 8 Effect of GM-CSF on the distribution of $cPLA_2$ between the cytosol and membranes

Neutrophils (3×10^7) were incubated without (a, b) or with (c, d) 500 pM GM-CSF for 15 min at 37 °C. Cells were washed and sonicated in 0.4 ml of lysis buffer without (a, c) or with 2.5 mM Ca^{2+} (b, d). After sonication, the post-nuclear cytosolic and the membranous fractions were subjected to SDS/PAGE and immunoblotted with anti-$cPLA_2$ antibody as explained in the Materials and methods section. The results represent one of three identical experiments. Lanes: a, b, $cPLA_2$ from the cytosol of unstimulated cells in the absence and presence of Ca^{2+}, respectively; c, d, $cPLA_2$ from GM-CSF-stimulated cells in the absence and presence of Ca^{2+}, respectively; c′, d′, $cPLA_2$ from membranes obtained from c and d in the absence and presence of Ca^{2+}, respectively.

Translocation of $cPLA_2$ to the neutrophil membranes

It is well established that Ca^{2+} ions play an important role in the cell activation and the mobilization of AA. We examined the effect of the Ca^{2+} ionophore A23187 on the phosphorylation and the distribution of $cPLA_2$ between the cytosolic and the membranous fractions of neutrophil homogenate. As shown in Figure 7, it is apparent that most of the $cPLA_2$ in resting cells resides in the cytosol. Addition of Ca^{2+} to the lysis buffer before homogenization resulted in the association of both forms (phosphorylated and dephosphorylated) of $cPLA_2$ with the membranes. Stimulation of the cells with A23187 resulted in the phosphorylation and the association of the enzyme with the neutrophil membranes. Homogenization of the A23187-stimulated cells with lysis buffer containing 1 mM EDTA and 1 mM EGTA, and with no added Ca^{2+}, failed to solubilize $cPLA_2$. When cells were stimulated with GM-CSF, most of the enzyme was recovered in the cytosolic fraction of the neutrophil homogenate (Figure 8). Homogenization of the GM-CSF-stimulated neutrophils in a Ca^{2+}-containing buffer resulted in the translocation of the enzyme to the particulate fraction. Since GM-CSF primes neutrophils for AA release induced by fMLP,

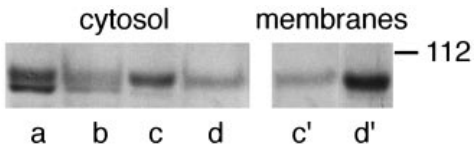

Figure 9 Effect of fMLP on the distribution of $cPLA_2$ in GM-CSF stimulated neutrophils

Neutrophils (3×10^7) were incubated without (a, b) or with 500 pM GM-CSF for 15 min, followed by incubation with 0.1 μM fMLP for 3 min (c, d). Cells were washed and sonicated in lysis buffer without (a, c) or with 2.5 mM Ca^{2+} (b, d). The homogenate was centrifuged at 15000 ***g*** for 10 min, and the supernatant obtained was centrifuged at 150000 ***g*** for 30 min. The pellets were suspended in lysis buffer without or with Ca^{2+}, and 80 μg samples of protein from the supernatants and the pellets were subject to SDS/PAGE and immunoblotted with anti-$cPLA_2$ antibody. The results represent one of three experiments with identical data. Lanes: a, b, $cPLA_2$ from the cytosol of control cells lysed in the absence and presence of Ca^{2+}, respectively; c, d, $cPLA_2$ from the cytosol of GM-CSF- and fMLP-stimulated cells in the absence and presence of Ca^{2+}, respectively; c′, d′, $cPLA_2$ from the membranes of GM-CSF- and fMLP-stimulated neutrophils in the absence and presence of Ca^{2+}, respectively.

which increases intracellular Ca^{2+} concentrations, the effect of fMLP on the distribution of $cPLA_2$ in GM-CSF-stimulated neutrophils was examined. Cells were incubated with GM-CSF for 15 min, followed by 2 min incubation with fMLP. The results presented in Figure 9 show that fMLP caused translocation of the enzyme to the cell membranes in the GM-CSF-stimulated neutrophils.

DISCUSSION

The experimental data presented in this paper contribute to the understanding of the molecular basis of GM-CSF priming of human neutrophils. Previous reports have appreciated the role of GM-CSF in augmenting the stimulation of neutrophil functional activities by other stimuli [6–10]. GM-CSF alone up-regulates fMLP receptors and the G-protein in the cell membrane [21,22]. However, GM-CSF does not increase the Ca^{2+} concentration in the cell, nor does it activate phospholipase C. Despite the dramatic priming effect of GM-CSF on the release of AA and its metabolites by Ca^{2+}-mobilizing agents such as fMLP, GM-CSF by itself does not promote AA release, nor does it increase its metabolites. Since AA and its metabolites are at the centre of neutrophil activation, we were especially interested in examining the effect of GM-CSF on the recently discovered $cPLA_2$ activity, which is known to play a central role in the agonist-mediated AA mobilization in various tissues [29]. The data reported herein clearly show that, although GM-CSF is incapable of AA mobilization, it does phosphorylate and increase the activity of $cPLA_2$. $cPLA_2$ exists in two apparently interconvertible forms: an active phosphorylated form, and an inactive dephosphorylated form. It has been reported that the activated form is translocated to the cellular membranes, where its substrates, mainly phosphatidylcholine and phosphatidylethanolamine, are located [27,28]. The translocation of the active form of $cPLA_2$ to the membrane is a prerequisite for AA release by this enzyme. In the present study we demonstrate that GM-CSF alone is capable of phosphorylating and activating $cPLA_2$, but does not cause translocation of the enzyme to the membranes. This translocation process is Ca^{2+}-dependent, since addition of Ca^{2+} to neutrophil homogenate, or treatment of neutrophils with Ca^{2+}-increasing agents such as fMLP after GM-CSF stimulation, or stimulation of neutrophils with A23187, result in the association of the enzyme with the membranes. These results might explain the great enhancement of AA release by Ca^{2+}-mobilizing agents

following GM-CSF stimulation [19]. The Ca^{2+} increase exerted by these agents causes an immediate translocation of the already phosphorylated and active $cPLA_2$ to the membrane, where its activity is expressed. Similar results showing fMLP-induced up-regulation of $cPLA_2$ associated with the neutrophil membranes were obtained by Durstin et al. [37]. Our results show that Ca^{2+} causes the translocation of both forms of $cPLA_2$ to the membranes. However, we assume that the phosphorylated form is the one which is responsible for the release of AA. The exact mechanism underlying the translocation of $cPLA_2$ is not understood. However, since EDTA and EGTA were unable to solubilize the translocated enzyme from the membranes, it is reasonable to assume that the association of $cPLA_2$ with the membrane is not due to mere ionic strength exerted by the Ca^{2+} ions.

Under our assay conditions, where 4 mM DTT was present to inhibit any contaminating activity of other phospholipases [30,31], only the $cPLA_2$ activity was measured. Anti-$cPLA_2$ antibody completely abolished the measured $cPLA_2$ activity of control and GM-CSF-stimulated neutrophils. The inability of GM-CSF to provoke AA release, which is the rate-limiting step for prostaglandin and leukotriene (primarily leukotriene C_4) synthesis in the activation process of neutrophils, is because GM-CSF does not cause an increase in cytosolic Ca^{2+} concentration. The phosphorylation of $cPLA_2$ is probably due to an increase in MAP kinase activity, which was reported to be dramatically increased by stimulation of neutrophils by GM-CSF [18]. Recently it was reported that MAP kinase is responsible for the phosphorylation and activation of $cPLA_2$ [27]. The results reported in the present paper that genistein, a tyrosine kinase inhibitor that inhibits MAP kinase, is capable of inhibiting the phosphorylation and the increased activity of $cPLA_2$ elicited by GM-CSF, speak in favour of an involvement of MAP kinase in the phosphorylation and activation of $cPLA_2$. The release of AA from the *sn*-2 position of phospholipids results also in an increase in the concentration of the corresponding lysophospholipids. This is an important metabolic step for the biosynthesis of PAF [9]. PAF accumulation in neutrophils in response to $cPLA_2$ activity was implicated in the control of degranulation and superoxide generation [6,10]. The present study suggests that GM-CSF primes neutrophils for subsequent stimulation by other Ca^{2+}-mobilizing agents through the activation of $cPLA_2$. The synthesis of agents that control $cPLA_2$ activity will be of importance as a pharmacological tool in regulating the activation of neutrophils, and therefore the general inflammatory conditions.

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