Cytosolic phospholipase A₂ and its mode of activation in human neutrophils by opsonized zymosan

Correlation between 42/44 kDa mitogen-activated protein kinase, cytosolic phospholipase A₂ and NADPH oxidase

Inbal HAZAN*, Raya DANA*, Yoseph GRANOT† and Rachel LEVY*¹

*Infectious Disease Laboratory and Clinical Biochemistry Department, Faculty of Health Sciences, Ben-Gurion University of the Negev and Soroka Medical Center, 84105 Beer Sheva, Israel, and †Department of Life Sciences, Ben-Gurion University of the Negev, 84105 Beer Sheva, Israel

The role of cytosolic phospholipase A₂ (cPLA₂) and its mode of activation by opsonized zymosan (OZ) was studied in human neutrophils in comparison with activation by PMA. The activation of cPLA₂ by 1 mg/ml OZ or 50 ng/ml PMA is evidenced by its translocation to the membrane fractions on stimulation. This translocation is consistent with dithiothreitol (DTT)-resistant phospholipase A₂ (PLA₂) activity detected in the membranes of activated cells. Neutrophils stimulated by either OZ or PMA exhibited an immediate stimulation of extracellular-signal-regulated kinases (ERKs). The inhibition of ERKs, DTT-resistant PLA₂ and NADPH oxidase activities by the MAP kinase kinase inhibitor PD-98059 indicates that ERKs mediate the activation of cPLA₂ and NADPH oxidase stimulated by either OZ or PMA. The protein kinase C (PKC) inhibitor GF-109203X inhibited epidermal growth factor receptor peptide

kinase activity, the release of [³H]arachidonic acid, DTT-resistant PLA₂ activity and superoxide generation induced by PMA, but did not inhibit any of these activities induced by OZ. PKC activity was similarly inhibited by GF-109203X in membrane fractions separated from neutrophils stimulated by either PMA or OZ. In the presence of the tyrosine kinase inhibitor genistein, ERKs, PLA₂ and NADPH oxidase activities were inhibited in cells stimulated by OZ, whereas they were hardly affected in cells stimulated by PMA. The results suggest that the activation of cPLA₂ by PMA or OZ is mediated by ERKs. Whereas PMA stimulates ERKs activity through a PKC-dependent pathway, signal transduction stimulated by OZ involves tyrosine kinase activity leading to activation of ERKs via a PKC-independent pathway.

INTRODUCTION

Recent studies have indicated the role of phospholipase A₂ (PLA₂) in stimulated phagocytic cells. Arachidonic acid (AA), as well as some of its metabolites, can act as first and second messengers [1]. AA by itself stimulates NADPH oxidase activity, resulting in superoxide production in intact cells and in a cell-free assay [2,3], but in both cases the relatively high concentration of AA calls into question the physiological relevance of AA. In neutrophils and macrophages an increase in PLA₂ activity and a release of AA have been reported to occur in association with stimuli that also activate the oxidase [4–6]. In a series of studies we [7–9] and others [10,11] have demonstrated the necessity of PLA₂ in the transduction pathway stimulating NADPH oxidase activity in peripheral blood monocytes and granulocytes.

In the past decade, several mammalian PLA₂s have been found, purified and cloned in phagocytic cells. (1) The mammalian type II, 14 kDa non-pancreatic secreted PLA₂ (sPLA₂) [12,13] was found in neutrophil and monocyte microcosms [14]. sPLA₂ is Ca²⁺-dependent at millimolar concentrations and sensitive to reducing conditions [15]. (2) The Ca²⁺-independent PLA₂ (40 kDa), which was found in muscle cells [16], has recently been identified in the mouse macrophage cell line P388D [17]. (3) The 85 kDa cytosolic PLA₂ (cPLA₂), insensitive to reducing conditions present in the cytosol, has been purified from U937 cells [18]. It has a high specificity for the phospholipids that contain *sn*-2-arachidonate [19]. The cPLA₂, whose translocation to the membranes is Ca²⁺-dependent [20,21], is activated by

phosphorylation on Ser-505 mediated by mitogen-activated protein kinase (MAP kinase) [22].

MAP kinases are a family of serine/threonine kinases whose activation requires phosphorylation on tyrosine and threonine residues. The MAP kinase family includes the 42–44 kDa extracellular-signal-regulated kinase (ERKs) and the stress-activated protein kinases (SAPK): the 38 kDa reactivating kinase (RK) and the 54 kDa *c-jun*-N-terminus (JNK) [23]. The ERKs are phosphorylated by bifunctional MAP kinase (ERK) kinase (MEK), which itself is activated by phosphorylation [23,24]. New evidence suggests several pathways of MEK activation, including a recently cloned MEK kinase, but the best delineated pathway indicates the involvement of Ras and the Raf, which phosphorylates and activates MEK [25]. MAP kinase activity (42/44 kDa) has been shown to be activated by a wide variety of agents in neutrophils such as formyl-Met-Leu-Phe (FMLP), granulocyte/macrophage colony-stimulating factor or lipopolysaccharide [26–32], but the role of MAP kinase in neutrophils is totally unknown.

This work was designed to define whether cPLA₂ participates in the physiological stimulation of neutrophils by opsonized zymosan (OZ) and to study its mode of activation. The research was performed in parallel with the non-physiological stimulus PMA, which activates protein kinase C (PKC) directly. Because cPLA₂ was shown to be phosphorylated and activated by ERKs in other systems, the involvement of these MAP kinases was studied. Our results indicate that cPLA₂ is activated by both OZ and PMA in human neutrophils. The activation of cPLA₂ is

Abbreviations used: AA, arachidonic acid; cPLA₂, cytosolic PLA₂; dbcAMP, dibutyl cAMP; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; EGFR-P, epidermal growth factor receptor peptide; FMLP, formyl-Met-Leu-Phe; HBSS, Hanks balanced salts solution; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; OZ, opsonized zymosan; PKC, protein kinase C; PLA₂, phospholipase A₂; sPLA₂, secreted PLA₂.

¹ To whom correspondence should be addressed.

probably mediated by ERKs that are stimulated with PMA or OZ by alternative pathways.

MATERIALS AND METHODS

Materials

PMA, zymosan A, *p*-nitrophenyl phosphate, FMLP, ionomycin, cytochrome *c*, PMSF, leupeptin, *N*⁶,2'-*O*-dibutyryladenine 3',5'-cAMP (dbcAMP), histone (type III-S), 1,2-dioleoyl-*sn*-glycerol, phosphatidylserine, ATP, dithiothreitol (DTT) and genistein were obtained from Sigma Chemical Co. [³H]AA (100 Ci/mmol) was purchased from NEN-DuPont. [γ -³²P]ATP (3000 Ci/mmol) was purchased from Rotem (Dimona, Israel). Anti-(MAP kinase) (sc-153, K-23) antibodies were from Santa Cruz Biotechnology. Anti-(rabbit IgG)-horseradish peroxidase and the enhanced chemiluminescence detection kit for immunoblotting were from Amersham. GF-109203X (bisindolylmaleimide) was from Calbiochem-Novabiochem Co. The epidermal growth factor-receptor peptide T-669 was synthesized at Weisman Institute (Rehovot, Israel). PD-98509 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.).

Neutrophil isolation

Neutrophils were separated by Ficoll/Hypaque centrifugation, dextran sedimentation and hypotonic lysis of erythrocytes [33].

Superoxide anion measurements

The production of superoxide anion ($O_2^{\cdot-}$) by intact cells was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* [34]. Cells were suspended (5×10^5 cells per well) in 100 μ l of Hanks balanced salts solution (HBSS) containing 150 μ M ferricytochrome *c* and stimulated by the addition of the appropriate stimulus. The reduction of ferricytochrome *c* was followed by a change of absorbance at 550 nm every 2 min for 20 min on a Thermomax microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.). The maximal rates of superoxide generation were determined by using the value of $21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ϵ_{550} .

OZ was prepared as follows: 20 mg of zymosan was incubated with 1 ml of pooled human serum (lipopolysaccharide-free) for 1 h at 37 °C and washed three times with HBSS.

Isolation of membrane and cytosol fractions

Membrane and cytosol fractions were prepared as described previously [34]. Cells suspended at 10^8 cells/ml in relaxation buffer [100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$ /1.25 mM EGTA/10 mM Hepes (pH 7.4)] containing 1 mM PMSF, 100 μ M leupeptin, 10 μ g/ml aprotinin and 20 mM *p*-nitrophenyl phosphate, were sonicated at 4 °C three times for 20 s, resulting in approx. 95% cell breakage. Nuclei, granules and unbroken cells were removed by centrifugation (1 min at 15600 *g*) and to the postnuclear supernatant were added 1 mM Na_3VO_4 and 25 mM NaF from 20-fold concentrated and 100-fold concentrated stock solutions of the phosphatase inhibitors respectively. The supernatant was centrifuged in a Beckman Airfuge (60 min at 134000 *g*) to obtain a cell membrane pellet and a cytosol supernatant. Membranes were suspended at 10^8 cell equivalents/ml in relaxation buffer. Solubilized membrane and cytosol were stored at -70 °C.

PLA₂ activity

In cell fractions

Neutrophils (5×10^6 cells/ml) in HBSS were stimulated for 2 min at 37 °C by 1 mg/ml OZ or 50 ng/ml PMA; the reaction was stopped by 1:10 dilution with cold HBSS and immediate centrifugation at 4 °C. Neutrophils were sonicated and membranes and cytosol fractions were separated as described above. PLA₂ activity was performed immediately after fraction separation by using sonicated dispersions of 1-stearoyl-2-[¹⁴C]arachidonyl phosphatidylcholine (30 μ M, 50000 d.p.m. per assay) co-sonicated with 1,2-dioleoyl-*sn*-glycerol at molar ratio 2:1 in the assay mixture [35]. The assay mixture contained the phospholipid substrate in 80 mM KCl/5 mM $CaCl_2$ /5 mM DTT/1 mg/ml BSA/1 mM EDTA/10 mM Hepes (pH 7.4) [36]. The reaction was started by the addition of 50 μ g of neutrophil cytosolic or membrane protein (within the linear protein range of the reaction) and incubated at 37 °C in a shaking water bath for 10 min. The reaction was stopped by the addition of 2 ml of Dole's reagent [37] [isopropyl alcohol/heptane/0.5 M H_2SO_4 (20:5:1, by vol.)], which was followed by the addition of 1.2 ml of heptane and 1 ml of water. The solution was vortex-mixed and centrifuged (1000 *g* for 10 min). A 1 ml portion of the organic phase was added to 200 mg of silica with 2 ml of heptane and centrifuged to remove contaminating phospholipids and lysophospholipids. The radioactivity in labelled fatty acids was counted (Packard Spectrometer 1900CA). PLA₂ activity was defined as the percentage of [³H]AA in total radioactivity.

In intact cells

Incorporation and release of [³H]AA were performed as previously reported [9]. Neutrophils (10^8 cells/ml) were incubated for 30 min at 37 °C in Ca^{2+} - and Mg^{2+} -free PBS containing 1 μ Ci of [³H]AA. Neutrophils were washed once with PBS containing 0.1% fatty acid-free human serum albumin and twice with PBS. The pellet was resuspended to 10^7 cells/ml in incubation buffer [137 mM NaCl/5.4 mM KCl/5.6 mM glucose/10 mM Hepes (pH 7.4)] containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% human serum albumin. Neutrophils (10^7 cells/ml) were incubated at 37 °C with the appropriate stimulus in the presence or absence of inhibitors for 2.5, 5, 10 or 15 min. The reaction was terminated by centrifugation at 4 °C and samples of the supernatants were counted by liquid scintillation (Packard Spectrometer 1900CA). In every experiment 100 μ l aliquots were taken from each reaction mixture to measure superoxide production on a microtitre plate.

Preparation of antibodies against cPLA₂

A fraction of 410 bp from the 5' primer of the cPLA₂ cDNA, which was given to us by Dr. Knof (Genetic Institutes Inc, Cambridge, MA, U.S.A.) was engineered into pGEX1 (Pharmacia) and transfected into bacteria. The protein bound to glutathione transferase was amplified by the addition of isopropyl β -D-thiogalactoside and purified on a glutathione-agarose column. Polyclonal antibodies against this fraction were raised in rabbits and were efficient at recognizing the recombinant cPLA₂ (Dr. Knof, Genetic Institutes), whereas preimmunized serum could not detect this protein.

MAP kinase assay

Neutrophils (5×10^6 cells/ml) in HBSS were stimulated for 2 min at 37 °C with 1 mg/ml OZ or 50 ng/ml PMA; the reaction was stopped by dilution 1:10 with cold HBSS and immediate centrifugation at 4 °C. In every experiment 100 μ l aliquots were

taken from each reaction mixture to measure superoxide production on a microtitre plate. Neutrophils were sonicated as described above and centrifuged (1 min at 15600 *g*) to remove granules, nuclei and unbroken cells. MAP kinase activity in neutrophil lysates was assessed by alternative methods.

Phosphorylation of epidermal growth factor receptor peptide (EGFR-P) (T-669)

This was done as described in [38]. Samples (1 μ g of protein) were incubated for 25 min in 30 °C in a final volume of 30 μ l containing 20 mM Hepes buffer, pH 7.0, 2 mM DTT, 10 mM MgCl₂, 0.1 mg/ml BSA, 0.1 mM EGTA, 100 μ M ATP, 0.5 μ Ci of [γ -³²P]ATP per sample and 1 mg/ml EGFR-P. The reaction was terminated by the addition of 10 μ l of stop solution containing 45 % (v/v) formic acid and 25 mM ATP. The reaction mixtures were spotted on filters (1.5 cm² square of P-81 phosphocellulose Whatman paper) and the filters were washed twice with, per 10 filters, 100 ml of 1 M acetic acid containing 4 mM sodium pyrophosphate for 30 min each. After the final wash, each paper was air-dried and transferred to a scintillation vial. The incorporation of radioactivity was determined by liquid-scintillation counting.

Electrophoretic mobility of ERKs

This was detected by Western blot analysis (details are given in the next section).

Immunoblot analysis

Lysates, cytosol or membrane fractions were solubilized in five volumes of sample buffer and analysed by electrophoresis on SDS/7.5 % (w/v) polyacrylamide gels. The amount of protein in each sample was quantified with the Pierce bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA standards. The resolved proteins were electrophoretically transferred to nitrocellulose, which was stained with Fast Green to detect protein banding, and then blocked in 3 % (w/v) BSA in TBS [10 mM Tris/135 mM NaCl (pH 7.4)]. The blots were incubated overnight at room temperature with a 1:1000 dilution of primary rabbit anti-ERK-1 antibodies (K-23; Santa Cruz) or rabbit anti-ERK-2 antibodies (sc-153; Santa Cruz), or a 1:1000 dilution of rabbit anti-cPLA₂ antibodies in TBS-T (TBS containing 0.15 % Tween-20). After being washed with TBS-T (four washes for 20 min each) the membranes were incubated with a 1:3000 dilution of the second antibody, peroxidase-conjugated goat anti-(rabbit IgG) (Amersham) for 1 h at room temperature and developed with the enhanced chemiluminescence detection system (Amersham).

PKC activity

PKC activity was determined by the method of Isakov et al. [39] with some modification; 5 $\times$ 10⁶ cells/ml were pretreated with different concentrations of GF-109203X for 3 min at 37 °C and then stimulated for 2 min with 50 ng/ml PMA or 1 mg/ml OZ. The reaction was terminated by the addition of 2 vol. of cold buffer and the mixture was centrifuged at 4 °C. The pellet was resuspended in relaxation buffer and sonicated; the cytosol and particulate fractions were separated (as described in the Isolation of membrane and cytosol fractions section). The particulate and cytosol fractions were dissolved in seven volumes of buffer A containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF and 10 mM 2-mercaptoethanol. Triton X-100 (1 %, w/v) was added to the particulate fractions. The

cytosolic and particulate fractions were chromatographed on DEAE-cellulose columns (0.5 cm radius $\times$ 2.5 cm height) that had been equilibrated with buffer A. PKC was eluted with buffer A containing 100 mM NaCl. Enzyme activity was assayed at 30 °C for 10 min by measuring the incorporation of [³²P]phosphate from [γ -³²P]ATP into histone in the presence or absence of phosphatidylserine (100 μ g/ml), 1,2-dioleoyl-*sn*-glycerol (100 μ g/ml) and CaCl₂ (1 mM) in a final volume of 200 μ l. The reaction mixture contained 20 mM Tris/HCl, pH 7.4, 10 mM magnesium acetate, 10 μ M ATP (1 μ Ci of [γ -³²P]ATP per sample), 25 μ g/ml histone with or without lipids (dispersed by sonication) and Ca²⁺. The reaction was started by adding 50 μ l of particulate or cytosolic fractions and terminated by the addition of 0.25 ml of ice-cold BSA (2 mg/ml) and 0.5 ml of trichloroacetic acid (25 %, w/v). The mixtures were spotted on 0.45 μ m filters (Whatman GF/F), washed four times with 3 ml of ice-cold trichloroacetic acid (5 %, w/v) and dried. The amount of labelling was determined by scintillation counting.

Statistical analysis

The differences in means were analysed by Student's *t* test. The plots were drawn as least-squares regression lines and tested by analysis of variance. The results are expressed as means $\pm$ S.E.M.

RESULTS

Distribution of PLA₂ activity in fractions of stimulated neutrophils

DTT-resistant PLA₂ activity was studied in cytosol and membrane fractions of neutrophils stimulated with 1 mg/ml OZ or 50 ng/ml PMA. PLA₂ activity in a cell-free extract was assayed with 1-stearoyl-2-[1-¹⁴C]arachidonyl phosphatidylcholine as the substrate in the presence of 5 mM DTT, conditions that have been reported to be specific for cPLA₂ activity [20,22]. Neutrophils were stimulated for 2 min at 37 °C with PMA or OZ, cytosol and membrane fractions were separated, and the release of [¹⁴C]AA from labelled PC vesicles was determined. As shown in Figure 1(A), when the fractions were separated in the presence of 1 mM Ca²⁺ (and in the absence of EGTA) in the relaxation buffer, PLA₂ activity was detected mainly in the membranes. This activity was similar in the membranes of neutrophils stimulated with either PMA or OZ. When the fractions were separated in the absence of Ca²⁺ and in the presence of 1.25 mM EGTA in the relaxation buffer, the activity was detected mainly in the cytosol and was similarly induced by both agents (Figure 1B). In a parallel experiment, the location of cPLA₂ in cytosol and membrane fractions in resting and in neutrophils stimulated by OZ or PMA was studied by immunoblot analysis. Cells were stimulated and the cytosol and particulate fractions were separated as described in Figure 1(A). As shown in Figure 2, cPLA₂ was detected in the cytosol of resting cells. On activation with either PMA or OZ, cPLA₂ translocated to the plasma membranes, in accordance with PLA₂ activity detected in these fractions (Figure 1A).

Role of ERKs in the activation of cPLA₂ and NADPH oxidase by OZ or PMA

The translocation of cPLA₂ in accordance with the DTT-resistant PLA₂ activity in the membrane fractions suggests that cPLA₂ is the type of PLA₂ activated during the stimulation of neutrophils with either PMA or OZ. As it was shown that phosphorylation of cPLA₂ by ERKs is essential for its activation in different systems [22], we studied whether ERKs are activated by these agents. It has been reported that ERKs phosphorylate EGFR-P

Figure 1 [^{14}C]AA release from PC vesicles in neutrophil cytosol or membrane fractions

Neutrophils were stimulated with 50 ng/ml PMA or 1 mg/ml OZ for 2 min at 37 °C, after which the fractions were separated in the presence of 1 mM Ca^{2+} (A) or in the absence of Ca^{2+} and in the presence of 1.25 mM EGTA (B). The results, expressed as percentages of control, are means $\pm$ S.E.M. for three experiments performed in duplicate. There is a significant difference ($P < 0.001$) between the activities of resting and activated neutrophils in either cytosol or membrane fractions. There are no significant differences in the activities induced by OZ or PMA. [^{14}C]AA release (100%) in the cytosol or membranes of resting cells separated in the presence of Ca^{2+} ions (A) was 0.46 ± 0.06 or 0.095 ± 0.015 pmol/10 min per μg respectively, and in the absence of Ca^{2+} ions (B) was 0.47 ± 0.05 or 0.089 ± 0.018 pmol/10 min per μg respectively.

on Thr-669 [38]. We used a 19-residue synthetic peptide of this phosphorylation site as a substrate for ERK activity in neutrophil lysates stimulated by either PMA or OZ. As shown in Figure 3(A), ERKs were activated in a similar time-dependent manner by OZ or PMA, reaching maximal activation 1 min after stimulation, before the onset of [^3H]AA release or superoxide

Figure 2 Immunoblot analysis of cPLA₂ in cytosol and membrane fractions of resting neutrophils and cells stimulated with OZ or PMA

Protein (100 μg) was subjected to SDS/PAGE [7.5% (w/v) gel] followed by immunoblotting with anti-cPLA₂ polyclonal antibodies. Lane c, resting cells; lane p, PMA; lane o, OZ; lane rh, recombinant human cPLA₂. Abbreviation: kDa, kDa.

Figure 3 Time course of ERK activity in neutrophils stimulated by PMA or OZ

Neutrophils were stimulated for different intervals with 50 ng/ml PMA or 1 mg/ml OZ and the cells were lysed as described in the Materials and methods section. (A) Determination of EGFR-P kinase activity. The basal activity of resting cells, which was subtracted from the stimulated activity, was 0.35 nmol/min per mg. Results are shown from a representative experiment; two other experiments showed similar results. Inset, superoxide production and [^3H]AA release in intact neutrophils stimulated with PMA or OZ were determined in parallel. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ. Abbreviation: mOD, 1/1000 of an absorbance unit. ERK-1 (B) or ERK-2 (C) electrophoretic mobility. Lysates (100 μg /well) were subjected to SDS/PAGE [7.5% (w/v) gel] followed by immunoblotting with anti-(ERK-1) or anti-(ERK-2) polyclonal antibodies. Abbreviations: ', minute; '', second; kDa, kDa.

generation (Figure 3A, inset). Because the EGFR-P has been demonstrated to be the substrate for other serine/threonine kinases recognizing the -Pro-Leu-Thr-Pro- motif [40], we studied the time course of activation of ERK-1 and ERK-2 stimulated with either OZ or PMA by determining their electrophoretic mobility shift. As shown in Figures 3(B) and 3(C), the polyclonal antibodies anti-ERK-1 and anti-ERK-2 recognized two polypeptides of molecular masses approx. 44 and 42 kDa respectively in quiescent human neutrophils. Stimulation of cells with PMA or OZ decreased the electrophoretic mobility of the immunoreactive material, resulting in a clear mobility shift. The shift was induced in a similar time-dependent manner by OZ or PMA and was detected 20–40 s after stimulation. ERK activity measured by phosphorylation of a synthetic EGFR-P and by electrophoretic

Figure 4 Effect of PD-98059 on EGFR-P kinase activity, cPLA₂ activity and superoxide production in neutrophils stimulated with OZ or PMA

The cells were preincubated at 37 °C for 30 min with PD-98059 before stimulation with 50 ng/ml PMA or 1 mg/ml OZ. EGFR-P kinase activity (A), cPLA₂ activity (B) and superoxide production (C) were determined as described in the Materials and methods section. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ. The results in all panels, expressed as percentages of control, are means $\pm$ S.E.M. for three experiments performed in duplicate; 100% EGFR-P kinase activity was 0.79 ± 0.08 or 0.47 ± 0.07 nmol/min per mg stimulated by PMA or OZ, respectively; the basal activity of resting cells was 0.21 ± 0.02 nmol/min per mg; 100% DTT-resistant PLA₂ activity was 1.09 ± 0.3 or 0.93 ± 0.1 pmol/10 min per μ g in cytosol of neutrophils stimulated by PMA or OZ respectively; the basal activity of resting cells was 0.39 ± 0.1 pmol/10 min per μ g; 100% superoxide production was 15 ± 1.1 or 8.9 ± 0.5 nmol/min per 10^6 cells stimulated by PMA or OZ respectively; the basal superoxide production was 0.8 ± 0.02 nmol/min per 10^6 cells.

mobility shift of both ERK-1 and ERK-2, revealed similar results. In accordance with the higher ERK activity induced by PMA (Figure 3A) this agent converted all of the ERK-2 into its more slowly migrating form by 5 min (Figure 3C), whereas OZ was a less effective agonist.

To study the role of ERKs in mediating the stimulation of cPLA₂ and NADPH oxidase by OZ or PMA, the effect of the specific MEK inhibitor PD-98059 [41] was analysed. As shown in Figure 4, preincubation of neutrophils with PD-98059 in a concentration range of 25–100 μ M, for 30 min at 37 °C inhibited in a dose-dependent manner EGFR-P kinase, DTT-resistant

Figure 5 Effect of GF-109203X on EGFR-P kinase activity, [³H]AA release, cPLA₂ activity and superoxide production in neutrophils stimulated with OZ or PMA

The cells were preincubated at 37 °C for 3 min with GF-109203X before stimulation with 50 ng/ml PMA or 1 mg/ml OZ. EGFR-P kinase activity (A), [³H]AA release (B), DTT-resistant PLA₂ activity (C) and superoxide production (D) were determined as described in the Materials and methods section. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ. The results, expressed as percentages of control, are means $\pm$ S.E.M. for five experiments performed in duplicate; 100% EGFR-P kinase activity was 0.8 ± 0.15 or 0.5 ± 0.05 nmol/min per mg stimulated by PMA or OZ respectively; the basal activity of resting cells was 0.31 ± 0.05 nmol/min per mg; 100% [³H]AA release, expressed as a percentage of total, was $15.4 \pm 1.7\%$ or $14.5 \pm 1.4\%$ stimulated by PMA or OZ respectively; the basal activity of resting cells was $2.5 \pm 0.5\%$ of total; 100% DTT-resistant PLA₂ activity was 0.95 ± 0.1 or 0.98 ± 0.09 pmol/10 min per μ g in cytosol of neutrophils stimulated by PMA or OZ respectively; the DTT-resistant PLA₂ basal activity in resting cells was 0.41 ± 0.07 pmol/10 min per μ g; 100% superoxide production was 14 ± 1.3 or 9.5 ± 0.9 nmol/min per 10^6 cells stimulated by PMA or OZ respectively; the basal superoxide production was 0.75 ± 0.03 nmol/min per 10^6 cells.

Figure 6 Effect of preincubation of GF-109203X on PKC activity in enriched membrane fractions of neutrophils stimulated with 50 ng/ml PMA or 1 mg/ml OZ

The results, expressed as specific activities (pmol of ³²P/min per 10⁶ cells) are from a representative experiment; two others showed similar results. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ.

PLA₂ and NADPH oxidase activities stimulated with either 1 mg/ml OZ or 50 ng/ml PMA.

Role of PKC in the stimulation of neutrophils by OZ or PMA

Next we compared the role of PKC in the activation of ERKs, AA release, DTT-resistant PLA₂ activity and NADPH oxidase by OZ and by PMA. The effect of bisindolylmaleimide (GF-109203X), a specific inhibitor of PKC [42], on these activities stimulated by both agents was studied. As shown in Figure 5, pretreatment of neutrophils with GF-109203X (0–2 μM) for 3 min inhibited EGFR-P kinase activity and [³H]AA release, DTT-resistant PLA₂ activity and superoxide production in neutrophils stimulated by 50 ng/ml PMA in a dose-dependent manner, but did not affect these activities when stimulated by 1 mg/ml OZ. The resistance of the activities induced by OZ to the inhibitory effect of GF-109203X suggests that OZ activates a PKC-independent pathway. Thus we studied the effect of preincubation with GF-109203X on PKC activity in PMA- or OZ-stimulated neutrophils. PKC activity was assayed in enriched membrane or cytosol fractions (as described in the Materials and methods section). In resting cells more activity was detected in the cytosol (19.7 ± 0.59 pmol of ³²P/min per 10⁶ cells) than in the membrane fraction (8.6 ± 1.8 pmol of ³²P/min per 10⁶ cells). In cells stimulated by PMA or OZ the activity was detected mostly in the membrane fractions, 23.4 ± 6.4 and 21.6 ± 1.68 pmol of ³²P/min per 10⁶ cells respectively, compared with 7.1 ± 1.15 and 4.96 ± 0.72 pmol of ³²P/min per 10⁶ cells in the cytosol fractions. PKC activity in the membrane fractions was inhibited in an identical dose-dependent manner by preincubation with GF-109203X in cells stimulated with either OZ or PMA (Figure 6). PKC activity in the cytosol fractions separated from cells treated with GF-109203X was low, similar to the activity in cytosols of stimulated neutrophils in the absence of GF-109203X (results not shown). The concentrations of GF-109203X needed to inhibit PKC activity were higher than those needed to inhibit EGFR-P kinase activity, [³H]AA release, cPLA₂ activity or superoxide production stimulated by PMA (Figure 5). This difference might be due to the dilution of the inhibitor during the PKC purification procedure.

Figure 7 Effect of dbcAMP on superoxide production and on EGFR-P kinase activity

Neutrophils were preincubated with dbcAMP for 10 min before stimulation with 1 mg/ml OZ, 50 ng/ml PMA or 100 nM FMLP. (A) Effect of dbcAMP in the concentration range 0–2 mM on superoxide production. Superoxide production was studied during 20 min and the rate was determined. The results, expressed as percentages of control, are means ± S.E.M. for three experiments performed in duplicate; 100% superoxide production was 13.2 ± 1.2 or 9.9 ± 0.8 nmol/min per 10⁶ cells stimulated by PMA or OZ respectively; the basal superoxide production was 0.69 ± 0.05 nmol/min per 10⁶ cells. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ; ▲, 100 nM FMLP. (B) Effect of 2 mM dbcAMP on EGFR-P kinase activity. Neutrophils were preincubated with dbcAMP for 10 min and then stimulated by the different stimuli for 2 min. The reaction was stopped and EGFR-P kinase activity was determined as described in the Materials and methods section. The results, expressed as specific activities (nmol/min per mg), are means ± S.E.M. for three experiments performed in duplicate. The basal activity of resting cells was 0.48 ± 0.06 nmol/min per mg. (C) Effect of dbcAMP and GF-109203X on superoxide production and on EGFR-P kinase activity in neutrophils stimulated by OZ. Neutrophils were preincubated with dbcAMP for 10 min and/or GF-109203X for 3 min before stimulation with 1 mg/ml OZ. The cells were stimulated for 2 min for the determination of EGFR-P kinase activity and for 20 min for the determination of superoxide production rates. The results are means ± S.E.M. for three experiments performed in duplicate.

Effect of cAMP in the stimulation of neutrophils by OZ or PMA

ERKs might be activated by Ras through Raf-1, independently of PKC [43]. cAMP has been shown to prevent the transmission of signals from Ras to Raf-1 [44] and to inhibit the activation of Raf-1 stimulated by FMLP in human neutrophils [38]. Thus the

Figure 8 Effect of genistein on EGFR-P kinase activity, [³H]AA release and superoxide production, in neutrophils stimulated with 50 ng/ml PMA or 1 mg/ml OZ

The cells were preincubated at 37 °C for 30 min with genistein in the concentration range 0–0.3 mM before stimulation with 1 mg/ml OZ or 50 ng/ml PMA. EGFR-P kinase activity (A), [³H]AA release (B) and superoxide production (C) were determined as described in the Materials and methods section. The results, expressed as percentages of control, are means $\pm$ S.E.M. for five experiments performed in duplicate; 100% EGFR-P activity was 0.9 ± 0.15 or 0.7 ± 0.05 nmol/min per mg in cells stimulated by PMA or OZ respectively; the basal activity of resting cells was 0.25 ± 0.04 nmol/min per mg; 100% [³H]AA release, expressed as a percentage of total, was $15.1 \pm 1.2\%$ or $13.5 \pm 1.5\%$ stimulated by PMA or OZ respectively; the basal activity of resting cells was $2.5 \pm 0.5\%$ of total; 100% superoxide production was 15 ± 1.7 or 9.1 ± 1.2 nmol/min per 10^6 cells stimulated by PMA or OZ respectively; the basal superoxide production was 0.52 ± 0.03 nmol/min per 10^6 cells. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ.

effect of dbcAMP on MAP kinase activity, measured by EGFR-P kinase activity stimulated by OZ or PMA, was studied and compared with that stimulated by FMLP. In addition, the effect of dbcAMP on superoxide production stimulated by OZ or PMA was studied. As shown in Figure 7(A), preincubation of neutrophils with dbcAMP for 10 min in a concentration range of 0.1–2 mM did not affect superoxide production stimulated by PMA and had a slight and non-significant effect on this activity stimulated by OZ. dbcAMP caused a dose-dependent inhibition of superoxide generation stimulated by FMLP that was significant from 0.5 mM ($P < 0.001$), achieving a maximal inhibition of $38 \pm 12\%$ at 1 mM dbcAMP. The effect of dbcAMP on EGFR-P kinase activity stimulated by these three stimuli (shown in Figure 7B) was consistent with its effect on superoxide generation. Whereas dbcAMP caused a significant ($P < 0.001$) inhibition of EGFR-P kinase activity stimulated by FMLP, it did not significantly affect this activity when stimulated by OZ.

Figure 9 Effect of genistein on ERK-2 shift induced by 50 ng/ml PMA or 1 mg/ml OZ

Cells (5×10^6 /ml) were preincubated at 37 °C for 30 min in the absence or in the presence of various concentrations of genistein (Gen) before the addition of the stimulant. Lysates (100 μ g per well) were subjected to SDS/PAGE [7.5% (w/v) gel], followed by immunoblotting with anti-(ERK-2) polyclonal antibodies. In the right-hand lane lysates of resting cells (Con) and PMA-stimulated cells were loaded together to distinguish between the phosphorylated and non-phosphorylated forms. Abbreviation: KDa, kDa.

dbcAMP did not affect EGFR-P kinase activity when stimulated with PMA. Combination of the PKC inhibitor GF-109203X with dbcAMP did not affect EGFR-P kinase activity or superoxide production induced by OZ, as shown in Figure 7(C).

Role of tyrosine phosphorylation

Preincubation of neutrophils with the tyrosine kinase inhibitor genistein [45] (0–0.3 mM) for 30 min at 37 °C caused a dose-dependent inhibition of EGFR-P kinase activity, [³H]AA release and superoxide production when stimulated with 1 mg/ml OZ (Figure 8). Under the same experimental conditions, EGFR-P kinase activity, [³H]AA release and superoxide production stimulated by 50 ng/ml PMA were only slightly affected. The effect of genistein on the electrophoretic mobility of ERK-2 was also studied. As shown in Figure 9, preincubation of the cells with genistein prevented the ERK-2 shift induced by OZ in a dose-dependent manner. This isoflavone did not affect the shift induced by PMA. Similar results were obtained with anti-(ERK-1) antibodies (results not shown). These observations are in correlation with the inhibitory effect of genistein on EGFR-P kinase activity (Figure 8A).

DISCUSSION

The presence and activation of both sPLA₂ and cPLA₂ have been demonstrated in phagocytic cells. The relative roles of these phospholipases in stimulated phagocytes are yet unknown. The results of the present study demonstrate that OZ and PMA induce a similar DTT-resistant PLA₂ activity in cytosol and in membrane fractions of neutrophils. The two agonists induced translocation of cPLA₂ to the membranes (Figure 2) in correlation with cPLA₂ activity detected in membrane fractions separated in the presence of Ca²⁺ (Figure 1A). These results suggest that the molecular form of PLA₂ activated by both OZ, an agent shown to increase intracellular Ca²⁺ levels, and PMA, which does not alter intracellular Ca²⁺ levels, is probably cPLA₂. The similar stimulation of [³H]AA release and cPLA₂ activity by PMA or by OZ and the similar effect of the PKC inhibitor GF-109203X on both activities (Figure 5) indicate that the release of [³H]AA from prelabelled neutrophils is mainly a consequence of cPLA₂ activity. This suggestion is consistent with a recent study [46] in rat neutrophils, reporting that the stimulation of PLA₂ OZ

was probably derived from cPLA₂ activity. In addition it has been suggested [47] that cPLA₂ is the enzyme that induces the release of AA in a permeabilized neutrophil model.

Several lines of evidence suggest a possible role of ERKs in the activation of cPLA₂ by PMA or OZ in human neutrophils. The two agonists induced an immediate activation of 42/44 kDa MAP kinase preceding the release of [³H]AA (Figure 3). ERK and cPLA₂ activities induced by either PMA or OZ were both inhibited by the MEK inhibitor. Furthermore, the correlation between ERK and cPLA₂ activities is also supported when using additional inhibitors. Both enzymes are inhibited by GF-109203X when stimulated by PMA and by genistein when stimulated by OZ. Consistent with this suggestion, the presence of cPLA₂ and its translocation to the membranes have been shown in neutrophils and macrophages stimulated with disparate agents such as FMLP, A23187, zymosan or PMA, all of which activate p42/44 MAP kinase [48–51]. Recently a number of studies [52–55] reported that the activation of cPLA₂ stimulated by tumour necrosis factor α is mediated by p38 MAP kinase and not by ERKs. In contrast, the activation of cPLA₂ stimulated by granulocyte/macrophage colony-stimulating factor is mediated by ERKs and not by p38 MAP kinase. Thus different agonists might activate cPLA₂ through distinct MAP kinase types. Furthermore, the stimulation of multiple subtypes of MAP kinase by the same agonist was also reported. Lipopolysaccharide was shown to trigger p42/p44 MAP kinase, SAPK/JNK and p38 MAP kinase pathways in the RAW 264.7 macrophage cell line [56]. Likewise, stimulation of neutrophils with FMLP resulted in the activation of both ERKs and p38 MAP kinase [57]. The decrease in cPLA₂ activity stimulated by OZ or PMA after inhibition of the MEK–ERK pathway, demonstrated in our study, indicates that this pathway mediates the activation of cPLA₂ and that other MAP kinase subtypes are probably not involved in cPLA₂ signalling by these agonists. The possibility that OZ or PMA activates p38 MAP kinase or SAPK/JNK, and their role in neutrophils, are currently being investigated.

It is well recognized that more than one signalling pathway can activate the same response in neutrophils [58,59]. The mechanism leading to activation of ERKs by PMA has been explored [60] by identifying the components required for the reconstitution of the signalling cascade from PMA to MAP kinase *in vitro*. The activation of MAP kinase by PMA requires the presence of PKC, c-Raf and the MAP kinase activator MEK, but the nature of the PKC–Raf interaction has not yet been defined. The potent selective inhibitor of PKC, GF-109203X, caused a dose-dependent inhibition of EGFR-P kinase activity, [³H]AA release, DTT-resistant PLA₂ activity and superoxide production (Figure 5) when stimulated by PMA, suggesting that in human neutrophils PMA activates 42/44 kDa MAP kinase by a PKC-dependent pathway.

Zymosan opsonized with serum opsonins (IgG, C3b) interacts with three kinds of transmembrane receptor in human neutrophils: the IgG receptors, Fc γ RII, Fc γ RIII and the receptor for C3b, C3bR (CR1) [61]. The signal transduction initiated by this agent in neutrophils remains to be elucidated. In the present study we demonstrate that OZ caused a significant stimulation of ERKs, similar to the effect of PMA. To the best of our knowledge this is the first report to show that binding to the above-mentioned transmembrane receptors stimulates ERK activity. Although OZ induced a high activity of PKC, the ineffectiveness of GF-109203X in impeding EGFR-P kinase activity, [³H]AA release, DTT-resistant PLA₂ activity and superoxide generation in neutrophils stimulated by OZ (Figure 5) implies that this agent might stimulate these activities by a PKC-independent pathway. To test this hypothesis, the effect of pretreatment with the

bisindolylmaleimide GF-109203X on PKC activity in neutrophils stimulated by either PMA or OZ was studied. The predominant PKC isoforms found in neutrophils (PKC α , PKC β I, PKC β II, and PKC ζ [62,63]) have all been shown to be inhibited by GF-109203X [64,65]. Preincubation with GF-109203X in neutrophils stimulated with OZ caused an efficient dose-dependent inhibition of PKC activity, similar to that obtained in neutrophils stimulated with PMA (Figure 6), without inhibiting EGFR-P kinase activity, AA release, cPLA₂ activity and superoxide generation (Figure 5). These observations support the presence of a PKC-independent pathway triggered by OZ. A recent study [51] reported that PMA and zymosan trigger PKC activation, which leads to the activation of p42/p44 MAP kinase and cPLA₂ in peritoneal macrophages. In that study, although GF-109203X was similarly reported to inhibit p42/p44 MAP kinase activity induced by PMA or zymosan, PMA-induced tyrosine phosphorylation of p42/p44 MAP kinase was almost completely suppressed by GF-109203X, whereas it was only partly inhibited in zymosan-stimulated cells. This difference in the inhibition of p42/p44 MAP kinase tyrosine phosphorylation is consistent with the differential effect between OZ and PMA reported in our study.

Activation of ERKs through Raf-1 has been shown to be achieved also by Ras, independently of PKC [43]. cAMP, which prevents the transmission of signals from Ras to Raf-1 [44], did not significantly inhibit EGFR-P kinase activity stimulated by OZ (Figure 7C). The activity of ERKs in the presence of dbcAMP suggests that the Ras–Raf-1 transduction pathway is not the main pathway signalling the activation of ERKs by OZ, in contrast with the importance of this pathway for FMLP stimulation [38]. The high activity of ERKs stimulated by OZ in the presence of either GF-109203X or dbcAMP could imply that ERKs activation is mediated by alternative pathways: the PKC-dependent pathway or the Ras–Raf-1 PKC-independent pathway. In that case, the addition of both inhibitors should inhibit ERK activity. However, our results rule out this possibility, because these two inhibitors, when added together, affect neither ERK activity nor superoxide production induced by OZ (Figure 7C). These results indicate the existence of another, as yet undefined, PKC-independent mechanism triggered by OZ.

The rank order of activation of ERKs, PLA₂ activity and superoxide production (Figure 3) and the correlation between the effect of the different inhibitors on these activities (Figures 4, 5 and 8), although not conclusive, raise the possibility that cPLA₂ is activated by ERKs and releases AA, which, as suggested previously [9], participates in the activation of NADPH oxidase. The association between ERKs and superoxide generation is also shown by the inhibitory effect of dbcAMP on these activities stimulated by FMLP (Figure 7). The inhibition of FMLP-induced superoxide production by this agent is similar to that exhibited by others [66]. A recent study [57] has shown that the FMLP-induced release of superoxide anion is dependent on the activation of p38 MAP kinase. However, in agreement with our results, these authors also reported that PMA-induced superoxide release is dependent on ERK activation, thus supporting the conclusion that mechanisms dependent on different MAP kinase subtypes stimulation can mediate NADPH oxidase activity.

The inhibition of ERKs, [³H]AA release and superoxide generation by genistein in neutrophils stimulated by OZ indicates a role for tyrosine kinases in the signal transduction induced by this agent. Genistein was shown [45] to inhibit tyrosine-specific protein kinases and to be ineffective in inhibiting serine and threonine kinases such as PKC or cAMP-dependent protein kinases. The inhibition of superoxide production by tyrosine kinase inhibitors, erbstatin, α -cyano-3,4-dihydroxythiocinamide and herbimycin A, has been shown in FMLP- and C5a-stimulated

neutrophils [67–69] and in OZ-stimulated cells in one of our studies [70]. ERKs, which undergo phosphorylation on tyrosine and threonine residues, could be a potential target for the inhibitory action of genistein. However, because genistein was significantly less effective in inhibiting ERK activity stimulated by PMA (Figure 8), we suggest the presence of tyrosine kinase/s, essential for the effect of OZ and highly sensitive to genistein, which are located upstream of ERKs. Because the receptors for OZ (FcγR and C₃bR) have no intrinsic tyrosine kinase activity, non-receptor tyrosine kinases are implicated in participating in the OZ signalling pathway. Several putative target sites for genistein can be suggested, such as the FcγRII itself [71] or the Src-like protein tyrosine kinase Fgr, which is physically and functionally associated with FcγRII in human neutrophils [72]. In addition, the cross-linking of FcγRI [73] or FcγRII [74] in macrophages resulted in the association and activation of the tyrosine kinases Hck and Lyn or Syk respectively.

In conclusion, Ca²⁺-dependent translocation of cPLA₂ to the membranes in correlation with DTT-resistant PLA₂ activity suggests that OZ, similarly to PMA, activates cPLA₂ in human neutrophils. The rank order of stimulation of ERKs, [³H]AA release and superoxide production alongside the inhibition of ERKs, cPLA₂ and NADPH oxidase activities by the MEK inhibitor suggest that the activation of cPLA₂ by either OZ or PMA is mediated by ERKs and that cPLA₂ releases AA, which in turn might activate NADPH oxidase. Whereas PMA activates ERKs through a PKC-dependent pathway, signal transduction stimulated by OZ involves tyrosine kinase activity leading to activation of ERKs through an as yet unidentified PKC-independent pathway.

This research was supported by grants from the Ministry of Health, Israel, and from the Faculty of Health Sciences, Ben-Gurion University, Beer-Sheva, Israel.

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Received 3 December 1996/16 May 1997; accepted 22 May 1997